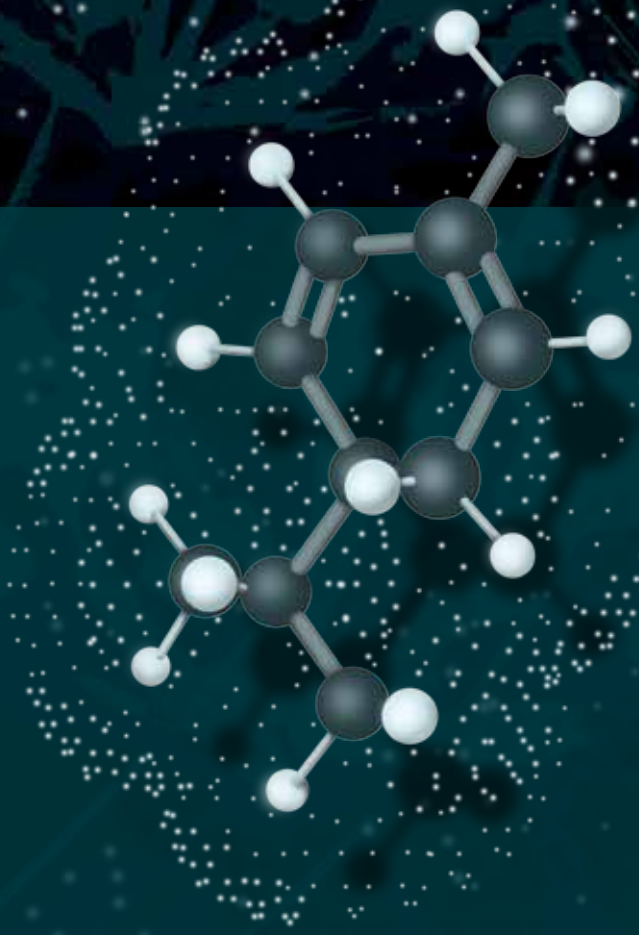
Marika Jestoi, Eila Järvenpää, Kimmo Peltonen, editors



First dice your dill

(Anethum graveolens L.*)*

– new methods and techniques in sample handling

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– new methods and techniques
in sample handling

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Foreword

This book is dedicated to celebrate the 60th birthday of Professor *Rainer Huopalahti*. Professor Rainer "Repe" Huopalahti has had, and in fact is still enjoying a distinguished career in the analysis of food and food related flavor compounds. One will find it hard to make any progress in this particular field without a valid and innovative sample handling technique and this is a field in which Professor Huopalahti has made great contributions. The title and the front cover of this book honors Professor Huopahti's early steps in science. His PhD thesis which was published on 1985 is entitled "Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors". At that time, the thesis introduced new technology being applied to sample handling and analysis of flavoring compounds of dill.

Sample handling is an essential task that in just about every analysis. If one is working with minor compounds in a sample or trying to detect trace levels of the analytes, one of the aims of sample handling may be to increase the sensitivity of the analytical method. On the other hand, if one is working with a challenging matrix such as the kind found in biological samples, one of the aims is to increase the selectivity. However, quite often the aim is to increase both the selectivity and the sensitivity. This book provides good and representative examples about the necessity of valid sample handling and the role of the sample handling in the analytical method.

The contributors of the book are leading Finnish scientists on the field of organic instrumental analytical chemistry. Some of them are also Repe's personal friends and former students from the University of Turku, Department of Biochemistry and Food Chemistry. Importantly, the authors all know Repe in one way or another and are well aware of his achievements on the field of analytical chemistry.

The editorial team had a great time during the planning phase and during the "hard work editorial phase" of the book. For example, we came up with many ideas on how to publish the book. After many long discussions, we decided to have a limited edition as an "old school hard cover book" – and to acknowledge more modern ways of disseminating knowledge by publishing an internet version of the book on the webpages of the University of Turku. Downloading the book from the webpage for personal use is free of charge.

We believe and hope that the book will be read with great interest by scientists working in the fascinating field of organic instrumental analytical chemistry. We decided to publish our book in English for two main reasons. First, we believe that in the near future, more and more teaching in Finnish Universities will be delivered in English. To facilitate this process and encourage students to develop good language skills, it was decided to be published the book in English. Secondly, we believe that the book will also interest scientists outside Finland – particularly in the other member states of the European Union.

The editorial team thanks all the authors for their willingness to contribute to this book – and to adhere to the very strict schedule. We also want to thank the various individuals and enterprises who financially supported the book project. Without that support, it would not have been possible to publish the hardcover book. ●

Helsinki, 15th October 2010

Marika Jestoi *Eila Järvenpää* *Kimmo Peltonen*

2
The Team Player
Rainer (Repe) Huopalahti

Does Repe play football in any team? No!
Does Repe play ice hockey in any team? No!
How can it be that he is such a good team player?

Sandy Fuchs, Engineer, Fuchs Konsultointi Oy
Päivi Laakso, Ph.D., Docent, Analytical Services Manager, Eurofins Scientific Finland Oy

Repe is a team player because he has succeeded in doing something in his life where most of us fail. To be able to manage the Home team and the Work team in such a perfect harmony that no one ever asks which team has the priority. Still it's obvious that his wife Eija and the children Sauli, Henri and Sanna-Mari are the top seeds in his eyes. The work team is built up of colleagues at the university (institution) and on the other hand, fellow workers from many different fields.

Here we enter a feature which might have a commercial sound but Repe is a real "Connecting people". To manage the joy of communication with people who deep inside are thinking in very different ways is his real forte.
Let's take some examples:

The sample handling expert says: for optimum sample analysis,
it is not just the chemistry it is the preparation.

The gas chromatography expert says: heat a 450 m long column
with 1.3 million plates, let's separate 970 components in a gasoline sample.

The liquid chromatography expert says: "alike – likes" is the method
where solvents do the real nice solutions – whether to follow or to stay.

The biochemist says: my interest is to know
how big my protein is – million kDaltons.

The mass spectroscopy expert says: by blowing up the protein
into small pieces (fragments) the computer calculates how big the protein is.

The collaboration with these groups with such different ways of thinking- confirms the wisdom of the saying "If you cannot beat them, work together with them" (– to obtain a mutual benefits) has been a cornerstone the actions of Repe. This quality is a way that he is using to narrow the gap in thinking between university teaching/educating and industrial price/performance thinking. Already as an assistant at University of Turku, teaching freshmen that evaluation is not just broadness integration of the sidewalk after having a party and trying to get home. Evaluation and integration is to count graph paper squares under each GC peak or laboriously cutting peaks out and weighing them in a laboratory balance.

At that time he was also called "The handy man" of the institution. We have to bear in mind that Perkin-Elmer held the patent on capillary gas chromatography from 1958 to1975. If the benefits of capillary gas chromatography were needed, then instrument modification had to be done by someone who had the right connections and know-how and could combine these two factors. "The handy man" did the modifications of packed column GC into capillary GC even though it was a long trial, error and success experiment.

One of the first borosilicate capillary columns in Finland were made at the "flight control tower" (University of Turku) as a co-operation between University of Turku and Åbo Akademi scientists. Repe was one of these pioneers from the very beginning. The columns were drawn with a D.H. Desty instrument followed by etching of the column's inner surface so that the stationary phase was mechanically bonded utilising the static method. Repe's columns were mainly FFAP type which was especially suitable for aroma component analyses. It was rather tricky to install the rigid glass column into a GC: both

Figure 1. Varian 2100 GC and Repe photographed at the University of Turku ~1976 at 10.34 o'clock. The size of the GC based on memory estimation was: oven height about 135–140 cm and width 40–45 cm, the height of the pneumatic compartment (incl. rotameters) was 20–25 cm situated on top of the GC oven. The measures of the young scientist leaning to the GC are estimated to be around 185 cm and 80 kg. This information is based only on estimations and the authors do not take any responsibility for the validity of these data.

ends had to be straightened using a Bunsen burner and then cut. When this had been done the most tricky thing was to tighten the fittings because a little 'click' might start the whole installation process again. Those were the days. In order to illustrate these "good old days" you can see one young scientist leaning against an up-to-date GC at that time in Figure 1.

Today fused silica capillary columns are easy to handle and install consumables. A wide selection of chemically bonded stationary phases for different kind of separations is commercially available. Hybrid phases represent the latest technology where stationary phases are bonded to functional groups incorporated into the base silica itself.

The official Repe can be described with the following achievements: Repe was born on 24th March 1950 at Uusikaupunki. He graduated in 1969 from Uusikaupunki

yhteislyseo. He completed his Master of Science degree in 1975, Master of Licentiate degree in 1984 and Doctor of Philosophy degree in 1985 at University of Turku. In 2002, he was appointed as Professor of Food Chemistry at University of Turku. Before that he had several teaching positions at the University of Turku, i.e. assistant of biochemistry 1975–1983, assistant of food chemistry 1983–1990, senior assistant of food chemistry 1990–2002, acting professor and acting associate professor of Food Chemistry for over 7 years during 1987–1999. He has been an Adjunct Professor of Food Chemistry at University of Turku 1994–2002 and Adjunct Professor of Pharmacognosy at University of Helsinki since 1994.

Repe has always been interested in new and innovative analytical techniques and how to apply them in the field of Food Chemistry. In his Ph.D. thesis, he studied dill aroma composition and its contents by applying GC and MS. He has also been interested in colour substances and their analyses utilising e.g. solid phase extraction and HPLC. Repe has been working twice at Jack Henion's laboratory at Cornell University, which is a well-known laboratory for its mass spectrometric expertise in combination with various chromatographic techniques including also supercritical fluid chromatography (SFC). We (or actually Sandy) have summarised the major timelines in separation science of GC, HPLC and other techniques presented in Chapter 10 Applications.

As a teacher Repe has always shared his knowledge with an enthusiastic way to students. He has been active in student exchange programs (Socrates and Erasmus), creating possibilities to visit/study at other universities or research institutions. He has arranged several visits for the students to see other laboratories and food industry plants. In addition, Repe has always encouraged his students to participate in scientific congresses and meetings and he has introduced them to his wide social network of friends.

Repe must also be described as "The Social organiser". He recognised at an early stage that university needs connections to the non-academic world, especially in the fields of applied sciences such as Food Chemistry. The key term is co-operation and understanding what are the needs of current food-related industry. Repe together with his colleagues recognised at early 1980's a need for seminars providing up-to-date information on various fields of food chemistry addressed especially to chemists and related specialists working in industry. A total of 13 seminars were organised during 1981–1993, the topics of which were related to current research projects at that time within the University of Turku. Each time the seminars attracted a wide audience, e.g. the one on food colours and their use in food industry in 1983 was attended by over 100 participants, mainly from industry. Other topics were the seminar on food rheology, and the seminar on fatty acids and lipids just to mention a few.

Repe has also been active in Association of Finnish Chemical Societies being a member of the organising committee in the years 2000–2003 planning the scientific program for Kemian Päivät. In addition, he was the chairperson of The Finnish Chromatography Discussion Group during 1996–1999.

Repe has always been and still is popular among his colleagues, students, leaders – and among sales people. His well known phrase to when talking about business was "We do not have money to buy, but if you let us use the instrument, then we could spend some time together with it". Repe is always open, treats all equally and is easy to get along with; during all these years we have known him. ●

3 Background

The general aspects of sample preparation
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The general aspects of sample preparation

Marika Jestoi | Eila Järvenpää

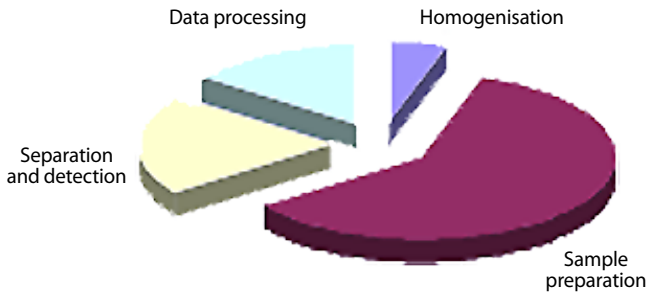
Introduction

Sample preparation is a process in which the analyte(s) of interest are extracted from a sample matrix, purified to remove any remaining interfering compounds and optionally concentrated to achieve an analytical system with method performance parameters fit-for-purpose. The whole process is a crucial and vital part of any analytical method and constitutes the basis for all separation-based research (1).

Sample preparation usually accounts for 50–75 % of the time used in the analytical method (Figure 1.). Therefore, advanced approaches for different steps of sample preparation are of great interest for each analyst to increase the laboratory efficiency. Additionally, in order to achieve reliable results and to maintain instrument performance, appropriate sample preparation is needed (2). During recent years, intensive development of laboratory techniques and instrumentation has occurred. The driving force in analytical chemistry is the trend towards simplification, automation and miniaturization of the total analytical system (3). Another significant change is the increased use of mass spectrometers and other sophisticated spectrometric devices which are becoming common tools in many applied science/chemistry laboratories.

Figure 1.

A typical segmentation of the time used for different steps of an analytical procedure.



The development of analytics for various 'omics' (4) will also increasingly emphasise the quality of sampling and sample preparation. These changes have also meant that more efforts to find more convenient and suitable sample preparation techniques are needed to fully utilise the possibilities offered by these technologies.

In this chapter, we briefly describe the aims of an appropriate sample preparation and outline typical problems related to it. Additionally, the chapter includes an overview of modern sample preparation techniques and related future trends. The perspective will focus on novel technological implementations of sample preparation relating to chromatographic and other instrumental methods.

Why sample preparation?

Different sample preparation steps are needed in order to remove any interfering matrix compounds and/or to concentrate the sample to achieve a proper signal on the analytical instrument. In fact, the major reason for sample preparation is usually related to the detection capability of the instruments. This means that the signal obtained may not be adequate unless the matrix components are removed. With respect to chromatographic methods, especially the conventional detectors, such as ultraviolet (UV)-detector, may suffer from a lack of selectivity and specificity in the presence of interfering compounds. Consequently, without proper sample preparation, the signal obtained from the analyte may not be recorded at an adequate level. With more selective/specific detectors, such as the mass spectrometer, the problems with the detection capability are usually different from the conventional ones. However, interfering compounds may hinder the signal obtained through a phenomenon known as the matrix effect. The matrix effect, the most usual form of which is ion suppression, may cause a dramatic loss of method accuracy, repeatability, trueness as well as induce higher limit of detection (LOD) and limit of quantification (LOQ). Therefore, an appropriate sample preparation is, in most cases, needed for mass spectrometric methods, despite the selective nature of the detector.

Due to the above mentioned problems of detection capability, one can list certain kinds of analyses which demand an extensive sample preparation to achieve a successful detection of compounds of interest. These analyses include residue analyses relating to food safety issues (drug residues, contaminants produced during food processing,

natural toxins etc.) as the sample matrices are most often very complex, consisting of proteins, carbohydrates and lipids, which all may have their own severe disturbing effects on the signal recorded either with conventional or modern detectors. Additionally, in most cases, the concentration levels are extremely low, even at ppt-level. Therefore, sample preparation is also a prerequisite for a successful detection of the analytes, even when mass spectrometric methods are being used.

In many circumstances, there are legislative or other demands for the method performance required for a successful analysis, e.g. not only the above mentioned food safety analyses, but also other approaches such as environmental and clinical analyses. As in many cases, the method performance parameters (e.g. repeatability, reproducibility, accuracy, trueness, LOD, LOQ) are dependent on the sample preparation (due to the instrumental problems described above), the analyst should not overlook the importance of this step in the analytical method to achieve results in compliance with the set down demands.

One issue which is not always perceived as part of sample preparation is the maintenance of the analytical instruments. Especially with the so-called destructive detectors (e.g. MS, light-scattering) in which the effluent is decomposed, any additional compounds may lead to contamination of the instrument. This will, in time, lead to the loss of detector performance. Consequently, there may arise demands for extensive maintenance or service of the instrument. Thus, the analyst should consider the importance of sample preparation in keeping the instrument running and avoiding any interruptions to the laboratory efficiency.

Many factors affect the choice of a proper sample preparation technique, and these will be presented in more detail in another chapter of this book. In brief, the most important determinants are the analyte itself and its chemical properties, concentration level, sample matrix and its composition as well as the expected interferences. In addition, the available sample amount and the analytical instrument applied will determine the techniques needed. Furthermore, attention should be paid to the nature of the analytical method: does one have a specific method for a single compound or should it be a more modern approach for multi-compound analyses, where compromises may be necessary to enable extraction, purification and detection of several chemically diverse compounds simultaneously.

There are many elements that can be used to assess sample preparation. In brief, a good sample preparation method should be effective (in all aspects), rapid, reliable, cost-effective and safe. The loss of the analyte(s) should also be minimal. Therefore in many cases a simple approach may be the best choice, if applicable. However, there are several occasions, where extensive sample preparation is needed to obtain good detection of the compounds of interest. In general, the sample preparation technique used should always be considered according to the "fit-for-purpose"-ideology.

Modern sample preparation techniques

As given in the title of this book, "New methods and techniques in sample handling" the authors of the following chapters will focus on many of the sample preparation techniques which are used prior to instrumental chemical analysis methods, such as chromatographic and spectrometric methods. In addition, we (as editors) chose methods which

we consider "modern". The third selection criterion was our interest in sample preparation and analytical methods related to complex food and biological samples.

In the literature "modern techniques" in the title of a presentation means something novel and new. For example, in 2007 two volumes of Journal of Chromatography A focused on novel sample preparation methods (5, 6). One could argue that kind of modern technique is "old-fashioned" by the next year, however in this book, we will focus on more durable modern techniques, which can be described as follows:

Modern sample preparation technique

- reduces time and effort the analyst needs to exert per sample, e.g. the modern method may combine two of the previous sample preparation methods into a single step: SPE is a combination of LLE and concentration steps
- is possible to be automated fully (integrated systems) or partly (sample preparation robotics)
- fits into the so-called green laboratory ideology. In comparison with the previous methods, it uses less energy and raw materials, uses less harmful solvents and produces less waste, environmental and occupational health risks
- facilitates microanalytics, i.e. the masses and volumes of samples and solvents are smaller than earlier

The search for the above attributes from literature databases would be somewhat problematic, thus more usual sample preparation –related keywords were chosen to illustrate the current sample preparation research papers. The search was conducted on 31.12.2009 using ISI Web of Knowledge database, and the publication years were limited to 2005–. Figure 2 illustrates the range of papers where "sample preparation" was mentioned in the topic. Figure 2A shows that of 10212 papers dealing with sample preparation, it is often combined with spectrometric and chromatographic techniques. As an example of all 'omics', proteomics was mentioned in 5 % (i.e. about 500 records) of the total sample preparation papers.

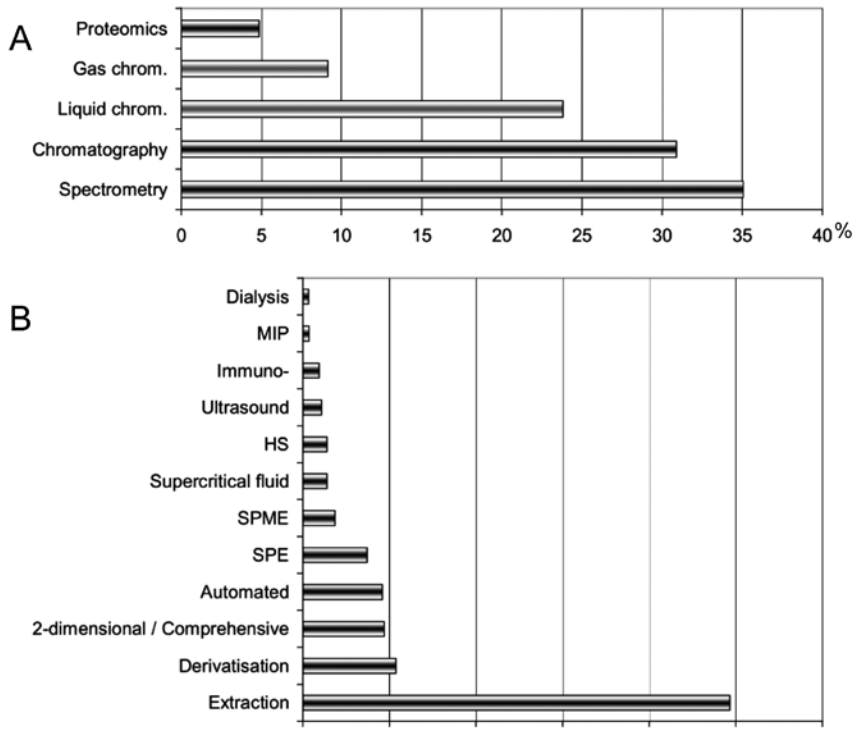
Figure 2B illustrates some of the actual sample preparation methods. Extraction as a sample preparation method exceeds the others, but also in a large number of research papers other techniques were mentioned, e.g. headspace was a key word in 3,8 % of the reports (about 140 reports).

Whether these methods are modern can be a matter of debate depending on the authors' or reader's viewpoints, However, if the same database is searched using the same keywords without time limit, some techniques seem to be more new than the others (data not shown). Molecular imprinted materials (MIP) and immuno-based techniques, as well as comprehensive techniques were clearly more popular in the recent research papers (Note: it was not evaluated whether immuno-based and comprehensive techniques were used as a sample preparation or a separation technique).

The editors are proud about the number of authors and chapters in this book. By inviting only domestic writers we were still able to cover various gas-phase, liquid, pressurised fluid and solid-phase extraction methods, derivatisation for chromatography, and automation. In addition, chapters describing issues related to the selection criteria and quality assurance of sample preparation methods as well as specific application areas will aid the reader to choose and validate perhaps the most important part of the

Figure 2.

Web of Knowledge database search using "Sample Preparation" as the term in topic (years 2005–). Abundance of "Sample preparation" in combination with terms on vertical axis is shown as percentage of total hits. A) Selected analytical methods B) Sample preparation methods. Search date 31.12.2009.



analytical methodology – sampling and furthermore sample preparation techniques determine what the analyst has to handle in the separation and/or what will be loaded onto the measuring devices which will determine the quality of the analytical result.

This book starts with selection of the sample preparation method and other fundamental considerations. As most of the papers and applications presented in this book relate to organic compounds found in biological materials, sample preparation has a major effect on the result, as a part of the total analysis procedure. Volatility is one chemical property special to organic compounds, except for permanent gases. If the analyte is already volatile, the analyst may be able to use the headspace sample preparation methods, including static and dynamic traditional headspace methods as well as purge-and-trap methods are gas extraction methods, as discussed in Chapter 5. It also describes the use of solid-phase microextraction for the analysis of volatile compounds, and focuses mainly on practical issues and headspace analysis of food-related materials. A good supplement to this topic is the recent review by Kataoka et al. (7), who critically considered other capillary microextraction techniques and evaluated their applicability for biological, environmental and food samples.

Most of the organic analytes however are non-volatile. Four extraction-specific chapters (Chapters 8–9) discuss fundamentals and modern extraction processes, using liquid, pressurised liquid or a fluid as well as solid materials as extraction media. The more novel techniques including ASE, SFE, microwave, ultrasound, and new, more selective or specific materials for solid-phase extraction and replacement materials for LLE are also discussed.

Actually, chapter 4 of comprehensive chromatography can be considered to be part of the extraction techniques i.e. chromatographic media as an additive. Individual chromatographic techniques such as gel filtration and gel permeation chromatography has been long used for sample preparation, as have thin layer chromatography and normal pressure liquid chromatographic methods. However, multidimensional chromatographic systems may be a specialised answer to very difficult analyte interference problems, and additionally this type of sample preparation could be automated and directly combined with analytical separation and quantification.

Most of the sample preparation methods are employed to isolate the analyte(s) from the matrix components. One important aspect is their concentrative effect. The handling and subsequent analysis (measurement) of the analyte quality and quantity is easier, when the analyte has a concentration well above the trace level. Additionally, derivatisation of the analyte(s) prior to or post separation can be used to enhance the signal obtained from the analyte and at least relatively reducing the signal obtained from the other compounds found in the sample aliquot (Chapter 6).

The first chapters discuss the sample preparation topics which can be used for common purposes, the last chapters of this book focus on specific sample preparation and analytical problems (Chapter 10). Although analyte or sample type specific, the reader may obtain some new ideas relevant to her/his own analytical field.

Problems relating to sample preparation

The key point for successful sample preparation and furthermore for a reliable analytical method is the appropriate sampling. Depending on the field of application, several legislative demands or guidelines exist to ensure the representativeness of samples to be analysed. These include documents such as European Commission regulations for the control of official analysis of mycotoxins (8) and operational procedures or guidelines issued by international and organisations and interest groups. Improper sampling will inevitably result in poor accuracy despite the selectivity and efficiency of the sample preparation method and the following instrumental methodology. Therefore the representativeness of the sample should be properly considered, taking into account any special physical or chemical features (heterogeneity, state, particle size etc.) of the whole sample.

Usually, different sample preparation steps account for 50–75 % of the total analysis time (Figure 1.). Accordingly, in many cases this can be considered as the most expensive part of an analysis, especially in those cases where sample preparation includes laborious steps demanding the involvement of a laboratory technician. Therefore, many of the recent developments in the sample preparation, especially different automation approaches, have focused on the reduction of labour in the steps necessary to increase the efficiency. Despite this fact, these modern approaches with special-use consumables may be more expensive as compared to conventional solutions, which can in certain cases reduce the potential economical savings. Regarding the automation of the sample preparation steps, it should also be considered that the development of an automated method is usually more laborious, and consequently more expensive, as compared to method development of a manual method. Accordingly, it should always be ensured that the automation really can confer advantages.

Usually, when the number of samples to be analysed is high enough, then automation will be profitable.

The stability of the analytes during the sample preparation process may also cause some problems. This applies, for example, to chemically active or volatile compounds. In some circumstances, the analyst should be aware of the possible interactions of the analyte(s) with the sample matrix, which may lead to poor method performance as measured in parameters such as recovery this being due to the inadequate extraction of the compound(s) from the sample matrix. In this certain case, the problems may be avoided with additional sample preparation steps such as hydrolysis that will enable the breakdown of the chemical bonds between the analyte of interest and the sample matrix. Overall, the chemical composition of the sample matrix may strongly affect the success of the sample preparation and the analytical method as a whole. Therefore, the sample preparation technique used should always be tested and validated for each analyte-matrix –combination separately. For instance, if a solid-phase extraction protocol has been earlier developed and described for using plasma as a matrix, the same protocol may not be appropriate for other kind of matrices, e.g. urine and the method performance cannot be the predicted. Consequently, the method protocol should be modified according to the application in question. However, in many cases, it may be difficult to specify and differentiate the reasons for dissimilar behaviour of sample preparation technique for different matrices and profound knowledge of the sample preparation technique as well as the chemistry of analyte/matrix which is needed to solve the possible problems. For example, this difficulty was demonstrated by Jestoi et al. (9) who reported that each sample matrix has its own distinct behaviour and this means that specific extraction or sample preparation protocols have to be adapted and optimized for each analyte/matrix-combination.

One of the main disadvantages of sample preparation is the loss of the analyte(s) during the process. Usually, the more complex and multi-phased sample preparation that is used, the higher the loss. Therefore the "fit-for-purpose" approach should always be kept in mind when developing a sample preparation method. A good way of avoiding sample loss is to use very simple approaches such as sample preparation minimisation. However, there is a continuous demand for multi-compound approaches to increase the laboratory efficiency, and in many of these cases minimisation and/or simplification of the sample preparation is not applicable due to the matrix effects described earlier. For these kinds of applications, there are some modern solutions, such as generic sample preparation using the QuEChERS-approach described elsewhere in this book.

Furthermore, the considerations of sample preparation include the balancing of the time consumed for cleaning of the sample and that of data processing or instrument maintenance. More extensive sample preparation and cleaning will, in most cases, result in cleaner extracts and furthermore have the advantage of easier signal detection (data processing) as well as less time spent on cleaning of the instrumental devices, especially in the case of destructive detectors as described above.

Future trends of sample preparation

As mentioned in the earlier chapter, there has been a move from "slow" manual sample preparation techniques to faster techniques (2). This can be achieved for instance by

using high-throughput -systems, such as solid-phase extraction using 96-well plates. The most widely used of these fast approaches are automated techniques that can be carried out either as off- or on-line with instrumental analysis. Especially the development of on-line systems (hyphenated techniques) can be predicted to become more popular. Automation confers several advantages as summarised in Kinsella et al. (2).

There is a constant need for multi-compound applications, which are now available when utilising the developments in analytical techniques, especially mass spectrometry. Nowadays, dozens or even hundreds of analytes may be determined simultaneously. In the future, the need for these multi-applications will be increased as the demands for laboratory efficiency increase. However, as there still are many problems, such as the matrix effect, which complicate multi-compound analyses, this being especially true in the case when compounds with diverse chemical properties need to to be analysed. Therefore generic sample preparation methods (e.g. QuEChERS) can be expected to become popular in the future, with broadening of the application area from pesticides to other compounds. However, this may mean, that some compromises may have to be accepted with regard to method performance parameters. These generic sample preparation methods will also help in fulfilling the other common demand of modern chemical analyses: low limits of detection and quantification (LOD/LOQ), as is the case in some food safety applications.

Future trends in sample preparation may include also simpler methods which are "just enough" prior to analysis (10) as more steps may introduce more errors as earlier stated. This phenomenon, known as minimisation of sample preparation, however, may cause some potential problems. These include the serious matrix effects observed in MS. In addition, to fully utilise the selectivity of the mass spectrometer (with regard to ion suppression), the use of selective and/or specific sample preparation techniques such as MIPs and immunoaffinity-phases for SPE will also become more popular in the future.

The current need to reduce the burden of environment with laboratory waste will lead to sample preparation techniques that use less solvent. This green laboratory-ideology includes the use of micro-extraction methods such as SPME, as described.

Conventional sample preparation techniques (SPE, LLE and protein precipitation) are still the most widely used in routine laboratories. However, their performance may well be surpassed by new modern approaches and their development should be encouraged, following high-throughput, low volume, ease of use, automated and environmental trends in order to reduce the gap between them and the fast LC and fast GC approaches (10).

One driving force in analytical chemistry is the trend towards simplification, automation and miniaturization of the total analytical system. Technologically, this development has resulted in lab-on-a-chip or μ-TAG (micro total analysis systems) applications. In addition, separation techniques using micro- and nano-scale chromatographic and electromigration techniques have been developed; e.g. fast chromatography systems have now matured sufficiently to be useful for real samples (or, routine and control analytical laboratories), although sample preparation and the introduction of a representative sample into the analytical system are still demanding issues. (3). However, it is likely, that more research and development in the field of sample preparation will be needed before more complex samples can be analysed, because at

present, these kinds of applications have not been demonstrated. There are (at least) two major concerns related to sample preparation:

- how to conduct the sampling and sample preparation procedure including enrichment of analytes with a very small amount of solvent, resulting in a representative sample aliquot which can be introduced into the analytical system
- the very small dimension systems do not tolerate any particulate matter in the sample solution

Thus careful selection of the suitable solvents, enhancement of dissolution and filtration of biological samples may again become important tasks of the analyst.

The analytical methodologies for the various 'omics', in biochemical, nutritional, toxicological and biotechnological research fields usually demand high productivity, i.e. a lot of data has to be collected from a single "shot" or "dot". Thus, the 'omics' involved in analytical chemistry development will include direct quantitative and/or qualitative tools, which are fast and effective, such as multidimensional chromatographic and spectrometric instruments. Although more attention has so far been paid to data handling and information technology (4) the 'omics' era will demand additional productivity from sample preparation and representative sample introduction methods. Some examples are given in the references (11–13).

Despite the current trends in sample preparation methods and techniques, the analyst should in all cases bear in the mind that the analytical method should be of high quality in all aspects. This includes, representative sampling but also all other aspects of the method performance parameters. To fulfil these criteria, a careful selection of a suitable sample preparation steps needs to be implemented with fully validated systems.

Quality and measurement reliability issues regarding the sample preparation are discussed in detail in Chapter 7. ●

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Sampling and sample handling for food composition database

Anna-Maija Lampi | Velimatti Ollilainen

Sampling

Sampling is a process in which the characteristics of the chosen samples are generalized to offset the whole population. The ideal sample equates to the whole material but ideal sampling is very difficult to perform. In practice, the sampling criteria will be set so that an acceptable sample is achieved. Sampling of the material and any related actions forms one of the greatest sources of error in bioanalysis. No reconstructive actions after improper sampling will be able to correct for this error.

Methods of sampling

Probability sampling

If the sample population is small, every unit of the population can be examined. In this case, the whole population will be measured. Total sampling is theoretically ideal but difficult to realize when the size of the population increases. A large population can rarely be described so that every sample unit has an equal probability of being collected. Therefore it is usually not worthwhile to examine the whole population, instead subsets are formed which represent the whole population as extensively as possible.

The simplest type of sampling is random sampling. The choice of unit collection is based on probability calculus and every unit has the same and equal probability for being selected (Horwitz 1990). In interval random sampling, every nth unit is taken starting with a unit randomly selected from a certain subgroup. According to sampling procedure, random samples can be further classified as simple random samples, stratified random samples, systematic random samples. The selection of sample is an objective procedure, and therefore independent from the collector. Random sampling can be utilized when there is sufficient proof for the homogeneity of the population and it enables the statistical evaluation of the results. Sample collection is done according to a certain procedure e.g. based on time or sample mass in the systematic sampling and the sample collection is performed based on certain criteria. This diminishes the error caused by sorting of the material. Systematic sampling requires good advance information about the material to be collected.

In stratified sampling, the sample units are first divided into groups (geographical, age), and samples are collected from these groups (stratum). In stratified random sampling, the samples are gathered randomly from those stratum. Stratified sampling confers higher accuracy if the sample units are of similar quality. The more variation that is present between those stratum, the higher efficiency that can be attained. By this means, a better population coverage is achieved compared to simple probability sampling. However, some drawbacks are inherent in this method. Difficulties may be encountered in the formation of groups, the method can be laborious to put into practice and the evaluation of the results may be complicated.

Nonprobability sampling

Samples are chosen on the basis on a subjective consideration to represent the population as well as possible. In the case that the population is heterogenic and the number of collected samples is small due to the low analysis capacity then purposive sampling can be considered. In this case, there can be no statistical. Selective sampling can be used in the analysis of contaminants whereas convenience sampling is limited to only a few cases. Purposive sampling requires extensive basic data on the sample material and population. A lack of this necessary information may restrict the applicability of the method.

Food sample selection and size

The sampling plan is a systematic and documented task which describes all the processes and their backgrounds in the sample collection. The personnel involved in sampling should be properly trained and experienced in this delicate task. A general protocol for food sampling has been described by Greenfield and Southgate (2003). The sampling plan should cover all the steps so that the sample chain can be traced from the sampling site to the testing laboratory. The key food concept was first launch by USDA. Key foods are classified as major contributors of nutrients of public health significance and they are utilized for setting priorities for foods used in nutrient analysis. The selection of the key foods is done according to the market shares and other consumption data. Other minor food items, such as wild foods, though less important in total consumption, can be included. Different brands of packed food items are usually available. Different brand types can be classified into several types;

the brand is owned by the producer and delivered to different retail sale chains, the same product sold in different package sizes are considered as different brands having their own market shares or the brand may be owned by one retail sale chain. If one is including all the brand types, then the leading brands of the food item will be selected according to their market shares. Bulk items are collected as available for the consumers. Meat, fish, and vegetables are collected from retail stores and market halls. The leading types of meat or fish species can be specified. However, the leading producers of bulk items cannot be specified countrywide, because there may well be a large number of different growers, for example, whose products are delivered locally. Despite the probable regional variation, the sampling can be carried out locally and the bulk items are collected from the leading retail sale chains as packed items. Certain wild foods, e.g. game (elk, reindeer), fish, and wild berries are available only at certain periods of year and they are collected from market halls, market places or wholesale traders.

The regional variation in nutrient composition of the samples needs to be taken into account as the variation between consumer foods depends notably on the food item. In this case, the regional stratification of the sampling may be considered. Production of certain types of foods may be largely centralized meaning that there may be only a few manufacturers in a country. On the other hand, raw food materials, berries, vegetables and wild food can show significant regional variation.

Example:

Sampling in National Food and Nutrient Analysis Program

National Food and Nutrient Analysis Program (NFNAP) is conducted by the Nutrient Data Laboratory (NDL), Agricultural Research Service, United States Department of Agriculture (USDA) in collaboration with several other governmental institutes or centers in USA. NFNAP was founded in 1997 to improve the quality and quantity of data in USDA food composition databases. Haytowitz and coworkers reviewed the activities of NFNAP in 2008 (3), and pointed out that sampling was considered as one of the main topics in their program.

In order to be able to identify and prioritize foods, it is important to identify key foods for sampling. Key foods are the major contributors of nutrients of public health significance to the US Diet; all together they provide 75 % of nutrients. Key foods are chosen based on food composition and consumption data. At the beginning of NFNAP, it was noted that the existing data contained old values and was poorly documented, and thus an expert system for evaluating data was developed. Sampling was one of the three features evaluated. As a result of the evaluation, the key food list was revised, and it is constantly being updated. Once the key foods have been selected, a probability-proportional-to-size sampling design was developed. The design had three levels. At first, geographically different and representative counties were chosen. Thereafter, within each county, a set of grocery store outlets or, for example, fast food restaurants and, finally, specific food products were selected.

The sampling plan can be modified to be better fit for the purpose for the specified study. Moreover, the number of samples can vary based on the variability of the nutrient or the consumption level as revealed during the key food process. Prior to being

used, the sampling plan is assessed by a contractor with tested selection protocols in retail outlets. In NFNAP, food samples are shipped to the Food Analysis Laboratory Control Center for sample preparation including homogenization, composing, storing and shipping to the contract laboratories which perform the analyses. Each processing and preparation step is carefully controlled and documented to meet the quality criteria of the program.

Sample size

Selective error in sampling depends on the heterogeneity of the population. Sampling error as standard deviation is the true variation of the material divided by the square root of the number of sample units. Therefore, the greater the number of primary units, the smaller will be the standard error. A large sample size reflects better the population. Statistical means for determining the sample size have been discussed by Proctor and Meullenet (4). In their practice, the optimum sample size is derived from the sample and population means, and the variation within the sample. This procedure requires that the preliminary information of the sample material is already available. In certain cases, sample size is calculated from data in the literature. In practice, analytes with high variability in food samples, like cholesterol in which variation is much large than moisture content, need higher number of samples. This may mean that tens or even hundreds of samples are needed for the analysis. This has been highlighted by Greenfield and Southgate (2). Most often food sampling is based on 10 sample units though the optimum sample size may not be fulfilled. For nutritional labelling, 12 sample units are required in the United States.

In addition, the sample size (in mass units) should be adequate to overcome the heterogeneity present in the material. Generally, the size of a primary sample should be at approx. 0.5–2kg, the size of a portion for composite sample 100g, and the size of the analytical sample 1–10g (for an example see Figure 1a). A composite sample consists of two or more primary samples which are first independently homogenized and then the same size of portions are pooled to form one composite sample. In food analysis, the number of subsamples to form a composite sample varies normally between 6 and 12.

The sampling and pooling procedure for a composite sample should be repeated to verify the validity of the sampling. The repetition is carried out over a short period of time (1–4 weeks) to avoid any seasonal variation between the sample pools.

Evaluation of data quality to be included in a database

In order to be able to rely on the information in a food composition database that has been obtained from various sources namely the food industry, research agencies or scientific literature, the nutrient contents in the food items need to be evaluated and rated for their scientific quality. Pioneering work in developing a quality evaluation system has been done at USDA NDL, and a multi-nutrient data quality evaluation system was created in 2002 being later modified in 2005 (5, 6). As another example, a European database on bioactive compounds in foods, EuroFIR-BASIS, is presented.

Evaluation of data quality for the USDA database

The quality evaluation consists of five categories, three of which belong to the scope of this chapter: Sampling plan, number of samples and sample handling. The two other categories are analytical method and analytical quality control (6). The evaluation system enables also documentation of information about the five categories. Each of the categories is evaluated with several criteria that are built up as distinct and specific questions. After evaluation, each of the categories is given rating points based on the answers to the individual questions. Each category uses a scale of 0–20 points, with the highest rating being 100 points. For each food item and nutrient combination, all ranking points from the categories are summed up to yield an overall rating, i.e. Quality Index (QI). It is worth highlighting that issues related to sampling can contribute up to 60 % to the QI. Data for a certain food and nutrient content in a database may derive from a set of sources, and thus a mean of QIs of all of them is calculated in order to be able to evaluate the overall confidence of the data value (5). Confidence code A or "exceptional" is given if the mean QI is 75–100, B or "above average" if the mean QI is 50–74, and C or "average" if the mean QI is 25–49. Lower mean QIs are designated with code D that is "below average".

The sampling plan focuses on the representativeness of the food sample units collected for analysis of a specific study. The sampling plan based on "convenience" that is buying food samples from local markets is of low value while a plan based on statistical theory or probability sampling achieves higher ratings. A good sampling plan includes consideration of geographical variation, market statistics for production, sales and consumption, and also the product description (6). Sampling according to NFNAP (National Food and Nutrient Analysis Program) would be awarded the highest rating (3). Composite samples, where primary sample units are collected from different locations and pooled together according to a controlled procedure, are commonly used to reduce the analytical work-load. These samples are more representative than individual samples, but on the other hand, they lose the knowledge on the natural variation present in the individual samples. The representativeness of the set of primary sample units must be defined relative to the primary objective for the database.

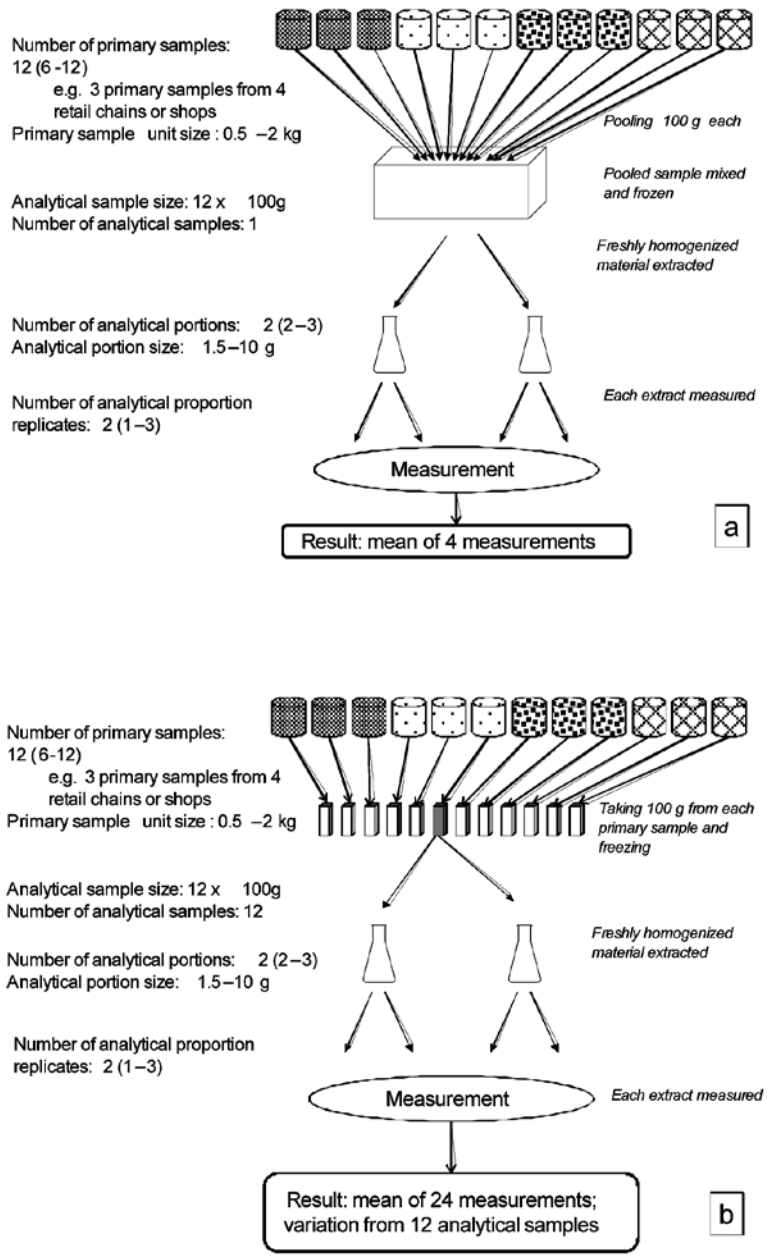
Sample handling includes all steps after the primary sample units have been bought or collected prior to analysis. The main focus is to verify that the nutrients to be analyzed remain stable, and that the representativeness of the analytical sample is not lost (5, 6). Sample handling includes sample dissecting, homogenization processes and storage conditions. In addition, one should verify that the moisture content does not change. The demands associated with sample handling clearly depend upon both the food material and the stability of the nutrient. Rapidly decomposing compounds, such as vitamins, need to be carefully processed prior to analysis. These aspects are very important when composite samples are prepared from primary sample units.

Number of samples is a critical value when evaluating data (6). If the number of samples is small, it is difficult to estimate the representative mean value of the food and nutrient combination to be included in the database. Moreover, not much is

known about the variability of contents. Data collected from multiple samples is more relevant and reliable. The number of samples should be separated from the number of analyses performed from the same analytical sample. When one pooled sample is analyzed twice (Figure 1 a), the number of samples is only one, but when twelve individual samples are analyzed separately (as shown in Figure 1 b), the number of samples is 12. Although the rating for the example of composite sample in Figure 1 a gathers only a low rating in number of samples, the sampling plan achieves a high rating, and thus a much higher QI than a single sample bought from a local store.

Figure 1.

Examples of sampling and sample handling when a) a composite sample and b) all primary samples are analyzed.



Evaluation of data quality for the EuroFIR-BASIS database

In Europe, a database on composition and biological activities of bioactive compounds has been created by the EuroFir Consortium, a network of excellence that was financially supported by EU PF6 "Food Quality and Safety Programme". At first, the EuroFIR-BASIS database focused on major food plants and their edible parts, and also on selected plant-based foods. Currently, the database houses data on 12 compound classes, such as carotenoids, flavonoids, phenolic acids and phytosterols. EuroFIR-BASIS is the first attempt to include data on the biological effects of the compounds in addition to compositional data (7).

The principles used in the evaluation of compositional data for the EuroFIR-BASIS database were rather similar to those used in the USDA database. Since the EuroFIR-BASIS was built up on the structure and organization of a database for natural toxicants in foods (www.foodcomp.dk/basis/) that focused on potentially toxic compounds, there were some differences in the priorities. Only data from scientific peer-reviewed literature was included (7).

There were six data quality criteria: plant/food description, representativeness of sample, sample handling, component description, analytical methodology and analytical performance (7). Three of these were related to sampling and sample handling (Table 1). The criterion "plant description" was not found as such in the USDA evaluation system, but had a more significant role in this database. Identification of the specific plant according to scientific name and botanical tissue was considered to be very important, because the contents and composition of bioactive compounds may vary considerably for example in different varieties, plant parts or individuals plants grown under different environmental conditions. Specificity in plant description inevitably reflected other criteria, and representativeness of the sample did not gain as much attention as is the case in the USDA evaluation system. There were lower demands for sampling plans, and the number of samples was included in the same criteria. These differences can be explained by the fact that in the EuroFIR-BASIS database, composition data from different sources are not combined to yield a representative value. Instead, each value is presented separately in the database, which means that levels of bioactive compounds and variation present in plants and plant foods can be better evaluated by the end-user.

Table 1. Data quality criteria concerning sampling and sample handling in the EuroFIR-BASIS /used for evaluation of food compositional data on bioactive compounds (Modified from 7).

Quality criterion	*Response*	*Examples of questions for the evaluation*
Plant/Food description	1–5	Is the plant name correct? Are the plant cultivar, season and year of growth and geographic origin included? Was the varietal type reported? Was the processing of the food properly described?
Representativeness of sample	1–5	Have appropriate sampling procedures been applied? Is this a representative sample for this food plant? Were there a suitable number of samples taken?
Sample handling	1–5	Was handling before analysis (and after sampling) appropriate? Could it affect the level of the component?

In Finland, National Institute for Health and Welfare (THL), the authority that is in charge of the national database Fineli (www.fineli.fi), follows currently the model of USDA in the evaluation of data to be included in the database. Their evaluation criteria include sampling, number of samples, sample handling, analytical method and quality control. In the future, THL is intending to adopt the evaluation system of EuroFir (Heli Reinivuo, personal communication 26.11.2009). ●

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How to select and develop a good sample preparation method?

Juha-Matti Pihlava | Merja Eurola | Veli Hietaniemi

Introduction

The process of selecting an analytical method for a particular analyte or analyte-matrix combination can quite often be a complicated sum of simultaneous events. Some chemists trust more on their instinct and insight gathered with a long experience in the laboratory work, while some may adopt a more systematic approach. Whatever the approach of selecting and developing a method, there are some general requirements for the methods that need to be considered.

We will narrow the scope of this paper mainly to the sample preparation methods for low molecular weight organic compounds that can be analyzed using gas or liquid chromatography. This article is based on numerous sources, review articles (1–10) and our own experience. More comprehensive and in-depth reviews on the analytical methods can be found in the following chapters of this book. Some of the recent reviews are listed as references. Other general sources for information, which are not mentioned in the list of references, have been the LC-GC magazine and its web pages, Reporter magazine, web pages of various suppliers of chromatography products and instrument manufacturers. The aim of the paper is to provide

an overview of various practical aspects in selection and development of sample preparation methods.

Characteristics of a good method

Safety is always underlined heavily in sample preparation and chemical analysis. Reagents used should provide efficient and accurate method but not be explosive, carcinogenic, teratogenic or otherwise very toxic. The chosen method should be efficient (good recoveries of the analytes), accurate, repeatable, robust and preferably suitable for multi-component analysis. The method should be also economical and suitable for high sample throughput, low labour in-put, cheap reagents (e.g. methanol in extraction) with low volumes, capable for preparing several samples simultaneously, suitable for multi-component analysis and automation.

First of all, the method should be as simple as possible, not including unnecessary steps in sample clean-up. For example, when the method includes liquid-liquid extraction (LLE), an additional clean-up by solid phase extraction (SPE) is not usually necessary. The expected lifetime of the method should be considered critically, especially in the case when totally new instrumental technique is used.

If the number of samples is huge and the analytes permit, it could also be reasonable to exert a considerable amount of effort in devising a non-destructive high-throughput technique, such as near Infrared reflectance/transmittance (NIR/NIT).

Requirements for the method set by the analyte and the matrix

Sample preparation methods depend on the analyte and the matrix. Matrixes having different compositions, such as in the amount of fat, moisture or protein, can require different approaches to prepare the analyte in a form suitable for instrumental determination.

The chemical and physical properties of the analyte

The chemical and physical properties of the analyte are the first key issues in developing an analytical method. Issues to be considered are the volatility of the analyte (higher volatility offers advantage to GC determination), polarity and solubility of the analyte (greater water solubility offers advantage to HPLC), how the analyte is to be detected and the thermal stability of the analyte (thermal sensitivity offers advantage to HPLC, or to on-column or PTV-GC). In addition, reactions of the analyte with other components present in the matrix, possibility to enzymatic breakdown (e.g. by polyphenol oxidase or myrosinase), sensitivity to oxidation by air or sensitivity to light (e.g. tocols and certain pesticides) or pH are topics which could be important.

There are also some ways to modify the chemical and physical properties of the analyte. By derivatization, the analyte can be transformed to more volatile form enabling analysis by GC (e.g. compounds containing hydroxyl groups such as sugars or hydroxyl and carboxyl groups such as phenolic acids) or to make it applicable to some other detection method (e.g. oxidizing ascorbic acid to its dehydro form and addition of a fluorescent tag, OPDA).

From an economical point of view, stable samples/analytes can be stored in a freezer in order to collect larger batches to the next step of the sample preparation method, e.g. clean-up or the analysis step. However, often the analyte needs to be determined immediately after sample preparation. Solvents and other reagents with suitable purity should be used. Either overdoing or underrating the question of purity of reagents can be uneconomical. However the consequences of underestimating could be more severe. It should be kept in mind that however high-quality the solvents are, they have limited shelf-lives even when stored properly.

Sample type, amount and concentration of the analyte in matrix

The sample type imposes requirements on the method. If the sample contains fat or oil, extracts made with organic solvents usually have to be cleaned-up prior analysis by GC or reverse phase HPLC. For limited sample sizes miniaturized or scaled-down methods may be needed. The concentration of the analyte might affect to the choice of the sample preparation method. For example tocols in vegetable oils can be analyzed directly by normal phase HPLC after dilution of the sample with non-polar organic solvent. When the analytes are present in the sample at lower levels, enrichment of the sample is usually needed.

Background work – what has been done before

Fortunately in the method development one does not usually have to start from zero. In most cases, a considerably body of work concerning analytical methods has been done before. It is worth the time and efforts to check whether suitable methods have been published for the analyte or similar compounds. It should also be wise to check if there are any official methods available e.g. from EU, AOAC, NMKL, AOCS, EPA, ICC and Pharmacopoeia. Using an official/standard method can ease the validation process considerably.

The pros and cons of the published "non-official" methods need to be considered and how the methods would fit ones own requirements. Unfortunately sometimes the published methods are too good to be true. Authors may have omitted to describe some crucial points, which would have been needed for the method to work properly.

When selecting and developing a new method, the best way is to try to keep things as simple as possible. Additional steps are included to the method only in very extending circumstances. It has to be considered whether extensive sample clean-up is needed, or if the specificity of the detector in use and the separation power of the column will permit the less cleaned sample extract to be analyzed. This use of less purified samples in HPLC needs gradient elution solvents to wash the co-extracts from the column between sample runs. Sometimes more cleaning is needed to reduce the matrix effect in GC and to keep the GC inlet clean from the excessive build-up of non-volatile co-extractives.

Methods already in use in the laboratory

One of the first thing to consider when a need for analysis of a new compound is arisen, could it be included in the method selection already used in the laboratory (in this you have to understand the chemistry of the analyte as well as the method). If the analyte requires its own specific sample preparation method, the question arises could it be possible to analyze it with the same instrumental methods used for the other compounds (e.g. flavonoids and phenolic acids).

Infrastructure of the laboratory

The infrastructure of the laboratory is the basis for selecting or developing a method. In addition, it is important to estimate does the laboratory have the skilled personnel capable of conducting the sample preparation.

The economical feasibility to develop a method has also to be evaluated: how many samples are to be expected, how labour intensive the method is, what is the price of the chemicals needed, are there special instruments required etc. Sometimes it can be more economical to purchase the analysis from some other laboratory.

Quality control and validation of a method

In SFS-EN ISO/IEC 17025 standard it is stated that "The laboratory shall have quality control procedures for monitoring the validity of tests and calibrations undertaken" (11). The responsibility of the above-mentioned quality assurance in a method development and sample analysis are shared among laboratory management, research scientists, chemists and technical staff. If the analytical work and the results obtained are going to be published in a journal, the reader has the right to know that the relevant quality-control tools have been used. For example, the quality control should at least cover sample preparation, instrumentation and validation of the method (12).

The validation process is easier with the official standard methods than with in-house methods. The performance of the method is usually determined by using reference standards or materials, comparing the results with other methods, inter-laboratory comparisons, assessing the uncertainty, assessing the factors influencing the results. In daily work, use of replicates, commercial or in-house reference materials, daily controls and recovery checks are needed. Of course there are many cases when a reference material is not available or it is impossible to use due to the sensitivity of the compound. In these cases, quality control has to be done in some other way, for example with recovery tests.

Whenever possible it would be recommended to use an official method rather than trying to build everything from scratch. Unfortunately, sometimes the official methods may be based on the old techniques when at the same time more efficient and faster methods for sample preparation are available. To speed up the validation process the commercial method development and validation softwares are available. These are worth while of consideration especially if there are many new methods to be developed annually. Sometimes a less stringent validation process will suffice though in other cases, the full validation of the method is needed.

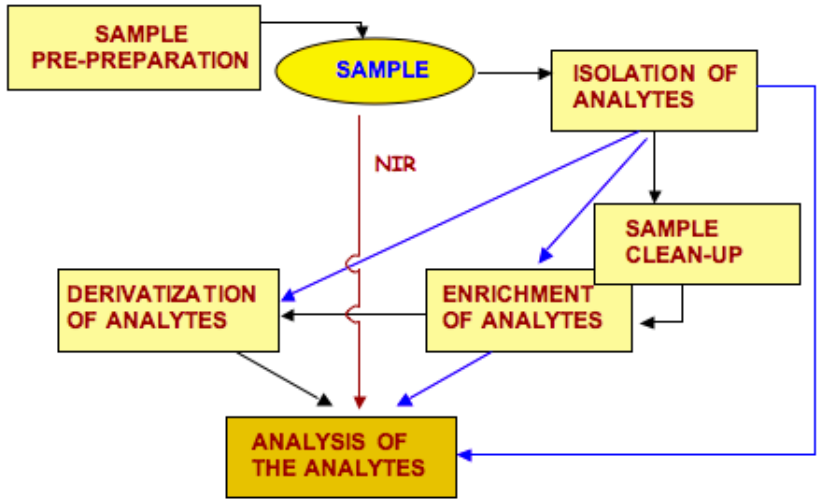
Anatomy of a sample preparation method

The sample preparation method can usually be divided roughly into two parts. Firstly "trimming" or pre-preparation of sample and secondly preparing the analyte by using isolation, clean-up and possibly derivatization into a form from which it can be measured. In the following chapters, some issues and suggestions concerning

the sample pre-preparation, isolation by various extraction and clean-up methods are discussed. An example of the anatomy of typical methods is presented in Figure 1.

Figure 1.

Possible steps in sample preparation method.



Sampling and sample pretreatment

The sampling process begins with the problem definition, aim and purpose of the study. The main goal is to collect a representative sample. Many times, sampling is the most critical point of the analyses. Therefore the European Commission has laid down several directives concerning the sampling methods for the official control levels for certain contaminants such as mycotoxins in foodstuffs.

It is important to know why the sample is being taken, what kind of sample and how it should be taken and stored. The best sampling method depends on the problem (size of the lot, homogenous or heterogenous distribution of the analyte, matrix and particle size, only one sample, several increment samples to be combined, a composite sample, multi-stage sampling). Precautions to avoid changes must be considered. Both biotic (cultivar, plant diseases, pests) and abiotic differences (soil, temperature, day-length, rainfall, fertilization) can cause changes and differences. Combined (aggregated) samples reduce the number of analyses and save costs. Often pre-treatment procedures are needed to prepare the final laboratory sample. These procedures include cleaning, washing, brushing, peeling, chopping, drying, freeze drying and homogenisation. Therefore preserving sample integrity during handling must be considered and component losses, breakdown of analytes, contamination and enzymatic changes should be minimized.

Representative sub-samples can be formed e.g. by chopping and mixing (solid samples such as potatoes, carrots, apples and cheese) and then homogenizing the combined sub-samples. Homogenization can be performed by hand-held mixer (such as Bamix), high speed dispenser (such as Ultra-Turrax), ball mill, hammer mill, cryo mill or bowl cutter mixer (such as Robot Coupe), depending on the sample type and stability of the analyte. It is always important that the sample is adequately ho-

mogenized. Large particles of peel or fruit skin may cause high analyte and chemical analysis variations, which could be problematic especially in scaled-down methods. Coarse particles may also lead to poor recoveries of analytes e.g. in cereal samples.

Some analytes may undergo rapid enzymatic changes during homogenization in water containing plant material. The intrinsic enzyme activities, most commonly polyphenoloxidase (PPO) in case of phenolic compounds or myrosinase in case of glucosinolates, have to be controlled. Enzymes may be de-activated by blending the sample with a water miscible organic solvent prior to the final homogenization. The other way is to freeze-dry the sample. An example of a somewhat more complicated sample pre-treatment method is PPO and the phenolic compounds in apples. The apples are cut into halves and after freezing chopped into smaller pieces while taking care that the sample will not melt during the chopping process. Then the sample is freeze-dried and homogenized to a fine powder. It has to be emphasized that the addition of water to freeze-dry material will restore the enzyme activity. Freeze-drying is also a method for enriching the analyte.

Even a dry sample cannot be considered as a stable matrix. Some changes can take place, especially with longer storage time at room temperature. For example ground cereal samples undergo lipid auto-oxidation and the formed radicals can also destroy the analyte. Lipid auto-oxidation can be slowed down by storing the sample in a freezer. If several compounds have to be analyzed by using different sample preparation methods, one needs to take into account the fact that re-freezing and re-melting the sample can destroy sensitive compounds. Careful synchronizing of different sample preparations is necessary when parallel samples are not available.

Isolating the analyte from the matrix

Usually the analyte and matrix have to be separated before the chromatographic analysis. Sometimes liquid samples with analytes at high concentrations, such as anthocyanins in juices or tocols in vegetable oils, can be analyzed directly. Extraction of the analyte from the matrix can be based on liquids using aqueous and/or organic solvents, supercritical fluid or gas in case of easily volatile compounds.

Open liquid extraction

Open liquid extraction is performed at room temperature, or at slightly raised temperature and at normal pressure. Depending on the polarity of the analyte, the solvent used for extraction of the analyte can be organic or water/buffer or a combination of organic solvent and water (e.g. acetonitrile or methanol and water). The organic extraction solvent can be polar or non-polar, either pure or a combination of two or three solvents.

Extraction can be combined with sample homogenization with a hand-held mixer, a high speed dispenser or a ball mill. With certain samples, extraction can be enhanced by using a magnetic stirrer, shaker or ultrasound treatment. If an extended extraction time is needed, e.g. overnight in magnetic stirrer in an erlenmayer flask, and the analytes are sensitive to oxidation, it is recommended to flush the sample and solvent containing flask carefully with nitrogen or argon and then seal the flask properly. External antioxidants may also be added to the extraction solvent, such as BHT, BHA, pyrogallol or ascorbic acid, as long as they do not interfere with the analysis. Some compounds may be photosensitive, thus with longer extraction times, brown glassware or other form of protection from natural or artificial light may be needed.

It is possible to use liquid extraction as a clean-up method by removing fat or oil and leaving the more polar analytes within matrix. After removal of the non-polar solvent and drying of the matrix, polar analytes can be extracted with polar solvents.

In the case of glucosinolate analysis, elevated temperature and alcoholic solvent can be used to enhance the extraction and to denature enzymes that may otherwise rapidly decompose the analytes. In liquid extraction, the choices of the solvent, liquid/solid ratio, extraction time, temperature and number of extraction cycles have to be considered. Sometimes the extraction of the analytes from the matrix is slow, especially if the polarities of the analyte and solvent differ. If this situation is suspected, different extraction times (e.g. 0,5 h , 2 h and overnight) should be tested. The remaining insoluble part is usually separated from the extract by filtration or centrifugation.

Extraction at the boiling point of the solvent by Soxhlet, Soxtec and Soxflo equipments

This extraction type is performed on solid samples by equipments for this special purpose. Automation of the extraction process and simultaneous parallel extractions are possible with the newer sophisticated instruments. Soxhlet-extraction is traditionally used to extract organic pollutants, such as PCBs and organochlorine compounds in the sediment and soil samples. The need to enhance and speed-up the Soxhlet-extraction has lead to the development of Soxtec- and Soxflo-techniques.

Pressurised liquid extraction at elevated temperatures

The most known equipments in this application are ASE (Accelerated Solvent Extraction) from Dionex and SpeedExtractor from Buchi. The technique is fast, a low amount of solvents is needed and automation is also possible. In addition specific multi-sample evaporators have been developed together with these instruments.

The extraction temperature is more important than the pressure and extraction solvent plays a key role. Because of the high temperature, there is a greater solvent capacity to extract the analyte. Since the diffusion velocity is increased due to the reduced viscosity of the solvents, the extraction can be conducted with smaller volumes and shorter extraction times than in the open liquid extractions. The selectivity of the extraction can be increased by careful selection of appropriate solvents, temperature and also by using adsorbents such as alumina in the extraction vessel.

Microwave Assisted Extraction (MAE)

The microwave extraction is performed in pressurised vessels at high temperatures. Extractions are relatively fast but the cooling of the vessels may take a long time. Selection of a suitable extraction solvent is very important. The problem is that a solvent efficient in Soxhlet-extraction is not necessarily efficient in MAE. The MAE-technique has traditionally used for soil and sediment samples. It is claimed that polyaromatic hydrocarbons (PAH) can extracted in 6 min with MAE compared with 4 h in Soxhlet.

Hydrolysis and hydrolytic extraction

Hydrolysis and hydrolytic extraction can be also considered as a clean-up step. Hydrolysis can be conducted at elevated temperatures, as with flavonoids at the boiling point of acidic alcohol, or at room temperature as in the case of carotenoids. Acid hydrolysis is used in order to cleave sugar residues from flavonoids, phenolic acids or phytosterol glycosides. Concentrated acid can be used to hydrolyse fat or oil in PCB and OC-compound analysis. These harsh conditions can be used only for chemically stable compounds.

Alkaline hydrolysis is commonly used to cleave esterified fatty acid residues from phytosterols and cholesterol, to cleave various phenolic acid esters and to break lignanpolymer in order to liberate SDG-lignan units in flaxseed. Concentrated alkaline hydrolysis is used to dissociate the protein containing matrix in the polyaromatic hydrocarbon (PAH) method. Alkaline hydrolysis (4 M NaOH) combined with microwave assisted extraction at high temperatures (170° C) can be used to cleave the ether bound phenolic acids in cereals in only three minutes.

Acid and alkaline hydrolysis can be undertaken without separate extraction of analytes with direct hydrolysis methods. Alkaline direct hydrolysis may be used for phytosterols and fat-soluble vitamins. Usually, it is recommended that oxygen is removed from the hydrolysis vessel by nitrogen or argon in order to protect the compounds.

The third hydrolysis method is enzymatic hydrolysis. Examples of enzymatic hydrolysis are the digestion of starch with amylase and the breakdown of plant cell wall structures by pectinase and cellulose. Enzymatic treatment may also be directed to change the analyte into less polar aglycon form by the cleavage e.g. of sugar residues from isoflavonoids, lignans and other phenolic compounds. After the enzymatic treatment, the sample can be extracted by solvents (liquid-liquid partitioning) or transferred to the clean-up step.

Supercritical Fluid Extraction (SFE)

Carbon dioxide is the most common gas used in supercritical fluid extractions. This is mainly because it is non-toxic at low concentrations and can be transferred into a supercritical (SC) state under relatively mild conditions at 31° C and 74 bar pressure. However, the extractions are typically performed at 250–350 bar and 50–70° C. The SC-CO_2 is relatively non-polar, and thus in order to extract more polar analytes, ethanol can be added to enhance the extraction. Controlling the water in the sample is important and samples with high water content can be mixed with drying agent, such as Hydromatrix, prior to extraction. Generally SFE is more suitable for dry solid samples and for non-polar to mid-polar analytes.

Headspace extraction

Easily volatile compounds may be extracted from the gas phase of a liquid or solid samples using helium in a special extraction vessel. Headspace extraction can be performed on static or dynamic mode.

Headspace extraction is simultaneously a clean-up and an enrichment method. Analytes are usually enriched first into an adsorbent and then into the cold-trap followed by transfer to GC-column by increasing temperature.

Solid phase microextraction (SPME)

Analytes from gas or liquid phase can be extracted into a short coated silica capillary attached to a special microsyringe. After an equilibrium time, the SPME capillary containing analyte is transferred to programmed temperature injector of GC and the analytes are moved into the GC-column by thermodesorption. The main advantage of this technique is that it does not need additional solvents. Automation has become possible in recent years (e.g. LabHut autosampler).

Stir bar sorptive extraction

In stir bar sorptive extraction, the analytes are extracted from water phase (or biological fluids) into a coated magnetic stirrer. After the equilibrium time, the stir bar is put in a

thermodesorption unit to introduce the analytes for GC. For the HPLC-determination, the use of back-extraction of analytes is performed. The higher capacity makes this technique about 1000 times more sensitive than SPME. Automation is possible e.g. by Gerstel autosamplers.

Distillation

Distillation can be considered as simultaneous isolation, clean-up and enrichment method. The typical distillation techniques are (i) water or hydrodistillation in which the sample is immersed in water (ii) water and steam or wet steam, in which the sample is above the water surface but in same vessel and (iii) dry steam in which the water is boiled in a separate vessel and the steam is led through the sample in another vessel and (iv) simultaneous steam distillation-solvent extraction with the improved apparatus of Likens and Nickerson.

Extractives and steam are forwarded to the condenser. In the final step of the process, condensed water and water immiscible extractives containing the analytes can be separated.

Steam distillation with water is a classic and good way to extract easily volatile analytes, such as essential oils, from liquid or solid samples. However, the technique is not very suitable for extremely volatile or very heat-labile analytes. A drawback of the method is that it requires a relatively large sample amount. Perhaps the most common application of steam distillation is the Kjeldahl- method of nitrogen determination.

Clean-up methods

The sample extract might need clean-up procedures to remove co-extractive material. The clean-up can be done by liquids with or without the help of adsorbents or by liquid chromatographic methods. With all clean-up methods, the selectivity of the sample preparation will increase. Sometimes it is not clear if the step is an extraction or a clean-up procedure.

Liquid-liquid partition in an extraction funnel or in a test tube

Liquid-liquid partition is a traditional way to remove co-extractives, enrich the analyte and to transfer the analyte into an organic solvent suitable for chromatographic analysis. The most common cases in liquid-liquid extractions are:

(i) Transferring the analyte from the water phase into a water immiscible organic phase.
This process can be enhanced by salting out (e.g. pesticides) or lowering the pH (e.g. phenolic acids). Some organic solvents, such as ethyl acetate and diethyl ether absorb water, which has to be removed by a drying agent before evaporation of the organic phase. The polarity of the organic phase is chosen according to the polarity of the analyte, a series of organic solvent at increasing polarity may also be used (e.g. hexane, dichloromethane, diethylether and n-butanol).

(ii) Keeping the more polar analyte in the water phase and removing co-extracted non-polar material into a water immiscible organic phase.

(iii) Partitioning with two immiscible organic solvents e.g. methanol or acetonitrile and hexane. An example of this approach is separation of polar phenolic compounds in vegetable oils into methanol.

Depending on the sample and solvents used, shaking of the sample can be done vigorously and casually or with outmost care. When the partition has to be done very carefully to prevent the formation of emulsion, enough time should be given to the analyte to transfer to the other phase. Partition is commonly repeated two or three times. After shaking, the separation of the phases can be enhanced by freezing, centrifugation, addition of a portion of iso-propanol or saturated salt water. Sometimes increasing the amount of organic phase relative to the water phase may bring the unseparated phases apart.

Usually liquid-liquid partitioning is done in an extraction separation funnel or in case of smaller volume in a test tube. The water phase can be separated from the organic phase also with silicone treated filter paper (Whatman, Schleicher&Schuell) without using a separation funnel.

The benefits of a liquid-liquid partitioning are inexpensive hardware and the automation is at least partly possible. The disadvantages of this technique are that it is labour intensive, large volumes of solvents are used and selectivity is limited.

Merck´s Extrelut and Varian´s ChemElut cartridges

Prepacked Extrelut and ChemElut cartridges are designed to replace traditional liquid-liquid partition. They are especially good if the sample tends to form emulsions easily in the liquid-liquid-extraction. In practise, a portion of the water based sample containing the analyte, is bound into the adrorbent (Hydromatrix) and after an incubation time, the analyte is eluted from the cartridge with a water immiscible organic solvent. The cartridges are available in many different sizes.

Precipitation of proteins and some water soluble fibers in water phases

In water containing samples, such as in biological fluids, co-extractives can be problematic during liquid chromatographic analysis of the analytes. Carrez solutions I (potassium hexacyanoferrate) and II (zinc acetate) are traditional way to precipitate proteins from water phase. However, this technique may not be appropppriate for all analytes. Another suitable way to precipitate proteins is addition of acetonitrile or methanol followed by centrifugation. Addition of water miscible organic solvent precipitates also some water soluble fibers in cereal based samples such as wort.

Solid phase extraction (SPE)

For analytes in a water-based liquid matrix, such as water, juice, liquid beverages or biological fluids, solid phase extraction may be considered as a technique for isolating the analyte from the matrix. In the case of analytes in a solid matrix, SPE is used as a clean-up method after the analyte is separated from the matrix into organic and/or water based solvent. As a sample clean-up technique, small solid phase extraction cartridges have in many cases replaced the classic open glass columns packed with adsorbent. The advantages of SPE as a clean-up method are the relatively small amount of solvents required, good selectivity and combination of clean-up with analyte concentrating step.

The SPE-process can be enhanced by pressure (with nitrogen) or by vacuum. A classic way to use the SPE-cartridge is to attach it to a disposable syringe and just pass the liquid sample through the cartridge in order to trap as least part of the co-extractives. Depending on the analyte chemistry, matrix and sample volume, a number of sorbents packed in various size cartridges are available. Some of the more recent

developments in this field are integration of SPE to microsyringe (SGE´s MEPS Micro Extraction by Packed Sorbent), solid phase membrane disks developed for water analysis and SPE in 96-well plate form. Nowadays there are number of manufacturers which provide automated liquid handling and SPE systems.

Matrix solid-phase dispersion (MSPD)

Matrix dispersion extraction is a variation of the SPE -technique, in a typical extraction, a solid sample is mixed or homogenized with a sorbent and packed into a cartridge. The analytes are then extracted from the matrix by eluting the sample-sorbent with suitable organic solvents. A recent development of this technique has been made in the field of pesticide multiresidue analysis in the form of QuEChERS (quick, easy, cheap, effective, rugged & safe) technique. In this technique the sample extract in acetonitrile is mixed with combination of adsorbents, such as PSA and GCB, and salt in order to remove certain co-extractives. Recently, a number of manufacturers have started to provide these adsorbents in pre-packed test tubes for more convenient and efficient sample preparation.

Immunoaffinity columns

The use of immunoaffinity columns for sample clean-up are perhaps most typical in mycotoxin applications. Immunoaffinity colums have been developed for single compounds such as DON, or groups of compounds, such as fumonisins and aflatoxins or for several groups of compounds simultaneously. There have been rapid developments in recent years with several manufacturers operating in this field.

Gel permeation chromatography (GPC)

Gel permeation chromatography is clean-up method of compounds by size exclusion by porous material using organic solvents as a mobile phase. GPC has been used for the separation of lipids and smaller analytes, such as pesticides, in fat or oil containing samples. GPC can be done as a low pressure application with Sephadex LH-20 or Bio-Beads S-X3 gel or at more elevated pressures e.g.with PL-gel and Envirogel -columns.

Sephadex LH-20 is a dextran based gel and in addition to molecular size it can undergo also other interaction with the compounds. Unfortunately, the flow characteristics of this gel can be quite poor. Bio-Beads S-X3 gel is a polymer of styrene-divinylbenzene and it can be used at low pressure separations. The main drawback of this gel, in addition to its softness, is its different swelling capacity in different solvents, and thus switching the solvents must be done with caution. The rigid polystyredivinylbenzene based gels, PL-gel and Envirogel, are suitable for operation at higher pressures and these gels are packed as HPLC-columns.

Flash chromatography

Flash chromatography was developed from open column chromatography for a faster separation and clean-up technique. The columns used are typically silica or C-18 and the system is operated at low pressures. In recent years, there has been major development in instrumentation and automation, also the number of manufacturers has been increased.

Semipreparative HPLC and multi-dimensional HPLC

In some cases the most selective technique has to be used to separate the analyte from the co-extractives prior to the final determination. Semi-preparative HPLC is an expensive technique, and in routine work it is economically justifiable only for special applications with a relatively high number of samples. Automation is possible

to some extent. The use of multidimensional HPLC with heart-cutting technique is sometimes needed in the case of very complex materials.

Enrichment of the analyte

The analyte in the solvent from the extraction or clean-up step is usually present at too low concentrations to permit direct determination. In order to achieve a lower detection limit, the analyte needs to be concentrated i.e. the volume of solvent needs to be reduced. However, there are some exceptions such as sugars and organic acids in vegetables which are present at such high concentrations that after the extraction, no enrichment is needed prior to HPLC -analysis.

Evaporation of the organic solvent is usually done in test tube volumes under a stream of nitrogen. Larger solvent volumes are usually evaporated with a rotary evaporator at reduced pressure in a warmed water bath. Multisample evaporators are available e.g. from GeneVac, Caliper and Buchi. For very polar compounds, it is recommended to silylate the glassware that comes into contact with the sample in the evaporation step. Especially if evaporation is done to complete dryness, polar analytes may adhere onto glass surfaces resulting in poor recoveries and high variations. In addition to concentrating the analyte, evaporation of the solvent may be needed for the chromatographic determination of the analyte. For example prior the RP-HPLC analysis of phenolic acids, the ethyl acetate-diethylether is evaporated to dryness and the sample is dissolved in a small amount of methanol.

Water samples preferably without organic solvent or volatile organic acid residues can be freeze-dried. Film evaporators and spray drying can be used for water based samples. Enrichment of the analyte can be also done by precipitation and filtration. Examples of this technique are precipitation of fiber component from buffer solution by addition of ethanol and precipitation of phospholipids by acetone at 0–4°C. In headspace extraction, the analytes are enriched from the "extraction gas" by an adsorbent such as Tenax and cryo-trap. A special enrichment method is a large-volume-injection in GC in which the evaporation of the organic solvent is achieved in the temperature programmable injector.

Derivarization or modification of the analyte for the determination

The need of derivatization depends on what kind of analytical instruments the laboratory has at its disposal, e.g. GC equipped with FID, MSD, on-column or PTV-injector, HPLC equipped with DAD, FLD, CAD, RID or MS. Derivatization of the compound may also be needed to achieve higher instrumental sensitivity. In derivatization prior to gas chromatographic analysis, the functional group(s) of a polar and poorly volatile analyte can be modified e.g. by addition of trimethylsilyl, methyl, acetyl or pentafluorobenzyl group.

In liquid chromatographic analysis, the derivatization of the analytes may be either a pre- or post-column process. Examples of the pre-column derivatization are transformation of ascorbic acid into quinoxalline derivate by OPDA and conversion of primary amino acids into a fluorescent form by OPA/thiol reaction. Post-column derivatization takes place before the analytes reach the detector, and it always requires a special set of equipment.

Some examples of the enzymatic modification of the analytes are desulfonation of glucosinolates, and conversion of ascorbic acid to its dehydroform prior to

derivatization. Enzymatic hydrolysis sets some limit on the method and the conditions such as pH, buffer content and temperature must be controlled carefully.

The analyte can be also changed into a conjugate with visible colour or UV-absorbing properties for spectrophotometric determination.

Final preparation just before analysis

In the final stage of the sample preparation procedure the sample-extract is in a liquid form in appropriate organic solvent. If the quantification of the analyte is done by an external standard method or if the internal standard is added at the final stage, the final volume of the sample-extract must be exact. In the latter case, the internal standard acts more as an injection control standard. In the case that the internal standard has been added at the beginning of the sample preparation, the exactness of the final volume is not so crucial.

For HPLC analysis, the sample extract is passed through a disposable 0.2 µm or 0.45 µm membrane filters attached to a disposable syringe into a sample vial. Depending on the consistency of the sample-extract, i.e is it organic or aqueous based solvent, different kinds of membranes have to be used. In order to reduce costs, sample extracts may be centrifuged instead of being subjected to membrane filtration.

In the case of the GC -analysis (excluding the on-column injection) the presence of small particles in the sample extract is not so problematic and the sample can be injected into the GC -system without filtration. Some samples however, may need centrifugation to clarify the liquid before injection.

Concluding remarks

It has to be kept in mind that no matter how good the method is, an elaborated sampling and a careful pre-treatment of the samples are the basis of whole the analytical work.

Deep understanding why certain steps are involved in the method and what happens to the analytes in each particular step is important. This understanding also allows one to make modifications to methods whenever they are justified.

When a properly validated method has been taken into use in the laboratory, the function of the method has to be checked all the time according to the quality assurance and quality control procedures. Safety issues of the method, starting from the reading of safety bulletins and using the proper protection gloves, can never be overemphasized.

Even the best sample preparation methods have certain life span. The pros and cons of the methods in use have to be re-evaluated from time to time, since new faster and more economical sample preparation methods and/or instrumental techniques are emerging rapidly on the market. ●

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4
Chromatography specific

Potential of multidimensional chromatographic systems in sample preparation

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Introduction

The determination of the chemical composition of complex samples is a challenging task, owing to myriad of different species of compounds, many of them present in only trace amounts. Frequently, chromatographic techniques are exploited in the analysis of complex samples, after suitable form of sample preparation prior to the separation step (1, 2). It should also be noted that chromatographic methods combined even with mass spectrometry do not allow the simultaneous separation of a large number of compounds in a complex sample in single run. The selectivity can be improved by removing disturbing matrix compounds. To achieve this goal, complex, multistep sample preparation procedures are typically needed, including steps such as extraction, fractionation/clean-up and concentration. Especially when the analytes of interest are present at a trace level and several other disturbing compounds of similar physico-chemical characteristics are also present, very careful fractionation is needed. The fractionation is typically done by manual multistep procedures, which is not only time-consuming and tedious but also increases substantially the risk of contamination, losses and alteration of the sample. Recently, multidimensional separation techniques

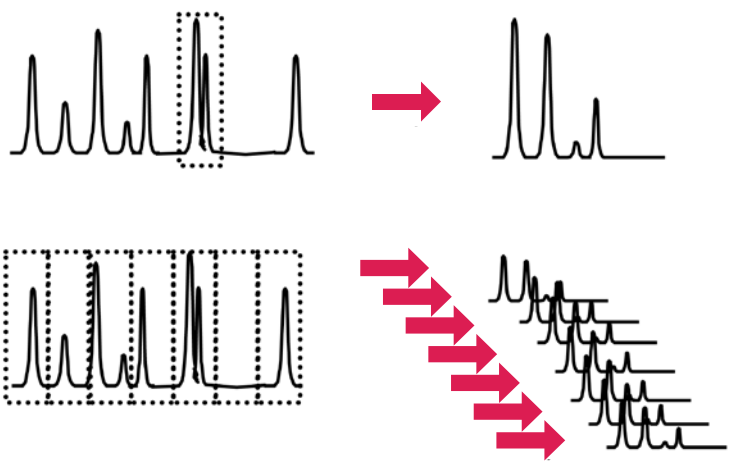
that can solve many of the problems related to sample preparation, separation and identification of analytes in complex samples, have attracted more and more attention.

Multidimensional chromatography can be performed both in off-line and on-line modes. The basic idea is to transfer a fraction or fractions from one chromatographic column to a secondary chromatographic column for further separation. The effluent of the first separation system can be transported to the next system manually (off-line) or automatically, e.g. with the aid of a robot (at-line), or via connecting tubing and/or a valve that directly transports the effluent stream to the next system (on-line). In general, the on-line combination of separation systems enables a significantly faster analysis of a complex matrix in comparison with an off-line or at-line combination. Multidimensional techniques can be used for further resolution of complex mixtures that cannot be separated entirely in a single column, for sample cleanup by removing matrix or interfering compounds, for increased sample throughput, and for trace enrichment of minor compounds of interest.

Multidimensional chromatographic techniques can be divided into two categories, namely heart-cut and comprehensive techniques. In the heart cut mode, only one or a few fractions are transferred into the second dimension separation while in the comprehensive mode, the whole sample passes through both separation dimensions. The difference is illustrated in Figure 1. The most popular version of multidimensional chromatography is two-dimensional (2-D) chromatography in heart cut mode (e.g. LC-LC). Recently also comprehensive 2D gas chromatography (e.g. GCxGC) has gained wide popularity. The technique has tremendous separation power, uses simple robust hardware, and provides similar analysis times to temperature-programmed high-resolution capillary chromatography. On the other hand, comprehensive 2-D LC (LCxLC) is still in its infancy and is more complex to perform. However, it is driven by user needs, especially in proteomics, and the topic is attracting more attention. In addition, the more traditional heart-cut GC-GC has been gaining more popularity again with the emergence of new technologies that make the instrumental operation more simple. In addition on-line coupled liquid chromatography-gas chromatography (LC-GC) is a valuable tool for the analysis of many types of complex samples.

Figure 1.

The difference between the heart-cut and comprehensive techniques.



Techniques

In the next chapters, the different techniques will be briefly described. More detailed information can be found in recent reviews (3–12) The selection of the proper methodology is dependent of several parameters, including type of analytes, type of matrix, concentration of the analytes, number of samples and goal of the analysis (target vs. comprehensive profiling). Figure 2 gives basic guidelines for the selection of proper multidimensional method and Table 1 summarises features of the sample preparation required for the different techniques.

Figure 2.

Selection scheme for a suitable multidimensional technique.

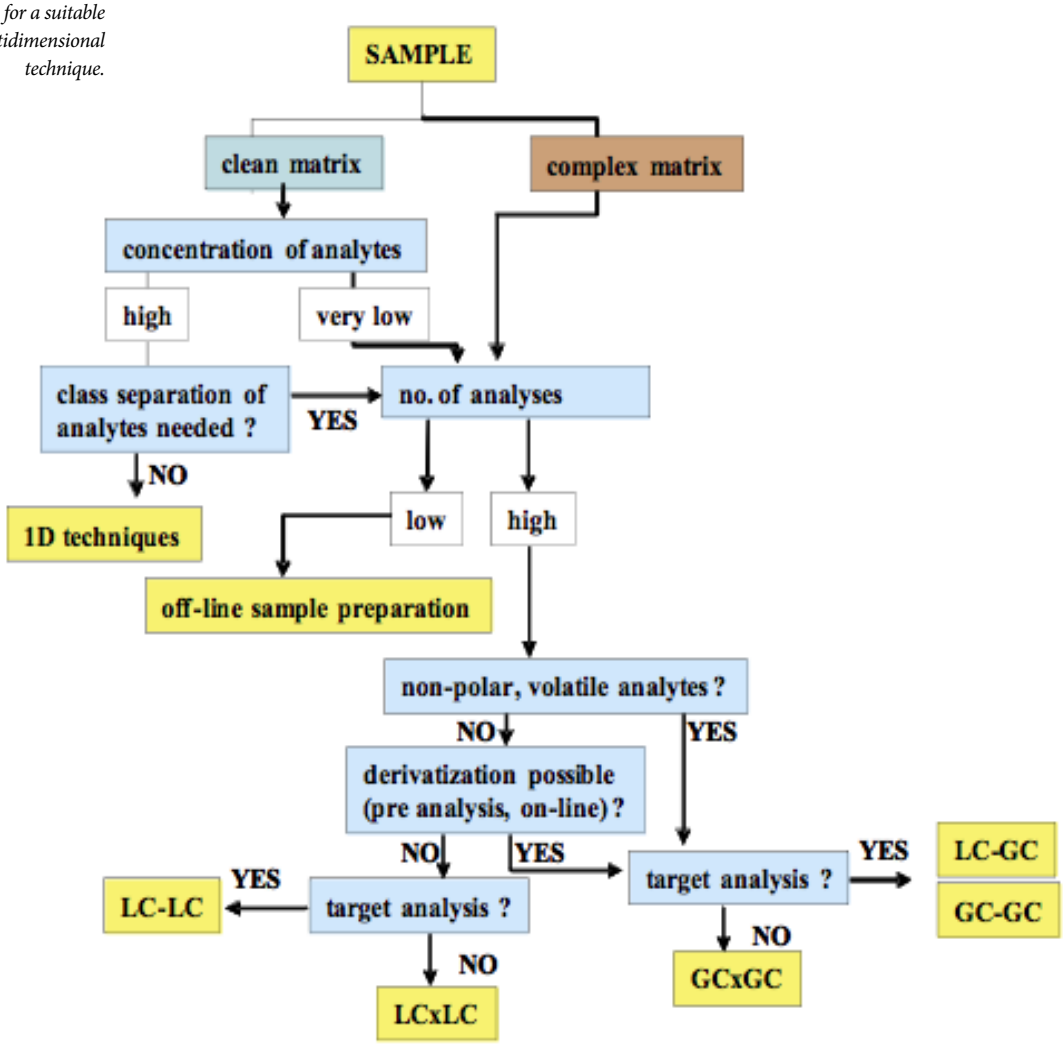


Table 1. *Summary of multidimensional chromatographic methods.*

Technique	*Analysis type*	*Sample requirements*	*Sample preparation prior the analysis*
LC-LC	target	Samples should be only in a suitable solvent	Liquid samples; often filtration, for solid samples, extraction into suitable solvent
LCxLC	profiling	Samples should be only in a suitable solvent	Liquid samples; often filtration, for solid samples, extraction into suitable solvent
LC-GC	target	Samples should be only in a suitable solvent	Liquid samples; often filtration, for solid samples, extraction into suitable solvent
GC-GC	target	Sample should be in a suitable solvent for GC, sample should not contain of nonvolatile material	Liquid and solid samples: extraction into organic, volatile solvent, removal nonvolatile material
GCxGC	profiling	Sample should be in a suitable solvent for GC, sample should not contain of nonvolatile material	Liquid and solid samples: extraction into organic, volatile solvent, removal nonvolatile material

LC-LC and LCxLC

Liquid chromatography is a versatile analytical technique that is amenable to a wide range of analytes, ranging from small molecules to very large biopolymers, and from very nonpolar to very polar compounds. In the quest for more separation efficiency for liquid chromatography, two-dimensional liquid chromatographic techniques (2D-LC) have been developed (5–8). Both heart-cut LC (LC-LC) and comprehensive two-dimensional liquid chromatography (LCxLC) are possible. The LC-LC approach is particularly suitable for targeted compounds and typically, the first column is used as a sophisticated sample preparation system. In the comprehensive mode (LCxLC), all fractions pass through the whole analysing system. The 2D-LC techniques utilise the several LC modes available, including reversed-phase liquid chromatography (RPLC), normal phase liquid chromatography (NPLC), ion exchange chromatography (IEC), ion pair chromatography (IPC), size exclusion chromatography (SEC), affinity chromatography (AC) and hydrophilic interaction liquid chromatography (HILIC). Liquid samples can often be injected into 2D-LC with virtually no sample pre-treatment due to the high selectivity of the methodology.

A typical on-line 2D-LC system consists of two pumps, two columns, an injector, an interface/modulator and a detector. The two columns are connected by means of the interface which usually is a high pressure switching valve. The instrumentation in both heart-cut and comprehensive modes is very similar. The 2D-LC systems can be easily constructed using conventional commercial high-pressure liquid chromatographic (HPLC) set-ups equipped with extra pump(s) and column(s) and an automated switching valve. For heart-cut mode, commercial systems are available.

In 2D-LC, the selection of the separation mechanisms, i.e. column materials and mobile phases in both dimensions must be chosen carefully so that separation in each dimension is based on a different mechanism. Various combinations of RP and NP, ion exchange, and/or size exclusion chromatographic techniques can be utilized in 2D-LC. In these combinations, the separation is based on the differences in the structural properties of separated compounds, such as size, polarity and shape, or the specific charge of ionic compounds. For example, the polar separation selectivity, which is the fundamental mechanism in NPLC, is based on the differences in the selective forces controlling the retention, such as the dipole–dipole or proton-donor/acceptor interactions, which depend on the combination of the stationary and the mobile phases used. The retention in ion-exchange systems is generally controlled by electrostatic ion-exchange interactions, but can be more or less affected by non-ionic interactions with the stationary phase matrix. Also the compatibility of the mobile phases in the two dimensions is essential in 2D-LC. The analytes should be more strongly retained in the second than in the first dimension to avoid excessive peak broadening and poor resolution in the second dimension. Therefore, the mobile phase used in the first dimension should have low elution strength in the second dimension. This is required because usually relatively large sample volumes will be collected and injected into the second column and thus, to minimize peak broadening, this sample should be focused at the inlet of the second column. Therefore, the effluent must be weak compared to the initial mobile phase composition used in the second column. Of course, the compatibility of the mobile phases in the first and second dimensions should also be considered – mainly mutual miscibility, solubility of analytes, adsorption of mobile phase components and viscous fingerprint. In addition, the second-dimension eluent must also be compatible with the detection system. Table 2 shows the compatibility of different LC modes.

In practise, the separation selectivities in different systems can be more or less correlated, due to the mixed character of the stationary phase and mobile phase interactions. This must be taken into account when developing real world 2D-LC separations.

Table 2. *The compatibility of different LC modes.*

	NPLC	*RPLC*	*IEC/ICP*	*SEC GPC*	*GFC*
NPLC	+++	+	+	+++	+
RPLC	+	+++	+++	+	+++
IEC/IPC	-	+++	+++	-	+++
SEC GPC	+++	+	+	+++	+
GFC	+	+++	+++	+	+++

Explanations:
+++ easy to combine
++ relatively easy
+ possible, but not convenient
- difficult

Heart cut LC-LC

The interfacing in on-line LC- LC systems can be divided into two classes: trapping via a loop or direct introduction on the top of the column of the second dimension, both in combination with one or more valves. Most separation modes can easily be combined if their mobile phases are compatible. However, combination is not always possible. The interfacing of NP and RP systems is particularly difficult, due to mobile phase immisciblities. In LC-LC, the first column is often short, and the second column is a long, high resolution column. The reason for this is that often the aim of first dimension separation is to separate the fraction containing the target analytes from the matrix and thus, the separation does not have to be highly efficient in this step. The final separation is then done with the second column. A typical example is the determination of drugs in a biological sample. The matrix compounds, such as proteins, uric acids, etc. in this type of determination are of no interest but they may interfere with the analysis and identification of the drug compound in a one-dimensional analysis. Moreover, other drug metabolites and small molecular mass compounds might also be of little or no interest. In LC-LC, the first column is used for the separation of matrix compounds from the fraction containing the drug(s) of interest, and this fraction is then transferred into the second column for final separation. It should be noted that off-line LC-LC is also a common approach. In the off-line mode, fraction collection is used after the first dimension, and then the collected fractions are injected into the second dimension using a normal LC injection system. These approaches are preferred when samples are extremely complex but available in a relatively large amount (e.g., plasma). Off-line separations also offer greater flexibility than on-line separations and can lead to an overall increase in separation power because each separation dimension can be independently optimized.

In most LC-LC applications, the most typical combination is RPLC and ion-exchange or another RPLC mode. Although in principle, the combination of RPLC to another RPLC separation does not meet the requirement of two different selectivities, in practise by careful selection of stationary phases and eluents, highly efficient separation can be achieved. In addition combination of restricted access column (RAM) with RPLC is usual, although this type of combination resembles more on-line SPE-LC because the first column is typically not a high resolution column. However, RAM-RPLC is a highly useful combination, particularly in bioanalysis, as it is possible to inject serum directly onto the column.

Comprehensive LCxLC

In LCxLC, several instrumental setups are available, but in most cases 8-port or 10-port switching valves have been used. The loop interface is the most widely used interface. It is based on a two-position 10- or 8-port switching valve equipped with two storage loops with identical volumes. Other configurations include dual second dimension columns, packed column interface and stop-flow interface. The interface ensures the collection of the first dimension effluent in aliquots of predefined volumes and enables transfer of these fractions on the secondary column in an automated way. The low flow rate employed in the first dimension enables the filling of one sample loop and at the same time flushing the second one by fast flow rate into the second dimension within one valve cycle. Prior to the introduction of a successive fraction onto the secondary column, the analysis of the previous fraction should be finalised. Thus, the second dimension analysis time should be equal (or less) than the duration of a modulation period. This sample cycle, i.e. the modulation time or sampling frequency is thus determined by the analysis time of the second column. To maintain the

separation achieved in the first dimension, a sufficiently large number of fractions (3–4) have to be taken from a peak eluting from the first column. Consequently, fast 2D analyses are essential in LC×LC. In addition, the speed of the second dimension is a key feature of successful separation and the separation should be as fast as possible, with sufficient resolution. Preferably, the second dimension analysis time should not exceed ca. 120 seconds. The second dimension separation time is the modulation time. The first dimension separation can then be optimized, with the flow rate optimized taking into consideration the modulation time. For example, if the second dimension separation time is 60 seconds, the flow rate in first dimension should be less than 0.1 ml/min, otherwise the volume of the fraction is too large. In LCxLC, on the other hand, the first column is long and is operated at lower flow rates than the second column which is short and employs a high-speed mobile phase. LCxLC is best suited to the profiling of complex mixtures, such as biological or food related samples.

In most LC×LC applications reported, various combinations of RPLC and NPLC, IEC, or SEC modes have been utilized. Since the mobile phases used in RP×RP, IEC×RP, RP×SEC or NP×SEC 2D-LC separation systems are usually fully miscible and have similar physicochemical properties, these are, in practice, the most commonly used combinations. For example, combinations of IEC-LC and RPLC with either salt concentrations or pH gradients can be used for separations of ionic compounds, acids or bases. The combination of SEC with either NPLC or RPLC is useful for the separations of macromolecules, such as synthetic polymers and biopolymers. An NPLC×RPLC combination is more demanding, due to eluent incompatibility, and is useful for the separations of samples with two or more types of structural elements differing in lipophilic and polar properties. For polar sample compounds, HILIC can be a useful mode of separation in the second dimension, using stationary phases that are less polar than silica gel (e.g. aminopropyl silica) because they allow the use of mobile phases containing up to 70 % water. Since the mobile phases used in RP×RP, IEC×RP, RP×SEC or NP×SEC systems are usually fully miscible and have similar physicochemical properties, these are in practice the most commonly used combinations. For example, combinations of IEC-LC and RPLC with either salt concentrations or pH gradients can be used for separations of ionic compounds, acids or bases. The combination of SEC with either NPLC or RPLC is useful for separations of macromolecules, such as synthetic polymers and biopolymers. An NPLC×RPLC combination is more demanding, due to eluent incompatibility, and is useful for separations of samples with two or more types of structural elements differing in lipophilic and polar properties.

LC-GC

On-line coupled liquid chromatography-gas chromatography (LC-GC) is an excellent example of the potential of multidimensional techniques (9,11). It combines the best features of liquid and gas chromatography and offers also further advantages over traditional methods. Typically, the LC part is used for sophisticated sample clean-up, fractionation and/or concentration. The benefits of LC are large sample capacity and the flexibility and selectivity of the analysis; the conditions can be easily adjusted on the basis of sample type. Capillary GC, in turn, is much more efficient in separation than LC, in addition to having many sensitive detectors available. An additional benefit of the coupled LC-GC is that, because of the efficient clean up by

LC, the whole sample fraction containing the analytes can be transferred to the GC. Since none of the sample material is wasted and the disturbing compounds are effectively eliminated, sensitivity is high. If necessary, several fractions can be injected to GC, using a stop-flow mode in LC. If fast GC is used, even comprehensive LCxGC is possible. Naturally, the combination of LC and GC can be done in off-line mode, as in LC-LC by using fraction collection. The sensitivity of this approach is, however, not as good as in the on-line mode. On the other hand, the solvent suitability is not as critical in the off-line mode, particularly if only a small volume injection is used.

LC-GC is best suited to heart-cut analyses of target compounds, when one or a few fractions in a very complex matrix is being analyzed, although it is also possible to perform comprehensive analysis of the whole sample using a LCxGC technique. Naturally, the target analytes should be suitable for the final GC analysis, i.e. they should be sufficiently volatile and non-polar or derivatisation should be possible either before the analysis or on-line. The main advantage of LC-GC over e.g. GCxGC is that it tolerates very dirty sample matrices, i.e. samples containing polar, non-volatile components as impurities. Liquid samples can often be injected to LC-GC with virtually no sample pre-treatment, while solid samples will require extraction to release the analytes from the matrix.

The role of LC in the LC-GC coupling is to perform selective clean-up, concentration and/or fractionation of the sample. In LC, the eluent should be suitable for the GC analysis. If the eluents are carefully chosen, the organic solvents used in normal-phase LC usually are not a problem. Reversed phase eluents, on the other hand, demand more complex solutions. In part, this is because the organic eluents used in NPLC are typically compatible with GC, making the coupling simpler. Another reason is that many of the samples analyzed by GC require extraction into organic solvent before analysis, and normal phase separation is the obvious choice.

A special interface is needed for coupling of LC with GC because the LC fractions to be transferred to the GC are typically several hundreds of microlitres while the conventional GC allows just a few microlitres. A number of interfaces have been developed for the (NP)LC-GC coupling, of which on-column, loop-type and vaporizer interfaces are commonly employed today (11). The on-column and loop-type interfaces are usually applied with retention gap techniques or concurrent solvent evaporation techniques, respectively, for the evaporation of the solvent. The choice of the interface and the evaporation technique depends on the application, the main parameters to be considered being the volatility of the analytes and the sample volume. For volatile analytes, on-column interface utilizing retention gap techniques is the best option. For less volatile analytes, the loop-type interface with concurrent eluent evaporation and the vaporizer interface can be applied. It should be noted that the coupling of RPLC to GC demands skill and special techniques since aqueous RPLC eluents are unsuitable for direct transfer to GC and the interfacing techniques used in NPLC-GC generally do not work well for RPLC-GC. There are two ways to solve the problems related to RPLC-GC coupling (4, 6). Direct solutions to the problem of aqueous eluents rely on special techniques, whereas indirect solutions avoid them by phase switching, i.e. replacing the water with suitable organic solvent before introduction to GC.

GC-GC and GCxGC

Comprehensive two-dimensional gas chromatography (GC×GC) is a powerful analytical technique which is an excellent choice for the separation of volatiles and semivolatiles in complex samples. The practicability of heart-cut mode of multidimensional gas chromatography (MDGC or GC–GC), was introduced several decades ago and it has convincingly been applied to the analysis of a variety of sample types. The main limitation of the MDGC approach is that only one or, at best, a few small fractions eluting from the first column are selected for further separation in the second column. In other words, the approach is successful in a target-analysis situation. For the analysis of the whole chemical profile (of semivolatiles/volatiles) GC×GC approach is a better choice. It should be noted that even though the 2D-GC systems reduce the amount of sample preparation compared to conventional methodologies, typically quite thorough sample preparation is still needed. The samples should not contain any involatile material which can cause problems in GC.

Heart-cut GC-GC

In a similar way than in 2D-LC, the heart-cut GC-GC is typically used for target analyses. The role of MDGC is clearly to target a certain poorly separated region and provide increased resolution. By selection of a heart-cut during a given region of a chromatogram, the components of interest are transferred to a second – more selective – column, whereupon the components are better resolved. This case is shown in Fig. 1. Here, two regions of unresolved components are selectively transferred to a second column, where they are now completely resolved. A major application area of MDGC is chiral analysis; the typical approach will be to use a conventional column in the first dimension, and a chiral column in the second dimension. When correctly employed, a single (unresolved) peak can be transferred and column 2 provides baseline resolution of the enantiomers, without any interfering overlapping peaks. Several devices that offer different mechanisms to the heart-cut process, such as the Live-T switching device, the Gerstel column switching system and the moving capillary stream switching (MCSS) device have been available already for decades. The use of MDGC has, however, been rather limited mainly due to complex instrumentation. Recently, the interest in MDGC has been growing due to improvements related to the field of microfluidics and this has given a new lease of life to MDGC (14). MDGC has become a more 'user-friendly' technique due to improved column connections, superior pressure/flow control, and the support of computer software to aid calculation of column dimensions and experimental conditions.

Comprehensive GCxGC

In GCxGC, the entire first-column effluent, cut into small adjacent fractions to maintain the first-dimension resolution, is subjected to further analysis on the second-dimension column. The instrumentation for GCxGC is simpler than for MDGC. The two columns are connected to each other with a simple column connector (e.g. press-fit) and typically the two columns are in the same oven. The modulator, which traps and transfers the fractions from the first column to the second one is placed in the beginning of the second column. The interface between the two GC columns, i.e. the modulator, should (i) accumulate and trap, (ii) refocus, and (iii) rapidly release, the adjacent fractions of the first-dimension column. At present, the modulation is based

on cooling e.g. with liquid N_2 or CO_2. Typically a non-polar stationary phase is used in the first dimension column in GC×GC while the second column has a (semi)polar stationary phase. It is also possible to use shape selective stationary phases, phases for enationmer separation or other special phases. The advantage of non-polar-polar column combination is that much information is available on the behaviour of a huge number of (classes of) compounds in non-polar columns in conventional 1D-GC and this can conveniently be used to optimize the first-dimension separation. With nonpolar stationary phase, the separation is mainly based on volatility of the compounds and, consequently, a boiling-point separation is obtained. With a non-polar first dimension column, in each individual narrow fraction analytes with mutually closely similar volatilities will elute from that column. In the second dimension, the separation is very fast (typically 2–5 seconds) and it is isothermal. Thus, in the second-dimension separation, for analytes of equal volatility – i.e., the analytes in each individual fraction – there will be no boiling-point contribution in that dimension. Then only the specific interactions with the stationary phase will govern the retention and the separation is orthogonal. In other words, the two dimensions operate statistically independently and the entire 2D plane of the GC×GC chromatogram, the so-called separation space, is available for peak separation. One main benefit of orthogonal GC×GC separations is that ordered structures – i.e., continuous bands or clusters – now show up in most GC×GC chromatograms for structurally related homologues, congeners and isomers.

Combining extraction and chromatography

Modern analytical strategies tend towards automatisation and integration of sample pre-treatment in the chromatographic systems as far as possible. Ideally, sample preparation is integrated with the final separation and detection system. In practise, most of the sample preparation procedures are done off-line. However, many of the extraction systems can be integrated as an on-line system, where the whole analytical procedure takes place in a closed, usually automated system (Table 3). The benefits are the increased sensitivity and reliability because the sample clean-up in an on-line system tends to be more effective. The most common on-line systems are on-line combination of solid phase extraction with LC (SPE-LC), and automated commercial systems are available. These systems closely resemble LC-LC. Also SPE-GC is relatively easy to perform. Other extraction techniques utilised in on-line systems are liquid-liquid extraction (LLE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave assisted liquid extraction (MAE), sonication assisted liquid extraction (SAE) and membrane-based sample pretreatment, such as supported liquid membrane extraction (SLM) and microporous membrane liquid-liquid extraction (MMLLE). The various approaches for on-line coupling have been reviewed recently (15, 10).

Conventional off-line sample preparation is flexible, and several samples can then be prepared in parallel. However, the off-line methodologies are often very tedious and time-consuming, and risks for sample loss and/or contamination are high. Many of the problems with off-line systems can be avoided by developing an at-line or in-line methodology, in which the sample preparation is done e.g. with an. autosampler, such as is possible with microextraction by packed sorbent (MEPS), or with robots, such as automated SPE systems. The at-line and in-line systems improve the repeatability of the procedure,

Table 3. *Summary of the most common methodologies for combining extraction on-line with LC or GC.*

Method	*Sample preparation based on*	*Advantages/ disadvantages*	*Suitable for: matrix/analytes*
SPE-LC	adsorption in solid support	commercial instruments available, wide selection of sorbent materials, quantitative recovery	Liquid/polar, nonvolatile
Dialysis-LC	dialysis	commercial instruments available, relatively slow extraction, recoveries rather low	Liquid/polar, nonvolatile
SLM-LC	two-phase liquid- liquid extraction with supported membrane	very selective extraction, simple apparatus, relatively slow extraction, recoveries rather low	Liquid/polar, nonvolatile
SPE-GC	adsorption	Quantitative recovery, efficient enrichment, fast extraction, drying time-consuming, relatively complicated instrument	Liquid/nonpolar /volatile
MMLLE-GC	partitioning in liquid-liquid extraction with microporous membrane	miniaturised systems also available, selective extraction for ionisable compounds, simple apparatus, relatively slow extraction, recoveries rather low	Liquid/nonpolar /volatile
LLE-GC	partitioning in liquid-liquid extraction	no recent applications in literature, rather slow extraction, sample capacity limited, relatively complicated instrument	Liquid/nonpolar /volatile
SFE-LC	extraction with supercritical fluid	used for relatively nonpolar analytes, quantitative recovery, selective, matrix dependent extraction, efficient enrichment, complicated instrument	Solid samples/ polar, nonpolar, nonvolatile
DMAE-LC	dynamic microwave assisted liquid extraction	careful choice of extraction solvent required, complicated instrument	Solid samples/ polar, nonpolar, nonvolatile
DSAE-LC	dynamic sonication asssited liquid extraction	quantitative recovery, efficient enrichment, large sample capacity	Solid samples/ polar, nonpolar, nonvolatile
PLE-LC	dynamic liquid extraction at high temperatures and pressures	in principle possible, no applications in literature	Solid samples/ polar, nonpolar, nonvolatile
PHWE-LC	dynamic extraction with water at high temperatures and pressures	can be used even with fully aqueoues phase in both extraction and LC, quantitative recovery, efficient enrichment, not for labile analytes, large sample capacity, complicated instrument	Solid samples/ polar, nonpolar, nonvolatile
SFE-GC	extraction with supercritical fluid	Quantitative recovery, selective extraction, efficient enrichment, relatively complicated instrument	Solid samples/ nonpolar, volatile
DMAE-GC	dynamic microwave assisted liquid extraction	Quantitative recovery, efficient enrichment, relatively nonselective extraction, complicated instrument as requires SPE trapping due to polar solvents,	Solid samples/ nonpolar, volatile
DSAE-GC	dynamic sonication assisted liquid extraction	Quantitative recovery, fast extraction, relatively nonselective extraction, relatively simple apparatus as direct connection possible for miniaturised extraction systems,	Solid samples/ nonpolar, volatile
PHWE-GC	dynamic liquid extraction at high temperatures and pressures	requires an intermediate trap (SPE/membrane unit), Quantitative recovery, efficient enrichment, complicated instrument	Solid samples/ nonpolar, volatile

and less labour is required. However, like in off-line procedures, with the at-line systems only a fraction of the sample is transferred into final analysis and consequently, the detectivity is not as high as in on-line or in-line systems.

On-line sample preparation can usually be automated, increasing the throughput of the analysis in most cases. However, the on-line sample preparation is done in serial manner, and in some cases off-line sample preparation with large amount of parallel samples can be faster. The main advantage of an on-line sample preparation is the higher detectivity because typically the whole extract is transferred into the final analytical system. Further advantages of the automated on-line sample preparation which takes place in a closed system are decreased problems encountered with contamination, sample loss and possible degradation of the analytes due to air or moisture. The main disadvantage is the complexity of the on-line systems. The optimisation of the on-line system is also more demanding and less flexible than with off-line or at-line systems. Several on-line systems have been developed (16–24). For liquid samples, even commercial on-line systems are available, such as SPE-LC and dialysis-LC. Several other, relatively simple non-commercial on-line systems are available as well, including SLM-LC and MMLLE-GC. Also on-line SPE-GC systems have been developed; however, these systems are rather complicated. With solid samples, the situation is different. Only a few on-line systems have been described, and in most cases they are quite complex. Examples of on-line systems include SFE-LC, SFE-GC, PHWE-LC, PHWE-GC, SAE-LC, SAE-GC and MAE-GC. Since with solid samples, exhaustive extraction is usually required to release the analytes from the matrix, the extracts tend to be dirty, making the on-line transfer of the whole extract very difficult. In most on-line systems, clean-up or fractionation must be added in between extraction and chromatographic instrument, making the system more complex. Typically, SPE or MMLE is used as the clean-up step.

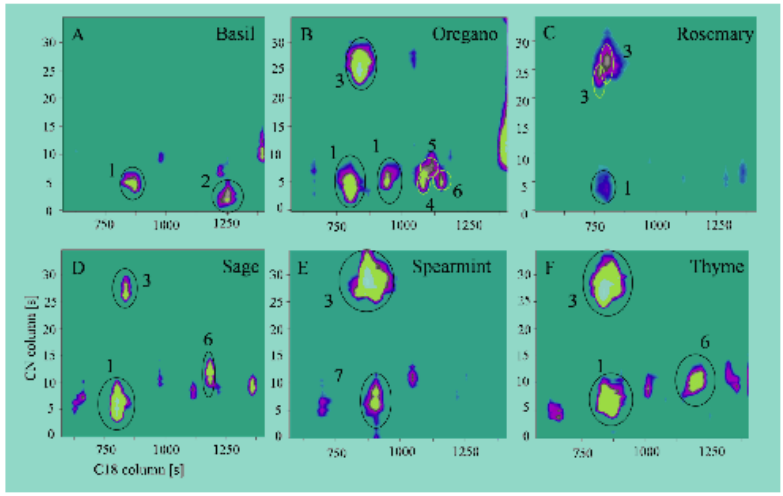
Selected applications

In this chapter, selected applications of the multidimensional techniques are highlighted. In tables 4 and 5, recent comprehensive two-dimensional applications are presented (25–59).

LC-LC systems have been used in various bioanalytical industrial and environmental applications, and in the analysis of food samples (60). Often, the only sample preparation needed for liquid samples prior LC-LC is a simple filtration. A typical example is the analysis of drugs by a combination of RAM and RPLC. In a recent application, a simple, rapid RAM-RPLC-MS method was developed for the determination of antiretroviral drugs in rat serum and urine (61). The samples could be directly injected into the RAM column which was used for the removal of proteineous part of biological matrix and trapping of drug analytes. The final separation of the fraction containing the target drugs was then carried out by the RPLC system. For very complex samples, more sophisticated separation is sometimes required. For example, in the determination of phenolic compounds in pyrolysis oils, off-line size exclusion chromatography was needed prior to the on-line LC-LC (62). Pyrolysis oils contain large amounts of high-molecular-mass lignin-derived compounds. In the first stage, the high-molecular-mass

Figure 3.

LCxLC analysis of herb extracts. For details, see ref. 70.



lignins were removed from the fraction containg phenols and other small molecules in an off-line step. Next, LC-LC utilising two different RPLC columns (cyano and C18) was utilised in the final separation for the removal of proteineous part of biological matrix and trapping of drug analytes first cyano column was undertaken for sample clean-up and pre-fractionation before introduction of the phenolic fraction to the C18 for the removal of the proteineous part of the biological matrix and trapping of drug analytes.

LCxLC has been employed in several applications in food analysis, including the characterisation of traditional Chinese medicines, the analysis of antioxidative compounds in herbs, beverages and olive oil (63–69). In most cases, RPLCxRPLC, RPLC-HILIC or NPLCxRPLC combinations have been used. An example of LC×LC-TOF-MS in quantitative analysis is phenolic acids in herb extracts and beverages (64). In these studies, a C18 column was used in the first-dimension separation and a cyano column or C18 in ion-pair mode in the second dimension. The benefit of the LCxLC for the herb analysis is clearly evident as shown in Figure 3 (70). Co-eluting compounds are separated in the second dimension making the results more reliable. For example, as can be seen from Fig. 3E, compounds 3 and 7 would coelute in the one-dimensional analysis of the spearmint extract but are well separated with the second, more polar cyano column. Visual comparison of the samples is also easy. Basil and rosemary are known to have a different chemical composition from the other herbs. A more complex LCxLC system was used for the separation of natural phenolic antioxidants in beer and wine (67). The first dimension separation was done with a C18 column and two parallel Zirconia Carbon columns were used in the second dimension. The combination of the two columns achieved great differences in separation selectivity in each dimension. High-temperature isocratic separation was employed in the second dimension of the comprehensive set-up, allowing improved fraction transfer frequency between the two dimensions and shorter 2D separation time in comparison to the earlier method.

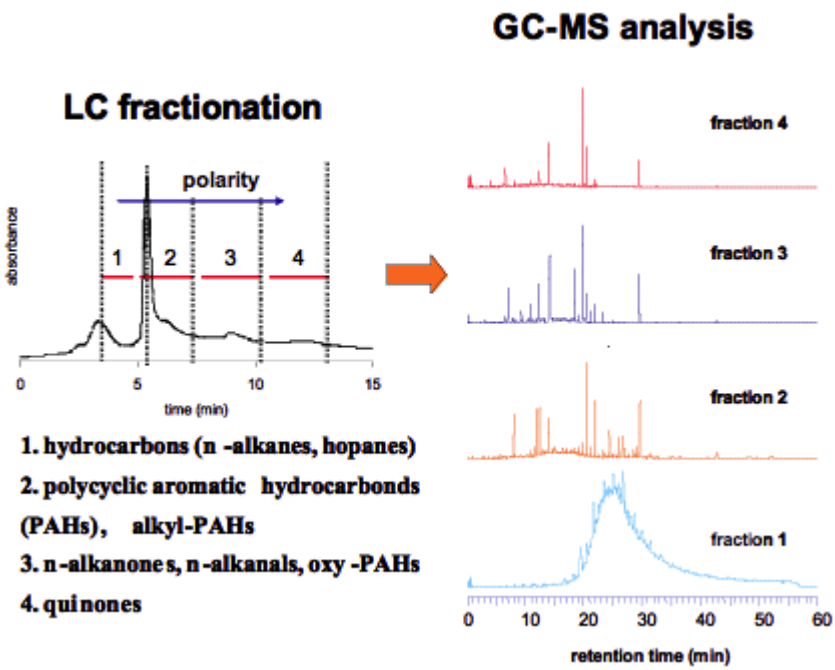
Most of the LC-GC applications involve the determination of target compounds or groups of compounds in a complex matrix, such as food, fossil fuel, agricultural, biological, environmental matrices. LC-GC has been widely applied in food analysis and particularly to fat-containing food matrices. These types of samples typically require lengthy saponication and clean-up before traditional (GC) analyses, making the method very tedious and time consuming. A major part of the sample pretreatment can be avoided by using on-line coupled NPLC-GC, where the LC is used for the separation of triglycerides from the analytes of interest. A good example of the separation power of NPLC-GC is the analysis of the origin of olive oils (71). In this case, the number of samples to be analyzed was high, and the traditional method was too laborious and the sample throughput was poor. The method that was developed to allow the analysis of sterols, triterpene alcohols and wax esters in a single run, something not possible by traditional methods. The ratio of the compounds acted as an indicator of the treatment of the oil (cold-pressed or extracted). The LC-GC method eliminated most of the manual preparation work and provided more information on the sample and excellent accuracy with substantially reduced analysis time (15-fold).

On-line coupled LC-GC is very useful when only a limited amount of sample is available, such as biological samples. Particularly, in the case of (living) patient tissue samples, the amount of sample is very limited and then the sensitivity of the method should be extremely high. In addition, often the number of samples is very high and automated methods are preferable. In a multi-centre control study of breast cancer, the concentration of DDE and PCBs was determined in subcutaneous fat, aspirated from buttocks of breast cancer patients and age-matched controls. The number of samples was >600 and the sample volume was limited to 200–800 µl of extract of the fat (72). Since the conventional methods would have been too tedious and displayed too low sensitivity, an on-line coupled NPLC-GC method was developed for the analysis of tissue samples. In this method, an aliquot of the tissue extract was injected to NPLC-GC without any pretreatment. The total analysis time was 80 minutes and the recoveries of target compounds were over 97 %.

It is also possible to combine extraction on-line to LC-GC thus further extending the possibilities of the system. An example of this type of system is on-line coupled SFE-NPLC-GC-MS which has been developed for the analysis of organic compounds in aerosol particles collected in filters (22–24). In this case, the system was not meant for routine analysis but for detailed characterization of the organic compounds in the particles. The aerosols consisted of a very complex mixture of compounds with very low concentrations and contained such a large number of analytes that it is difficult or even impossible to separate them in a simple analytical system. The idea of the SFE-LC-GC-MS system was to extract organic compounds from the filters with SFE, fractionate the extract with NPLC and then analyze the fractions in subsequent GC-MS runs. The whole procedure took place in a closed on-line system. It was also possible to include a derivatisation step with the extraction, which made it possible to extract and analyse of more polar analytes, such as carboxylic acids. The advantage of the on-line system over traditional GC-MS was that because of the efficient fractionation and highly sensitive on-line system, it was possible to use total ion monitoring in the MS. Typically, it has been necessary to use selective ion monitoring in MS because otherwise the analytes

Figure 4.

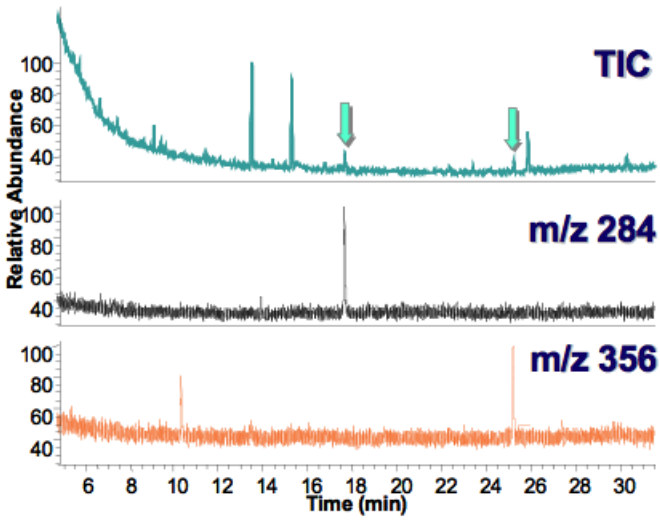
On-line coupled SFE-LC-GC-MS analysis of aerosol particulates. On the left, LC fractionation of the SFE extract and on the right, GC-MS separation of the four LC fractions. Note that the GC-MS chromatograms are not in the same scale, the scale in chromatogram of n-alkanes and hopanes is 10-fold smaller than in other three chromatograms (for more details, see ref. 23).



can not be identified due to the very high abundance of alkanes and trace level concentrations of other analytes in the particles. In addition, many of the compounds present in aerosols are reactive and it is beneficial to analyze the sample in a closed system where atmospheric oxygen and UV light are not present. Figure 4 presents the determination of organic compounds in aerosol particles by on-line coupled SFE-LC-GC-MS. As can be seen, the NPLC separation allowed accurate fractionation of the SFE extract into four main chemical fractions, the first fraction contained aliphatic compounds such as n-alkanes and hopanes, the second one polycyclic aromatic hydrocarbons and alkyl-PAHs, the third one n-alkanones, n-alkanals, oxy-PAHs and the last, fourth fraction contained quinones.

It is also possible to utilize pressurized liquid extraction in the on-line system where extraction is directly coupled to chromatography. In the determination of pesticides in grapes, pressurized hot water extraction was combined on-line with a membrane extraction unit which was further combined with gas chromatography-mass spectrometry (73). Grapes were placed in an extraction vessel and the extraction was performed with water at high temperature and pressure (120°, 20 bar). The extractant passed on-line through a membrane extraction unit, and the pesticides were extracted from the cooled water phase to an organic solvent (toluene) which was then directly injected into the GC using large volume on-column injection. This system provided very clean extracts and the on-line combination with large sample volume GC-MS

Figure 5.
PHWE-MMLLE-GC-MS analysis of pesticides in grapes. For details, see ref. 73.



gave an excellent sensitivity. Figure 5 shows a chromatogram of this on-line determination. Another recent application is the determination of phenolic compounds in herbal samples by on-line coupled ultrasound assisted extraction-liquid chromatography. In this system, the herb sample was placed into an extraction vessel which was placed into an ultrasonication bath. Extraction solvent (water:ethanol) passed through the extraction vessel, and the extracted analytes were then captured on a solid phase trap containing strong anion exchange material. From the solid-.phase trap, the analytes were eluted directly to a fast HPLC separation. The whole analysis took only 15 minutes. The system developed resulted in better sensitivity with shorter sample pretreatment and analysis times compared to off-line systems.

GC-GC has been widely used in the analysis of natural flavour extracts and concentrates, gasoline streams and environmental samples.(refs) In the analysis of essential oils, often the target analysis in MDGC is not directed to increased resolution of the majority of the sample, but towards specific components whose relative abundances may be of interest in order to study a particular aspect of the sample quality, history, source or biogenesis. Thus, MDGC provides the necessary separation to answer questions which cannot be addressed by single column analysis. An example of MDGC in the analysis of essential oils is the chiral separation in essential oils from Madagascar. Enantiomeric excess of five compounds – limonene, linalol, terpinen-4-ol, α-terpineol and citronellol – were reported (75) .The column set of choice for the chiral analysis was the first column set-up of DB-Wax coupled with a 2,6-dimethyl-3-O-pentyl-β-cyclodextrin – OV-1701 column for the separation of enantiomers of the first four components listed above, and the second column set-up of a 2,3-diacetyl-6-tert.-butyldimethylsilyl-β-cyclodextrin – OV-1701 column for the enantiomers of citronellol.

Table 4. *LCxLC applications.*

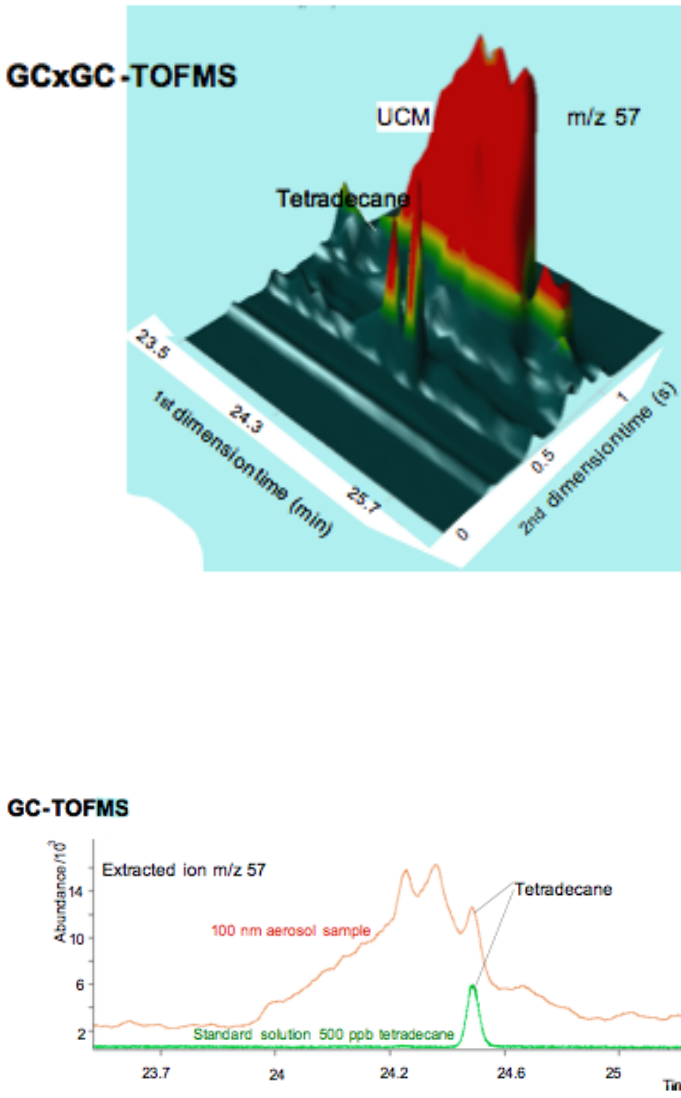
Application	*1. dimension: column, eluent*	*2. dimension: column, eluent*	*Ref*
Oligostyrenes	RPLC: C18, methanol	RPLC: Zirconia, acetonitrile	25
Plant extracts	RPLC: Cyano, methanol /H2O	RPLC: Monolithic C18, acetonitrile/H_2O	26
Herb extracts, phenolic acids	RPLC: C18, gradient of acetic acid and methanol	RPLC: Amino, water/ acetonitrile	70
Aromatic compounds	RPLC: Monolithic C18, tetrahydrofurane /H2O + 0,1 % acetic acid	RPLC: Monolithic C18, methanol /H_2O and acetic acid	28
Plant extract Stevia glycosides	RPLC: C18, gradient of water and acetonitrile	RPLC: Amino acetonitrile/H_2O	27
Aromatic and aliphatic compounds	RPLC: Amino,gradient of TFA and acetonitrile	RPLC: Cyano, trifluoro acetic acid + H_2O / trifluoro acetic acid + acetonitrile	29
Peptide purification	RPLC: C5, Gradient of acetonitrile and TFA	RPLC: C18 gradient formic acid, IPA, methanol, acetonitrile	30
Proteomics	RPLC: X-terra C18, Gradient of acetonitrile and TFA	RPLC: SB-Phenyl, Gradient acetonitrile, TFA	31
Orange essential oil and juice carotenoids	NPLC: Microbore silica, gradient of *n*-hexane and ethyl alcohol	RPLC: monolithic C18, gradient of 2-propanol, acetonitrile and water	32
Polymers: ethylene oxide-propylene oxide (EO-PO) (co)oligomers	RPLC: C18 Microbore, gradient of acetonitrile	NPLC: aminopropyl silica isocratic ethanol-dichloromethane-water	33
Tryptic peptides	IEC: PO4-zirconia, sodium phosphate gradient	RPLC: SB-C18, gradient of acetonitrile and TFA	34
Proteomics	IEC: PL-SAX, pH gradient	RPLC: PS-DVB RP, pH gradient	35
Proteomics	IEC: Polymeric beads bonded with diethylaminoethyl and sulfonic acid groups × Gradient KH2PO4	RPLC: C18, gradient of acetonitrile and TFA	36
Aerosols	IEC: Microbore SCX water (acetic acid) and acetonitrile gradient	RPLC: C18, gradient of water and acetonitrile	27
Peptides	IEC: Quaternary amine-SAX Stepped guanidine thiocyanate salt	RPLC: C18, acetonitrile gradient	37
Polymers: degradation product of poly(bisphenol A) carbonate (PC)	Alltech Platinum Silica, Chloroform	HSPgel-RT MB-L/M diethylether × chloroform	38
Polystyrene	Hypersil silica, Isocratic THF-hexane	Mixed-C, THF	39

GCxGC has been frequently applied to the analysis of petrochemical samples, food, environmental samples, biological and forensic samples (3, 4). Typically, the sample preparation steps can be minimised by using GCxGC. Fractionation is not needed prior to GCxGC analysis due to the very high separation power. In addition, also concentration steps are often not required due to the high sensitivity of the GCxGC. An example showing the high potential of the GC×GC is the determination of organic species in a rural atmosphere (76). In this study, a highly sensitive method was required, because a short sampling interval was required for the study of the nucleation of new particles in the atmosphere. Moreover, the low concentrations of organic species in this rural sampling site were very low. GC×GC–TOFMS with concentrating modulation improved the signal intensity, separation efficiency and MS spectrum quality relative to normal GC-MS analysis and allowed the identification of several compounds that had not been identified previously in aerosol particles. For example, in GC-MS analysis, it was not possible to identify sesquiterpene species due to their trace amounts and coelution. In addition, the spectral match was very low because of the many interfering fragments. In the GC×GC-TOFMS analysis, the concentrating modulation and the additional separation column in the second dimension improved the separation. As a result, the spectral match improved substantially. In addition to alkanes, over 50 compounds could be identified. Particularly, some volatile compounds that have been previously been found only in the gas phase could be identified with GC×GC-TOFMS in contrast to conventional GC-MS. In a recent study, GCxGC-TOFMS was compared with GC-TOFMS and GC-quadrupole-MS analyses in the determination of PAHs and alkanes in aerosol samples (77). The GC x GC-TOFMS proved to be the most sensitive chromatographic method, with limits of detection between 3- and 13-fold lower than those obtained with GC-TOFMS, and between 6- and 80-fold lower than with GC-QMS. Figure 6 shows the extracted ion chromatograms for n-alkanes (m/z 57) for the analysis of aerosol samples by the GC x GC-TOFMS and GC-TOFMS methods. In both chromatograms, it is possible to observe a region, called the unresolved complex mixture (UCM), containing mainly levoglucosan, which is a specific biomarker of biomass combustion. In the chromatogram obtained with GC-TOFMS, this region totally overlapped with the n-alkanes, preventing their identification and hence their quantification. The same overlap was observed for all the m/z ratios studied for the n-alkanes. Consequently, to achieve correct identification and quantification of all the n-alkanes with the GC-TOFMS method, it would be necessary to subject the sample to a pretreatment step – e.g. solid-phase extraction – prior to analysis by gas chromatography. With GCxGC-TOFMS, the UCM was separated from the n-alkanes, and reliable identification and quantification was easy.

The very high separation power of GCxGC is well demonstrated in the separation of PCBs in environmental samples. The complete separation of all PCBs (n = 209) and PCDD/Fs (n = 210) is a formidable task by conventional chromatographic methods. However, to obtain reliable toxic equivalents, it is sufficient to separate twelve WHO-PCBs and seventeen 2,3,7,8-substituted PCDD/Fs from each other and from other congeners present in the purified sample extracts. The task is further simplified by the fact that usually only 2,3,7,8-substituted PCDD/Fs are found in biota, owing to their

Figure 6.

Comparison of GCxGC-TOFMS and GC-TOFMS separation of aerosol samples, extracted ion chromatograms of tetradecane (m/z 57). For details, see ref. 77.



high persistence and biomagnification in comparison with non-2,3,7,8-substituted congeners. However, a complete separation is impossible using single-column GC. In a recent application, simple column chromatographic fractionation was used for the fractionation of the PCBs and PCDD/Fs according to their planarity, and the fractions were then analysed by GCxGC. Using a properly optimised GC×GC column assembly such as DB-XLB×LC-50, it was possible to completely resolve all WHO-PCBs and 2,3,7,8-PCDD/Fs from each other and from all other PCBs that were present in the same cleanup fraction (78).

Table 5. GCxGC applications.

Sample	*Columns*	*Detector*	*Ref*
Urban air / VOCs	50m x 0.53mm x 5 µm(BP-1) + 2.2m x 0.15mm x 0.2 µm (BPX50)	FID	40
Rural particles/SVOCs, VOCs		FID;TOFMS	76
Air / VOCs	30m x 0.25mm x 1 µm (DB-5) + 1m x 0.1mm x 1 µm (Carbowax)	FID; TOFMS	41
Roadside nanoparticles (29–58 nm)	30m x 0.25 x 0.25 µm (BPX5) c1.0 x x + 0.10 m x 0.10 µm (BPX50)	TOFMS&qMS; NPD&qMS	42
Urban aerosols	30m x 0.25 x 0.25 (SolGel-1) + 1m x 0.1mmm x 0.1 µm (BPX50)	TOFMS	43
Urban aerosols / semi-VOCs	30m x 0.2 x 0.5 µm (RTX-1MS) + 1 m x 0.1 mmx 0.1 µm (SolGel-WAX)	TOFMS	44
Urban aerosol / Partially oxidised organics	1.66 x 0.1 x 0.1 µm (DB-1701)	TOFMS	46
Urban aerosols PM 2.5	30m x 0.25 x 0.25 µm (BPX5) + 1.5 x 0.1 x 0.1 (BPX50)	TOFMS	45
PCBs	10 m x 0.1 x 0.1 µm (ChirasilDex) +1 x 0.1 x 0.1 µm (LC-50)	ECD	47
Toxaphenes	30 mx 0.25 mm x 0.25 µm (HP-1) + 1 m x 0.1 mm x 0.1 µm (HT8)	µ-ECD	48
Marine sediment / Pollutants	20 m x 0.25mm x 0.25 µm (DB-5) + 1 µm x 0.1m x 0.1 µm (BGB-1701)	TOFMS	49
Perfume	30 mx 0.25mm x 0.25 µm (SLB-5) + 0.75 x 0.1 x 0.1 µm (Swax10) LMCS	FID&Olf.;qMS	50
Essential oil rose geronium	30 mx 0.25mm x 0.25 µm (HP-5) +0.5 m x 0.32 mm x 0.25 µm (BP-20)	qMS	51
Grapes	60 mx 0.25m x 1 µm (Equity-5) + 2.5m x 0.1mm x 0.1 µm (Swax10)	TOFMS	52
Honey/volatile	30m x 0.25mm x 0.25 µm (DB-5MS) + 1.25m x 0.1mm x 0.1 µm (Swax10)	TOFMS	53
Roasted barley / Volatiles	30m x 0.32mm x 0.5 µm (DB-WAX) + 0.75m x 0.1mm x 0.1 µm (BPX50)	TOFMS	54
Can coating / Phenolic resins	30m x 0.25mm x 0.15 µm (PS-255) + 1.5m x 0.mm1 x 0.1 µm (SOP-50)	FID	55
Wine / Methoxypyrazines	30 x 0.25 x 0.25 µm (BPX5) + 1 x 0.1 x 0.1 µm (BP-20)	NPD; TOFMS	56
Olive oil / FAMEs	TWIN: 10 m x0.25mm x0.25 µm (BPX5) & 25m x 0.32mm x 0.25 µm (BP20) + TWIN: 1m x0.1mm x 0.1 µm (BP20) & 1m x 0.1mm x 0.1 µm (BPX-35)	FID	57
Diesel fuel Sulfur compounds	30m x 0.25mm x 1.0 µm (SPB-5) + 3m x 0.25mm x 0.25 µm (BPX50)	SCD	58
Oil spill	3.5m x 0.3mm x 0.1 µm (007-2) + 1m x 0.1mm x 0.14 µm (007-CW)	FID	59

Conclusions

Chromatographic analysis of samples from biomedical, petrochemical, environmental, and natural products as well as food and other important entities pose great challenges. In particular, rapidly growing areas in the life sciences, including proteomics, peptidomics, metabolomics, and glycomics, have to deal with biological samples of extraordinary compositional complexity. Effective sample preparation plays a crucial role in the analysis of this type of complex samples. The conventional methodologies, i.e. combination of conventional sample preparation and one-dimensional chromatography, are often inadequate for providing satisfactory separation of the thousands of components present in these samples. One new trend in many areas, and especially in molecular biology, is to move from target analyses to a type of profiling. In target analyses, the goal is to identify and quantify a few compounds in a sea of unknowns which are not of interest. In profiling, the whole sample is of interest and different approaches are needed in sample preparation and analysis. Multidimensional chromatographic systems are appropriate for the separation of very complex samples and often, tedious sample preparation can be avoided with the multidimensional techniques. The heart-cut methods are well suited to target analyses and of these, the LC-LC methods are already in wide use for a wide variety of applications. LC-GC and GC-GC methods are somewhat more complex, but these techniques have proven to be very useful, particularly in food related applications. Of the comprehensive two-dimensional techniques, the popularity of GCxGC has been rapidly growing. The instrumentation in GCxGC is simple to use and it offers very high separation efficiencies. LCxLC is still a relatively novel technique but it can be expected that the development of the technique will be continued in the future. ●

Abbreviations

DMAE	Dynamic microwave-assisted extraction
DSAE	Dynamic sonication-assisted extraction
GC	Gas chromatography
IC	Ion chromatography
LC	Column liquid chromatography
LLE	Liquid-liquid extraction
LVI	Large-volume injection
DMAE	Dynamic microwave-assisted extraction
MMLLE	Microporous membrane liquid-liquid extraction
MS	Mass spectrometry
NP	Normal-phase
PAH	Polycyclic aromatic hydrocarbon
PHWE	Pressurised hot water extraction
PCB	Polychlorinated biphenyl
PLE	Pressurised liquid extraction
PTV	Programmed-temperature vaporizer
RP	Reversed-phase
DSAE	Dynamic sonication-assisted extraction

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5
Headspace

Sample preparation in headspace analysis
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Applicability of SPME techniques for analysis of volatile compounds in complex matrices
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Sample preparation in headspace analysis

Mari Sandell

Introduction

The method for sampling volatile compounds depends on the analytical problem to be solved. Daily life and our environment are full of volatile compounds that differ in their molecular weights, polarities and volatilities. When the target is a quantitative analysis of compounds emitted from a sample, headspace is the best choice and also the method most commonly applied, because it allows the study of relatively volatile and free compounds.

With the headspace method, there is no need for any separate clean up procedures such as solvent extraction, solid-phase extraction or supercritical fluid extraction. The advantage of the headspace method compared to those extraction techniques is its nondestructivity and capability to isolate volatiles in their natural form. Headspace is actually a gas extraction technique where an inert gas is used. Profiles of the composition of volatile compounds obtained from the sample depend on sample preparation, collection techniques of volatiles, and also the respective analytical method. All steps can cause the losses of certain compounds or the formation of artefacts.

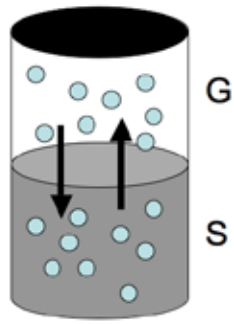
This chapter presents developments and applications of sample preparation methods for analysis of volatile organic compounds (VOC), mainly in food, air and water matrices. A typical volatile compound has an initial boiling point less than 200 °C measured at a standard atmospheric pressure. Static headspace (SH), dynamic headspace (D-HS) and high concentration capacity headspace (HCC-HS) techniques are introduced.

Basics of headspace analysis

Originally the word headspace was given to a gas content of the bulge that forms at the top of a can of food (1). A typical headspace sample is prepared in a sealed vial containing a consumer product or a biological sample. There is a need to have a gas volume above the original sample, which can be either a liquid or a solid. The gas phase (G) is commonly referred to as the headspace, and the original sample as the sample phase (S) as shown in Figure 1. The vial is then thermostatted at the constant temperature to reach the equilibrium between the gas phase and the sample phase. During this step, the volatile compounds should diffuse into the gas phase until a state of equilibrium has been reached. Then the headspace sample is taken from the gas phase and introduced in to the analyser. Usually the volatiles are analysed using a gas chromatograph (GC) combined with different detectors such as flame ionisation (FID) or mass spectrometry (MS).

Figure 1.

Gas phase (G) and sample phase (S) in headspace vial. Little dots symbolise volatile analytes.



To attain the best result, one should pay special attention to sample preparation and the equipment set up. It is very important to use clean vials for sample preparation and airtight materials for storage. The commercial possibilities for suitable headspace analysis products are very wide nowadays (2) .

Partition coeffiecient and phase ratio

Partition coefficient (K) and Phase Ratio (β) are important variables in headspace analysis (Equation 1 and Equation 2) (1). They could be used to optimise the sample preparation process and moreover, to increase the final concentration of volatile compounds in the the headspace (Equation 3). The theory behind the equations has been explained in detail by Kolb and Ettre (1).

Equation 1: $K = C_S / C_G$

Where C_S is the concentration of the analyte in sample phase,
C_G is the concentration of the analyte in the gas phase.

Equation 2: $\beta = V_G / V_S$

Where V_G is the volume of gas phase and V_S is the volume of sample phase.
A sum of V_G and V_S should be Vv, which is the volume of vial.

Equation 3: $C_G = C_O / (K + \beta)$

Where C_G is the concentration of the analyte in the gas phase,
and CO is the original concentration of volatile analytes in the sample.

Equation 4: $A \approx C_G = C_S / K$

Where A is the peak area in the headspace chromatogram.

Headspace samples need to be prepared in a way that maximum concentration of the volatile compounds can be reached in the gas phase. However, it is also important to minimize the number of potential unwanted compounds in the headspace. If the analyte is known, calculation of partition coefficient (K) is a useful tool for optimising the sampling method. K depends on the solubility of the analyte in the sample phase. In other words, compounds with a high solubility will have a higher concentration in the sample phase compared to gas phase. Compounds that have low K will be released from the solid phase faster than compounds that have high K values. Time is one of the physical factors that needs to be determined to achieve the optimum state of equilibrium in the vial.

From the analytical point of view, the sensitivity is increased when K is low. K can be lowered by changing the temperature of the vial. In addition to time, temperature is the second important physical factor in sampling. The higher the temperature, the more the K values will be reduced. However, it is very important to remember to maintain the original quality of the sample without destroying it by using excessively high temperature. Thermostating the prepared vials must be performed in a precise and reproducible manner. Moreover, if the original target has been to detect the profile of the volatile compounds at room temperature, heating may change the form of the original sample. This situation can most often be encountered in cases of consumer food products.

Instead of temperature changing, different matrix modifiers such as inorganic salts may be added to the aqueous sample phase. The type of matrix influences the volatile release significantly (3, 4). By using salting-out modifiers, the solubility of polar organic compounds in sample matrix can be decreased (5, 6). The matrix effect may be eliminated if the target is a very specific compound, but the effect of salts on K is not the same for all compounds. In general, the effect is highest with polar compounds in a polar matrix. Shaking and mixing the vial during the heating may also improve the movement of volatile analytes from the sample phase to the gas phase.

Usually the sampling is carried out in small vials or tubes with a constant diameter. If it is possible to increase the area on the surface of sample phase, this may also decrease the time needed reach equilibrium. The phase ratio (β) represents the ratio of the volumes of the gas phase and sample phase. Lower values for β increase the sensitivity. In other words,

a larger sample size in the vial will yield higher responses for volatile compounds. If the sample phase contains compounds that have high K value, only decreasing β will be useful. However, it is important to optimise the process in proportion to optimum K before changing β, to eliminate the matrix effect. In general, with lower K and β the concentration of volatile analytes will be higher in gas phase, in the way contributing to better sensitivity.

It is also important to remember the difference between the volatile analytes. In order to reach the sampling conditions for a complex sample such as food product, it is difficult to find the incubation time and temperature optimum for every compound. In these types of sample sets, the sampling is always a compromise.

Static headspace

Gas extraction techniques can be carried out in several variants. Static headspace is in general understood as a single step extraction technique (1, 2, 5, 6). Following the basic procedure of headspace sampling, the sample is located in a sealed vial and after a suitable time of thermostatting, the headspace sample is extracted with a gas-tight syringe. The sample is then transferred to the analyser.

Nowadays manufacturers sell also different GC-specific gas-tight syringe autosamplers for use with wide range of samples. In these types of automatic systems, the samples are thermostatted in an incubation oven with settings for temperature and time. After the equilibrium has been reached, the sample is extracted from the headspace and injected into the GC. Depending on the incubation temperature it is important to avoid recondensation of the analytes in the syringe. This potential problem can be solved by using a heated syringe in the system.

In addition to gas-tight syringe sampling, also a balanced pressure system can be used. Instead of suction of the aliquot, the vial is pressurised by the carrier gas to a pressure equal to the carrier gas inlet pressure of the GC-column. In other words, the vial is pressurised to the column inlet pressure. The headspace sample is injected using seamless injection into a stream of carrier gas. This technique is highly reproducible, because the number of moving parts is minimized.

The third typical system is the so called pressure-loop system, where the sample vial is first pressurised and then opened temporarily toward the sample loop. This techinique is typically used with a six-port system. After the loop has been filled, the valve will be turned and the sample will be flushed into the transfer line.

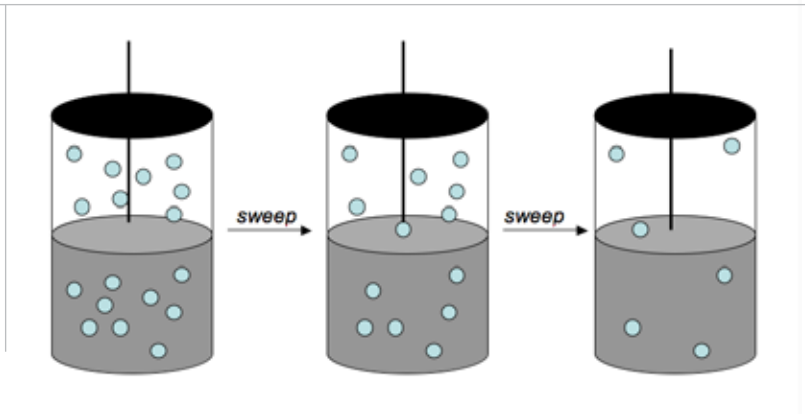
Dynamic headspace

Dynamic headspace (D-HS) is a widely used vapour phase sampling system. A simple D-HS approach is the dynamic system, where analytes are sampled from the flow stream passing over the matrix. Then the volatiles are liberated by thermal desorption or by solvent elution. Another typical dynamic headspace sampling approach is so called Purge and Trap technique (P&T). This separates the VOCs from a matrix, concentrates the VOCs and finally the concentrated sample is injected into the GC (7,8).

The basic operation is straightforward in the Purge and Trap system. The sample is placed in a sealed vessel. Then the sample is purged with an inert gas. Purging is the

Figure 2.

Headspace sample collection procedure in Purge-and Trap sampling. Sample phase is sweeped with an inert gas such as nitrogen or helium.



gas extraction and thus a gas supply of ultra-high purity is required. Either nitrogen or helium can be used as the purge gas but high purity is needed to avoid impurities contaminating the purging trap. During the purging, the volatile compounds are swept out of the sample (Figure 2). Then the volatile compounds are retained in an adsorbent trap by heating the trap. Usually the volatiles are transferred to GC by backflushing the adsorbent trap using the GC carrier gas.

During the purging the amount of each compound is proportional to both its vapor pressure and its solubility in the sample. Both of these are affected by the sample temperature. There are many commercial options for adsorbent traps, but usually the devices are filled with a charcoal or synthetic carbon. The purge efficiency of a sample depends on various factors such as purge volume, the temperature of the sample, the type of purging settings, the volatile analytes and the chemical composition of the original sample itself. Usually the extraction efficiency increases together with purging volume, i.e. the amount of purge gas used. It is important to optimise the purge time and flow rate of the purge gas.

After desorption, the trap should be heated or baked to remove residual analytes and water. Both the solid and liquid sample types are suitable for the purge and trap headspace sampling.

The development of automation has improved the control of P&T system parameters and process. Nowadays there are many manufacturers and commercial options for P&T instruments. The advantages and disadvantages of D-HS methods have been critically reviewed by Bicchi et al (9). They are also collections of D-HS applications for different sample materials.

In contrast to the static headspace, there is no equilibrium between gas phase and sample phase in D-HS. The volatile compound are removed by the purge gas. This means that the partial pressure of any compounds in headspace is essentially zero and this increases the removal of volatiles from sample to gas phase. There is a general linearity between the partial vapor pressure and the molar concentration of the analyte which is expressed by *Henry's law*:

Equation 5: $p_i = H \cdot Xi$

Where H is the Henry's law constant, p_i is the partial vapor pressure of the volatile analyte, X_i is the concentration of volatile analyte substituted for mole fraction in water.

It is quite normal to use a cryofocusing unit before GC transferring to refocus analytes before injection onto the GC column. The cryofocusing module cools a small portion of the column. For practical reasons, cryofocusing increases resolution and improves the peak shape in the GC chromatogram.

High concentration capacity headspace

In high concentration capacity headspace (HCC-HS) techniques, the volatiles are accumulated on liquid retaining polymers or even on solvents. This technique is based on either the static or the dynamic headspace sampling. Volatile analytes in the gas phase are dissolved in a liquid polymer and released by thermal desorption into the GC.

Several applications published for different type of samples have been widely reviewed by Bicchi et al. (9). Techniques based on HCC-HS approaches widely used in the field are in-tube solid-phase dynamic extraction (HS-SPDE), headspace sorptive extraction (HSSE), solid-phase aroma concentrate extraction (SPACE), headspace liquid-phase microextraction (HS-LPME), and large surface area HCC-HS sampling (MESI, MME, HS-STE).

One example of in-tube sorptive extraction consists of a needle coated with polymeric coating such as polydimethylsiloxane that is used as the extraction and preconsentration medium (10). Since this is really of an inside capillary adsorption trap, this method is also called INCAT. Sampling is performed on the solution headspace passage by passing the gas through the SPDE device actively. The in-tube sorptive extraction technique is effective with highly volatile compounds.

Headspace Sorptive Extraction (HSSE) in an extension of stir bar sorptive extraction. The analytes are accumulated onto a thick film of polymeric coating a glass coated magnetic stir bar. After sampling the stir bar is placed in a glass tube and transferred to a thermo-desorption system (11).

Solid phase aroma concentrate extraction (SPACETM) consists of a rod coated with a mixture of adsorbents. The rod is fabricated from stainless steel and the coating consists mainly of a graphite carbon. During the absorption, the rod is fixed in the head of a vessel for a predetermined time.Volatiles are released with thermal desorption into GC. The aim of this technique is to increase the area of the absorbent and to improve sensitivity (12).

Headspace liquid-phase microextraction (HS-LPME) involves a teflon rod or micro-syringe needle. Volatile analytes are attached on a drop of solvent at the tip of a rod or needle (13, 14). Before injecting, the drop is retracted back into the needle. This technique seems to be suitable to non-polar, polar and water miscible analytes. However, the vapour pressure of the solvent must be low enough to avoid evaporation during sampling. The system is suitable for selective sampling (15).

Large surface area high concentration capacity headspace sampling involves focusing on the area of the adsorbent. Membrane extraction sorbent interface sampling solution (MESI) combines the hollow fiber membrane, cryofocusing and thermal desorption technologies (16). The analytes are stripped from the flowing gas and concentrated in a sorption trap and are then injected into the GC after thermal desorption of the trap. A thin film membrane is filtering the analytes that are dynamically accumulated on a conventional polymeric trap.

Headspace Solid-Phase Microextraction

The headspace solid-phase microextraction technique (HSPME) was developed by Zhang and Pawliszyn (17). Thus utilizes a fuced silica fiber that is coated with a polymeric organic stationary phase. Volatile analytes are extracted and concentrated on the coating and releases by thermal desorption into the GC injector. SPME is a passive headspace method. The chapter "SPME techniques suitable for volatile compounds analysis in complex matrices" written by Nurmela and Järvenpää will focus on details of SPME.

Headspace sample types

The popularity of headspace techniques has grown during the last two decades. The wide range of different applications have been reviewed by Bicchi et al 2008 (9). The objective of the measurement can be flavour or off-flavour compounds in beverages or food products (9, 18, 19), fragrances and perfumes. In such cases the targets are usually odour or aroma, i.e. the key compounds correlating with the perceived sensation. However, the object may be also more health-oriented such as residual solvents (6), soil or in pharmaceutical products (10, 11), or alcohols in blood (20). Applications have been developed also for environmental topics such as air or water (21, 22) heating oil or diesel fuel (23). In general, the savings in time and money together with ease of operation are the main reasons for adopting headspace analysis as a sampling technique. ●

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Applicability of SPME techniques for analysis of volatile compounds in complex matrices

Eila Järvenpää | Kari Nurmela

Introduction

Solid-phase microextraction, SPME, is one of the simplest instrumental sample preparation techniques for chromatography. It was first developed for gas chromatographic analyses by Prof. Janusz Pawliszyn and his co-workers (see http://www.spme.uwaterloo.ca/) in the University of Toronto in the 1990's. Today, SPME-devices are commercially available both for GC and HPLC injections either for manual or autosampler use. The heart of this innovation was the design of the extracting phase so it became a reusable thin film on a narrow fibre inside of a syringe, which was directly transferable from sample vessels into the inlet of any commercial gas chromatograph, enabling solvent free release of concentrated sample compounds as sharp bands without complex and expensive trapping and focusing devices. It has been shown both theoretically and in practice, that by controlling certain sampling parameters, quantitative chromatographic analyses can be achieved with SPME devices.

The aim of our chapter is to inform the reader of the possibilities and limitations of the SPME and its application to volatile analysis in complex samples. After the original SPME invention, several parallel techniques have been developed. Nonetheless the origi-

nal coated fibre SPME variety is still clearly dominant in terms of number of publications and for this device there is the most experimental proof backed up by that is a valuable analytical sample preparation tool for real-life samples. Therefore we will focus on it here.

Overview of the theory of SPME techniques for practical use

Typically, analytical methods using SPME techniques are based on the equilibrium of chemical compounds between the existing phases in a closed vessel system. In Figure 1, an SPME device is illustrated, and under the tip of the injection needle, the SPME phase or fibre coating, is exposed to the headspace. In this example, an erlenmayer flask contains an aqueous suspension of a sample and headspace gas or vapour. The volatiles first transfer from the suspended sample particles to the water and from water evaporate into headspace gas. Volatile compounds will then distribute between the headspace gas and SPME phase.

Theoretical consideration of the SPME is that after the analytes are in the steady state and equilibrated between the existing phases, the amount of an analyte dissolved/adsorbed into SPME phase is in proportion to its concentration in the original sample.

Equation 1: $C_0 V_s = C_f^{\infty} V_f + C_h^{\infty} V_h + C_s^{\infty} V_s$

Where C is concentration of an analyte and V volume of the phase; 0 denotes initial and ∞ infinite (equilibrium) conditions, and s denotes sample, f fibre and h headspace phases (Pawliszyn, 1999)

In the equilibrium conditions, the amount of the analyte (n) extracted by the SPME phase can be expressed by the following equation:

Equation 2: $$n = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + V_s}$$

This equation is simplified by assuming $V_f << V_s$:

Equation 3: $n = K_{fs} V_f C_0$

Where K_{fs} is equilibrium partition constant.

In order to obtain comparable results from different samples using Equation 3, the sample amount, all volume ratios of existing phases and the temperature need to be kept constant and liquid phases should be stirred well to reach the equilibrium in a reasonable time. However, since the volume of SPME phase is very small (<1µl) and the volume of the contacting phase can be very large, the amount of an analyte adsorbed onto the SPME phase often does not change the concentration of the analyte in the contacting phase. Therefore, container-free SPME sampling of homogenous gases or liquids, such as room air or lake water, is possible.

SPME sampling by dipping the fibre into a liquid is often termed as using the direct or immersion mode. Immersion sampling is often used in SPME-HPLC analyses and for extraction of non-volatiles. Since GC is favoured for analyses of volatiles and complex matrices usually contain pyrolysable non-volatiles, immersion sampling in

these applications would lead to very high consumption of SPME fibres and artefact compounds in GC chromatograms. Therefore this paper will concentrate on headspace sampling. The third option, protecting SPME phase with thin semi-permeable membrane during immersion, is rarely used.

In addition to the abovementioned spot sampling methods for stable concentrations, SPME devices can be used to monitor averages of concentrations varying in time. Time-averaged sampling is done by keeping the SPME phase inside its needle during sampling, and compounds have to diffuse through the needle hole and along the coating of the fibre in the course of extraction. The variations of external concentrations in time are then reflected through the length of the coating, but all concentrations are averaged at the moment of injection. This is also less used application mode (2).

Typically, the time needed to reach an equilibrium state for the volatiles between sample and SPME phases ranges from 20 to 60 minutes in a headspace vial but for semi-volatiles it can be much longer. This can be considered as weakness by modern fast-paced scientists, but for them SPME also offers a faster alternative. If the sample is a homogenous solution already in equilibrium with a gas phase and the target component is relatively volatile and sufficiently abundant to avoid sensitivity problems, it is quite often possible to use rapid non-equilibrium extraction mode of SPME with good repeatability. Since the settling of the phase system into a new equilibrium state after insertion of the SPME phase is a sample concentration (C0) dependent repeatable dynamic process, it is possible to extract the same constant portion (n) of the full equilibrium extraction yield of a component (n_∞) by precise control of a constant extraction time (t), e.g. in 1 to 5 minutes (3).

Equation 4: $n = k\ KhsVhC0t$

This reduced equation proves, that if the above mentioned sampling time, and the volume of the headspace and the physico-chemical conditions of the sample affecting equilibrium constant are kept constant, the amount of analyte in the SPME phase is directly proportional to the initial concentration in the sample. Note that the basic assumption of the equation is that the partition between condensed and headspace is the rate-limiting step of the equilibrium, and the partition between headspace and SPME phase is a rather fast process. Thus, this consideration is valid in most food analysis systems. The theoretical and experimental evidence for the use of the reduced equation can be found in papers of Jiu Ai (3, 4).

In comparison to classical headspace sample preparation techniques, static headspace gas sampling and dynamic purge and trap (P & T) system, SPME occupies a position somewhere in between, being closer to static HS or dynamic P&T depending on the analytical conditions, analytes and their concentrations. Usually static headspace sampling will not change the concentrations of volatiles in the sample matrix and their concentrations in the gas phase and chromatographic responses are functions of the analyte's volatility and its matrix concentration. This relationship is also valid in SPME if the volumes of the sample and gas phases are relatively large and the concentrations of analytes are relatively high. The only difference compared to static HS gas sampling would be much amplified chromatographic responses due to the concentrative effect of

the extracting phase assuming that there is no chemical discrimination between volatiles attributable to the SPME phases.

On the other hand, an ideal dynamic P&T sampling will extract all volatiles out of the sample and transfer them into the GC column, causing enormous increase in the injected amounts of compounds and sensitivity of an analysis as compared to static HS gas sampling. In addition, peak areas are directly correlated with the concentrations of analytes in the sample matrix and independent of their volatilities. This needs to be remembered if the purpose is to evaluate aroma impact values of volatiles. Although SPME possess less sample capacity and thus sensitivity than P&T, in some conditions it can also approach this kind of exhaustive extraction. If extracting phase / matrix distribution constants strongly favor the extracting phase, if the concentration of a compound in the matrix is low and if the sample volume is small, then this compound can be almost completely extracted. When necessary, the exhaustive property of SPME can be increased by special techniques such as using an internally cooled fibre or by in-fibre derivatization.

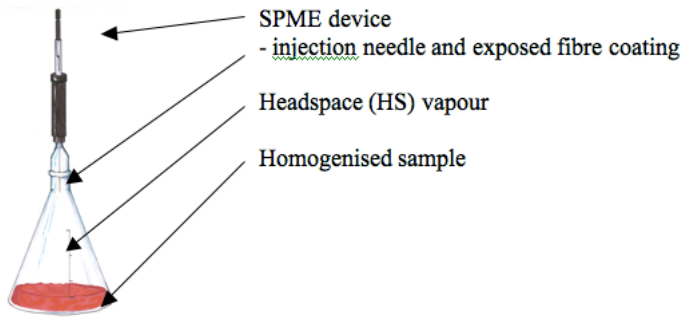
Headspace SPME in practice

SPME device

In the case the reader has never seen an SPME device, some of its properties may be difficult to comprehend from written text. Figure 1. is an illustration of the sampling scheme, and the reader is referred to the vendor's internet pages, because there one can view an animated illustration of the whole SPME sampling procedure (www.sigmaaldrich.com → SPME, read 17.11.09).

Figure 1.

Illustration of manual headspace SPME sampling. Original picture drawn by Anna Hillgren.



The device consists of the fibre assembly (fibre and metal or metal alloy needle) screwed with the plunger of a fibre holder. The first introduced SPME fibre was a thin silica rod partially coated with an extracting polymeric liquid phase. Recently, silica as a core material has been replaced by a metal alloy, which is more durable for modern autosampler use. In addition, the fibre assembly contains a colour-coded plastic screw hub, which indicates the type of coating, and a returning spring, if the device is for manual use. The standard length of coatings is 1 cm, but some phases are offered also as 2 cm versions, which obviously have higher sample capacities.

SPME devices with various fibre coatings and film thicknesses are at present available for GC and HPLC use. In addition, different needle assemblies are used for automated injectors than utilized with manual injection, and different needle types and sizes are provided for different injectors and GC inlet septa. However, pre-piercing of thick and firm septa is suggested prior to SPME needle piercing unless a self-sealing channel septa (Merlin) is used. One practical observation is that the SPME needle for GC use is somewhat thicker than typical liquid injection needles, thus septa especially of the "thermogreen" type, may need changing more often. In addition, special SPME sample vials with soft and thin cap septa are on the market as are SPME inlet liners (<1mm i.d.) for most common GC brands. From the analytical point of view however, the most interesting part is the extracting phase of the SPME fibre, the coating.

Coatings

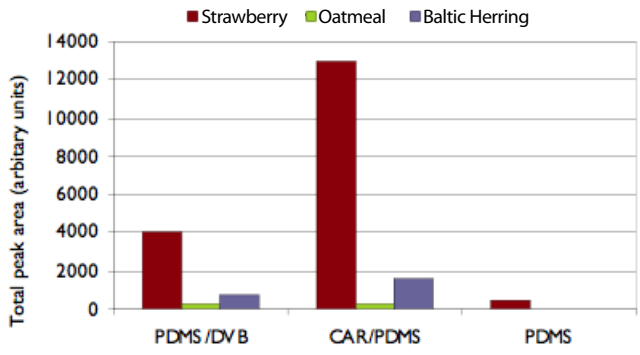
The first criterion of fibre selection is to evaluate the chemical properties of the analyte(s). When choosing a suitable SPME phase for a particular analysis need, one can review the published literature, as well as application notes and suggestions by the vendor. A list of currently available phases can be found in the vendor's printed catalogue and web-page (www.sigmaaldrich.com -> SPME, or www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spme.html).

SPME phases are most often categorized by polarity (e.g. 5), and the polarity reflects an important factor in sample preparation: "similar dissolves similar". Polydimethylsiloxanes (PDMS) are typical non-polar phases in SPME, but if they are mixed with porous solid polydivinylbenzene (DVB), semi-polar phases are formed. Polyacrylate (PA) and Carbowax (polyethylenglycol, CW) mixed with DVB are available as polar phases.

Recently, phases containing porous graphitized carbon (Carboxen, CAR) have been added to the commercial selection. They are especially suitable for very volatile small molecules and a combination of PDMS/DVB/CAR has become very popular as a general phase for untargeted screening of unknown samples. One drawback of porous solids in SPME coatings is their tendency to slow release of larger molecules in injections, which causes more peak tailing compared to SPME with homogenous liquid phases. While not underestimating the literature sources in search of the best phase for a particular application, the authors recommend that investigator tests experimentally the performance of a few different phases on his/her own samples. The results can be surprisingly variable, as illustrated in Figure 2.

Figure 2.

Comparison of different fibre phases using total peak area as criterion.



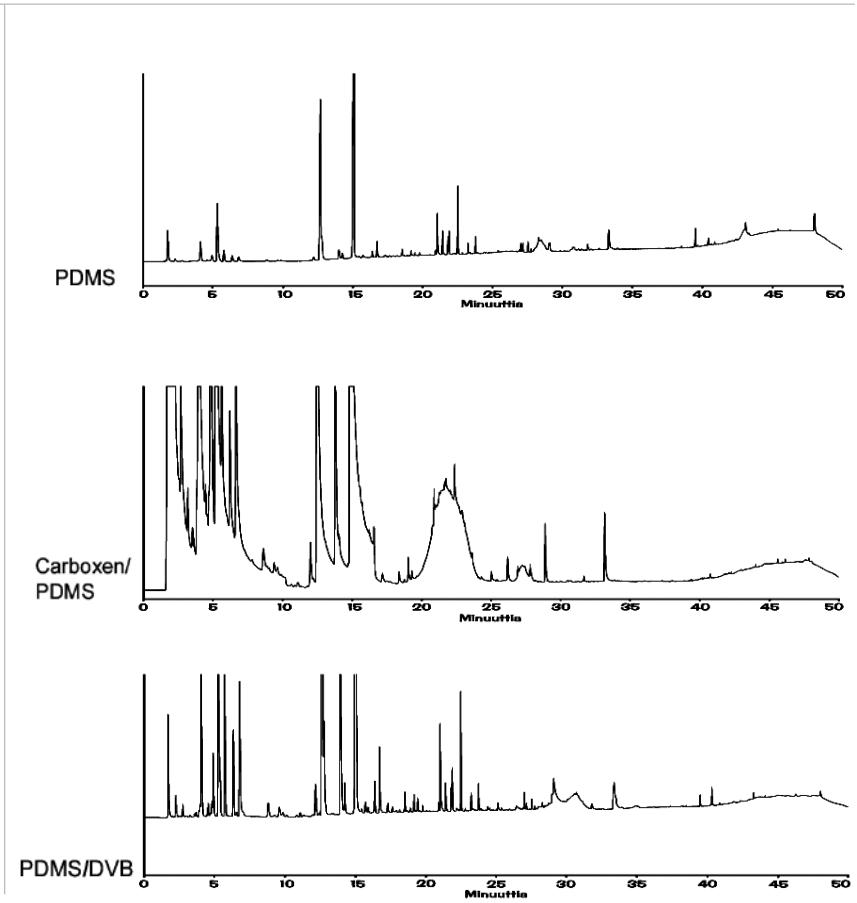
In this case the criterion was the total peak area. The analyst may also choose other criterion, e.g. area of the target analyte(s) in the chromatogram, or minimum number and/or small areas of interfering peaks in the chromatograms. HS-SPME-GC chromatograms of oatmeal and Baltic herring samples contained mainly aldehydes and ketones, which were derived from the oxidation of lipids, whereas the major compounds in strawberry sample chromatograms were different fruit esters as well as hexanal and linalool. One further selection criterion may be the strength of the retention of the analyte(s) onto the extractive phase, because analyte(s) may adhere too strongly, which may lead to underestimation of the amount and at least cleaning of the fibre is necessary between samplings.

In general, the thicker the phase film (df), the stronger the retention like in GC column. Therefore thin film fibres are recommended for semi-volatiles and thick film fibres for very volatile compounds. As mentioned above, if Carboxen and other porous particles are incorporated into the polymer films, this will increase the retention of most compounds.

Analysts may joke about the analytical result by SPME being a caricature of the sample drawn by the extracting phase, but it is more or less true and has to be considered when reporting the results. Figure 3 shows three GC-FID chromatograms of the same strawberry sample obtained using different fibre phases. For another type of samples, Vichi et al. (6) conducted a mathematical comparison of three coatings in the analysis of olive oil volatiles.

Figure 3.

An illustration of differences in GC-FID chromatograms of strawberry samples obtained using different SPME coatings.



As will be further discussed below, the SPME technique is useful and can be validated for regular use e.g. for manufacturing quality control or by the authorities. Especially in these applications, attention has to be paid to the stability and durability of the extractive phase and the fibre core material during long-term use and one must ensure the comparability of the results when using fibre assemblies from different batches. The manufacturer claims that some coatings tolerate organic solvents, but some of them may be damaged or fibre become more brittle and fragile if solvents such as water-soluble alcohols are present in the sample or are used in the sample preparation (see below). Even the amounts of ethanol found in wines (e.g. 7) seem to have this effect, thus dilution of these kind of samples is necessary in order to prolong the duration of fibre use.

Even in ideal conditions, every extraction (actually an injection) will wear out SPME phases to some degree and their sampling properties will change gradually. When the change is no more tolerable and the time when to replace the fibre, depends greatly on the purpose for which it is being used. The speed of wear is related to chemical compositions of coatings and samples, some are more deteriorating than others, but with headspace sampling in general, one can expect to be able to make several tens or even hundreds of extractions per single fibre. A novice user should realize that this wearing will also manifest itself as peaks in the chromatograms. During extractions, fibres adsorb some water, which forms steam in hot inlet of GC and hydrolyzes PDMS coating, producing various volatile siloxane derivatives. Degradation of DVB may be visible as small peaks of aromatic compounds such as toluene. Sometimes it may be possible to see a rather large peak of methoxyphenyloxime, which originates from the glue used in fibre assembly. Therefore blank samples are essential, when using SPME as a qualitative screening method without target analytes, but that may well be routine for all volatile analysts in any case.

Batches of the same fibre type or even single fibres from the same batch may differ slightly from each other. In addition to accelerating the wearing, sample components may change SPME phase properties (solvents, pH) or they may persist in the phase even after regular clean-up procedures described in the fibre package (e.g. fats, sugars). The analyst has to use quality control samples to study the stability and sampling capacity of the fibre. When using the standard to internal standard ratio for calibration the reduction of the fibre capacity is not always noticed. Fibre performance can be conveniently and inexpensively controlled by using an in-house control sample. The quality control sample should reflect the basic chemical composition and analyte concentrations of the typical analytical samples and should be stable during storage.

Sample preparation prior to SPME sampling

Does a sample preparation method, like solid phase microextraction need any sample preparation prior SPME sampling? Not necessarily, if the sample already is in a suitable phase (liquid or gas), and is sufficiently homogenous. Then put the sample into a vial, cap it and incubate under stirring (liquids) with the exposed fibre in the headspace – it can really be so easy. Solid samples, solutions containing particles and large non-homogenous or viscous liquid samples may require sub-sampling, homogenisation and/or dissolution/dispersion to aqueous solvents before SPME sampling.

Internal standards are often needed for quantitative determination of analyte(s) in complex samples. The analyst should pay attention to how much and what solvent the standards will bring along into the equilibrium system, and whether the added solvent or compound(s) can compete with the analyte(s) during the adsorption process and thus affect the equilibrium. The solvent may also affect the equilibrium partition constant K. For example, Górecki et al (9) and Grote & Pawliszyn (8) discussed the effect of one major volatile component on the analysis of trace volatiles from aqueous and air samples, respectively. These effects are both analyte and fibre phase specific.

Most sample preparations prior to SPME sampling aim at improving the extractability or the partition constant of an analyte, thus increasing the amount of intended analyte in the extraction phase and reducing the amount of interfering compounds. These kinds of preparations are typical for the LLE or other HS methods as well, such as derivatizations, pH changes and salt additions.

Sample introduction to chromatographic system

SPME technique is an excellent concentration method, because it concentrates the analytes from large volumes down to a very small volume (>1 µl). Thus desorption conditions, i.e. injection zone temperature, inlet dimensions and split programming need to be selected to avoid unnecessary sample volume expansion and to introduce analytes optimally (rapidly and sharply) into the column. Typically, desorption is started as splitless or using very low split flow, which is increased to the usual split values after a minute or two. The optimal procedure for desorption depends on what kind of analytes one is trying to separate and how the chromatographic and detection systems will respond to their feeding. A situation where the analytes entrapped in the extracting phase include both highly volatile and less-volatile compounds, such as that found in profiling of aroma and flavour of foods, could be problematic and may require a compromise solution.

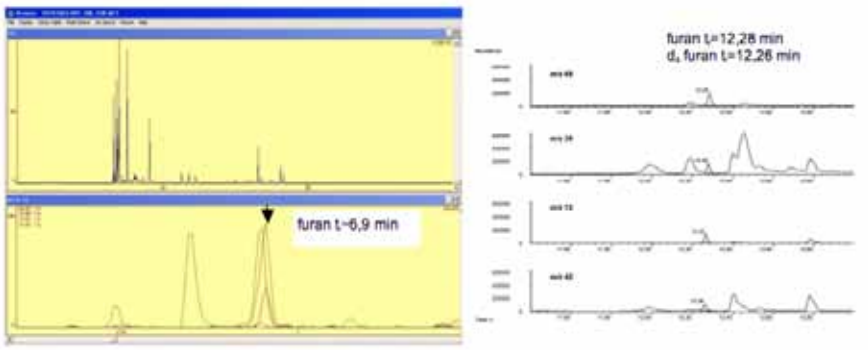
Desorption time, that is heating of the exposed fibre coating in the inlet, is relatively short when compared with other trap techniques. It has to be long enough to produce reproducible desorption of the majority of the analyte(s) from the fibre coating. The residual components are often removed by heating the fibre separately from the analytical system, e.g. using another GC injector or an automated desorptive cleaning device.

Sometimes in SPME-GC analyses, it is necessary to use relatively long desorption times in order to facilitate analysis of less-volatile compounds and compounds strongly adsorbed onto the SPME phase. Normally long desorption times result in broadening of the peaks, or even co-elution of early eluting peaks. Depending upon the analyst's requirements one may need to pay considerable attention to this phenomenon. There are several ways to compensate broad injection bands by chromatographic techniques, and the analyst may evaluate the usefulness of these following choices:

- cold trap (N_2(l), CO_2(s, l)) between injector and column
- lower column oven temperature at the beginning of GC run
- more retentive column stationary phase type
- more thicker column stationary phase

Figure 4.

Comparison of furan HS-SPME-GC-MS chromatograms. LEFT: TIC and SIM chromatograms using N2 trap (5 min) & RH-5ms+ column, df 0,25 µm (Restek). RIGHT: SIM chromatograms using HP-PLOT/Q column (J&W Scientific), df 25 µm. SIM ions 68, 39 for furan and ions 72, 42 for d4-furan, which elutes slightly before furan.



The following GC-FID chromatograms (Figure. 4) illustrate one example. Furan was analysed in baby foods using HS-SPME-GC/MS. During method development both liquid nitrogen cold trap and thick film GC column were evaluated. For routine analysis, the latter was found to be more convenient, because manual cold-trap resulted in small variation in retention times. Liquid N_2 is also a rather expensive coolant. Liquid CO_2 is cheaper, but it is not enough effective for compounds more volatile than octane.

Qualitative and comparative headspace analysis of volatile compounds in foods

At the beginning stage of the SPME development it was considered, that complex systems such as food matrices would be too complicated to allow any kind of quantification by SPME. In fact, all the quantitative considerations – both theoretical and experimental were performed using relatively diluted and simple mixtures of organic compounds in pure water (see below). However, immediately at start of SPME qualitative or comparative applications were popular, because a profile of volatile compounds could be obtained using relatively simple and cheap instrument with less effort than with previous techniques. Kataoka et al. (5) and Belliardo et al. (10) have tabulated most of the early SPME applications in the field of food, environmental and pharmaceutical applications.

Qualitative HS-SPME gas chromatogram is like an impressionistic chemical photograph of the sample volatiles under the conditions prevailing during SPME sampling. As discussed above, the sample preparation prior to sampling and SPME sampling conditions greatly affect the analytical result. By selecting the optimal conditions, the analyst like a photographer may adjust to a greater or lesser degree of what will be in the portrait of the sample, i.e. what the chromatogram will contain and how much each tone or shape alias compound will be highlighted or faded. When one is conducting a qualitative untargeted survey of an unknown sample, the style of art should be as non-discriminating as possible. In the case of volatiles, SPME cannot ever be as equitable as static headspace,

but it is still a much more sensitive camera. However, just as a photograph is only one of countless two dimensional projections of a real three dimensional object, this also reflects the chromatogram's relationship to the original sample. Nonetheless, we can use them like portraits to identify and to characterize our objects. The mass spectrometer as the detector will provide the third dimension and depth vision.

Although SPME sampling may favour some volatiles and discriminate against others, when it is used for qualitative profiling of a single sample, the question is what compounds exist within the sample – not their true ratios. If that is the goal, sensitivity is more critical factor than perfect impartiality, because in general, mass spectral identifications are more reliable with larger peaks. The question then arises why not use P&T or other more sensitive volatile collection method like stir bar sorptive extraction, SBSE? Indeed, if such instruments are available, the authors recommend their use as the first line tools for qualitative screening of unknown samples. However, after that is done, a more common task in a research project is to compare several or large number of similar but not identical samples. If one is not hunting foe vanishing traces, SPME may be a cheaper, faster and easier method for this kind of job.

The majority of the SPME analysis methods for food samples found in the literature can be categorised as un-calibrated comparative analyses. They have been used for several purposes, such as differentiating species or varieties of plants, revealing storage time or condition effects and spoilage e.g. microbial growth or oxidation. One very convenient application of HS-SPME for troubleshooting is the examination and identification of fault or taint odours, or other sensory defects. All these are examples where samples in the comparison have similar matrices but are by some non-chemical means have been classified as being different and correlations between classes and amounts of chemical compounds are explored. To find statistical correlations, peak areas can be used as variables instead of concentrations, if the sampling procedure is stable and constant for each sample. However, the comparability of sample matrices should always be born in mind. For example peak areas of hydrophobic volatiles in milks having different fat content should not be compared.

The finding of a statistical correlation does not necessarily represent a causal relationship. In an off-flavour case, the responsibility of a compound among a large selection of "correlatives" may be difficult to confirm without a sensory connection through an olfactometric port. Indeed, with HS-SPME, it is possible to inject for example enough cheese volatiles to simultaneously identify 1:1 split peaks with MS and to sniff their odour. Sometimes it will not be possible to sense a large peak, sometimes one can pick up a smell from a flat baseline. That can be true or simply imagination unless confirmed by a few other individuals who smell the sample.

In order to find reliable differences between samples, the conditions chosen have to be carefully tested and subsequently repeated, before samples can be compared with each other based on SPME analysis results. In general, the repeatability of a peak area using SPME expressed as relative standard deviation is better than 20 %, but it depends on the compound's chemical nature relative to the SPME phase and the GC stationary phase as well as its concentration. Usually homogenous solutions will evoke less variation than suspended solids. Equilibrium sampling is preferable, because it reduces the variation in the results.

Figure 5.

Formation of equilibrium between HS gas and sample by methyl butanoate and linalool.

Left panel pure compounds added to water. Right panel frozen strawberry purée samples.

Y-axis arbitary area units (GC-FID). Error bars indicate standard deviation (n=5). Arrow indicates 25 min stabilization time, which equals to SPME sampling time.

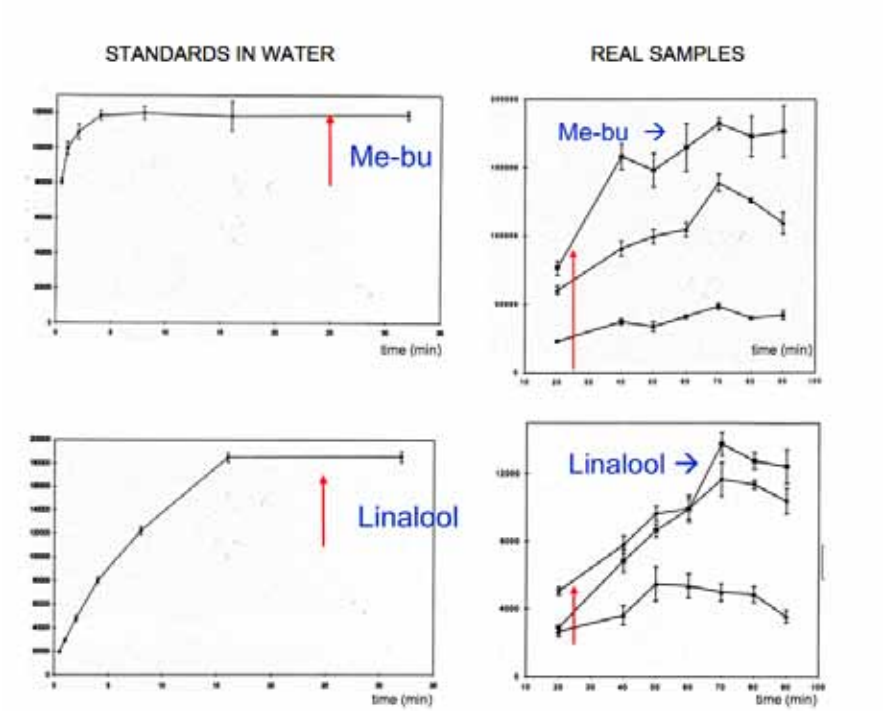
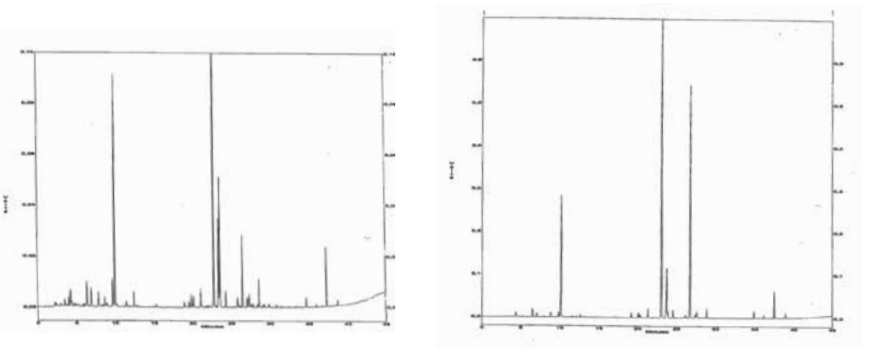


Figure 6.

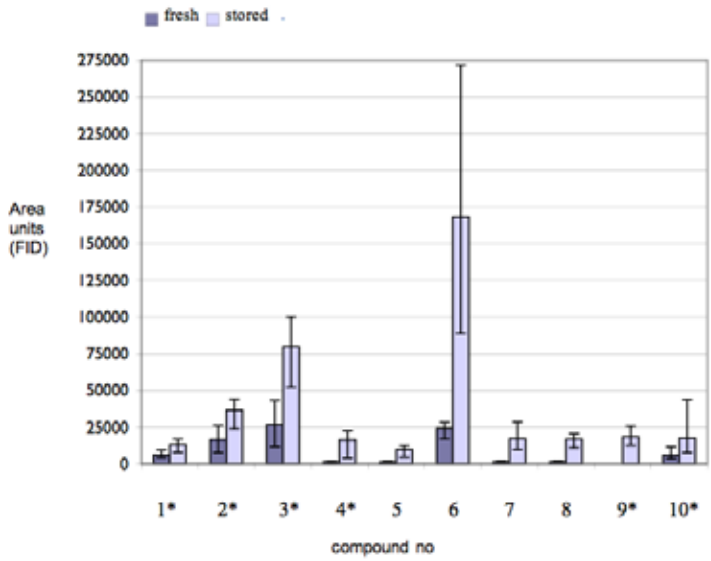
Comparison of two commercial basil leaf samples by HS-SPME. The GC-FID chromatograms are scaled to the tallest peak (t~23 min) Conditions: PDMS/DVB SPME fiber, 1 g dried leaves/100 ml HS bottle, 40 min stabilisation time including 20 min SPME sampling time. Desorption 2 min at 240 C. Column temperature programme 40–240 C .



The equilibrium time needed can be tested using a pure compound mixture, but the needs to be aware, that other sample components take part in the formation of equilibrium. Even homogenized food matrices, such as strawberry puree may slow down the partition dramatically, as illustrated in Figure 5. In this case, the volume of the headspace was large compared to the sample volume, which facilitated the stirring of the sample puree using a magnetic stirrer, but this resulted in a relatively slow process of equilibrium partition in the headspace. Note that the graphs on the right

Figure 7.

Refrigerated Baltic herring samples, fresh (stored one day) and stored (3 days) analysed using PDMS/ DVB fibre (n=3). Compounds: 1: 2-methyl propanal; 2: 3-methyl butanal*; 3: 2-methyl butanal*; 4: hexanal*; 5: heptanal; 6: pentamethyl heptane; 7: 2,3-octanedione; 8: octanal; 9: 2,4-heptadienal*; 10: heptadecane*. Error bars = standard deviation. Compounds with asterix* statistically different p=0,05.*



are of real samples and real concentrations, not spiked samples, which together with the presence of solids leads to larger variation.

The comparative analysis of volatiles by SPME does not necessitate the equilibrium; for example volatiles produced by an enzymatic reaction can be followed using SPME (11). The reader should note, that in such cases one has to think carefully about which conclusions can be drawn from the chromatograms, and the sample preparation and sampling conditions, especially the timing of actions has to be well controlled, preferably automated.

In this paper, we have chosen a few model applications in our areas of interest. An example of a fingerprint chromatogram is shown in Figure 6., where HS-SPME-GC/FID chromatograms of two commercial samples of basil leaves are depicted. The compounds were not identified in this study, but the areas of individual peaks were compared and statistical differences were found (unpublished results). The typical basil leaf volatiles are oxygenated monoterpenes and aromatic compounds, such as linalool, eugenol and methyl cinnamate (12).

Qualitative comparison using peak areas of fresh and stored Baltic Herring samples is given in Figure 7. Most of the identified compounds are typical fat oxidation products. Indeed, a relatively large number of different matrices has been analysed for lipid oxidation products (e.g. 6, 13), although the partition of aldehydes and ketones is relatively poor from lipid containing matrices into the headspace, as illustrated in Figure 2. In multiphase systems, fat usually competes for analytes with the SPME coating much stronger than the aqueous phase which means that many compounds in fatty foods are invisible in comparison with the same concentrations in water. From fats and fat-containing samples, the extractability of volatiles is enhanced and this can

be made more repeatable when the sample temperature is increased over the melting point of the fat, although extensive heating of the food samples may result in artefact formation. Figure 7. also shows capability of SPME fibres to entrap 2-methyl propanal (compound 1 in Figure 7), which is relatively difficult to analyze using other gas chromatographic methods. Detection of gaseous and highly volatile liquid compounds dissolved in food samples is also feasible via SPME sampling, because heating of the sample is not necessary. Another example is shown in Figure 8, where sulphur dioxide has been measured in the commercial grape juice concentrates using a titrimetric method and HS-SPME-GC/MS.

The Baltic Herring HS-SPME-GC/MS chromatograms revealed that in addition to degradation products of proteins and lipids, the untypical fish volatile compound limonene was found. It was clear to the analyst, that this compound was an artefact transferred to herring samples during storage in the refrigerator or extracted by the SPME fibre from the laboratory environment. An empty run of the fibre phase reveals that the artefacts originated from the fibre assembly and coating (see above), but additional compounds may be attributed to from glassware and plastic or aluminium closures of sample vials, plastic bags and containers where samples are kept prior analysis, and sometimes it is difficult to identify their origin. Based on our laboratory experiments, SPME phases seem to be very effective in concentrating these compounds. Another example in the literature is given by Diaz et al (14), who studied volatile compounds of truffles, and the philosophic question was raised about whether BHT could be identified as a volatile constituent of truffles (15). That paper is also a good example of how experimental design is used for SPME method optimisation.

Quantitative headspace analyses of volatile compounds in foods

The overall information about quantification methods for SPME is similar to that of other sample preparation techniques: the more complex the sample, the harder the analyst has to work to obtain the results. For a very simple aqueous system with known compounds, no standard curve is necessarily needed, if the physico-chemical properties of the analytes can be calculated or measured (18). However, this option seems very complex and even impossible if one is trying to analyse food and related samples, and it will not be discussed here further.

According to Equation 3, the quantification of headspace SPME analysis is possible if the compounds of interest are in equilibrium /steady state during the sampling. Steady state means, that the introduction of the SPME fibre phase into the headspace system does not greatly affect the equilibrium, or its effect is balanced during the SPME sampling time. In most cases, the volume of SPME phase is negligible compared to the other existing phases and thus linear calibration curve should be obtained over the concentration range of interest.

The initial and easiest option to produce a SPME calibration curve is external standardization (ES), which works well for homogenous liquid samples, as for solvent residues in water. However, as pointed out earlier, the partition coefficients of volatiles in equilibrium (K_{hs}) depend on sample matrix properties like pH, salin-

ity and amount of major compounds like fat, protein and carbohydrates. External standard curves cannot be realistic if they were made in a matrix not resembling the sample composition. It may be possible to build close imitations of soft drinks or vinegar, but quite hard to construct a matrix for meat.

Instead of attempting to create an artificial mimicry matrix for a complex sample, the use of standard additions to the sample itself is a simpler solution. Standard addition (SA) is the most reliable way to produce quantitative SPME calibrations for complex samples, but it is rather demanding and takes a long time. Calculations are not as simple as in ES, but they are usually included as an option in modern chromatographic software packages. An available blank sample without target analytes is not obligatory but useful.

Quite often there is extensive matrix variation between samples to be measured, in their fat content for example, or difficulties in maintaining every affecting factor as constant in sampling. In those cases, ES calibration is not recommended and SA can be too laborious, instead internal standardization (IS) is the most often used type of calibration in quantitative SPME. Naturally, the chemical nature of the internal standard(s) should mimic those of analyte(s). The amount of the internal standard(s) added to the samples has to be optimised or at least tested together with other parameters, because the additive may contribute to the equilibrium partition constants. In addition, for SA calibrations, the addition has to be evaluated sufficiently, that its effect on the SPME process can be controlled. If there is reason to suspect that an analyte and its internal standard may have different ratios of equilibrium coefficients in the sample from that in the calibration solution, IS can be combined with SA. If an analyst has the opportunity to use MS as the detector and isotope labelled analogs of target compounds as internal standards, the matrix equivalence of calibration and unknown samples is not necessary.

Using stable isotope labelled analogs it is also possible to utilize a very special SPME calibration technique, so called in-fibre standardization or kinetic calibration. In this case, the SPME fibre is first equilibrated in a vial with a known amount of labelled compound in a solution. Subseuently the SPME fibre is inserted into the sample vial. There the unlabelled analyte is absorbed by the coating while the labelled analog is desorbed from it. Since the absorption and desorption processes are isotropic, the concentration of the analyte in the sample can be calculated by using peak areas, if the detector response ratio of the analyte to the labelled analog is known.

The availability or prices of isotope labelled compounds is often a overwhelming obstacle for use of those kinds of internal standards in SPME, but there is another, although rarely used, option to eliminate systematic errors between samples caused by matrix differences. If the extraction is or can be arranged to be partially exhaustive, meaning that SPME sampling reduces significantly the concentration of the analytes in the sample, then by performing multiple extractions (3–5) from the same sample vial, an analyst can obtain a series of dampening chromatograms and from them it is possible to mathematically estimate, the sum of peak areas of a single compound if one would have conducted an infinite series of extractions. By doing the same for an external standard solution or injecting standard solutions using on-column inlet, the peak sum area/concentration ratio can be calculated (16).

Figure 8.

Sulphur dioxide analysis of grape juice concentrates. Logarithmic correlation was found between the standard method (titrimetry, x-axis) and HS-SPME-GC/MS (y-axis). Equation: y = 1E+07Ln(x) - 3E+07, regression R2 value = 0,9992.

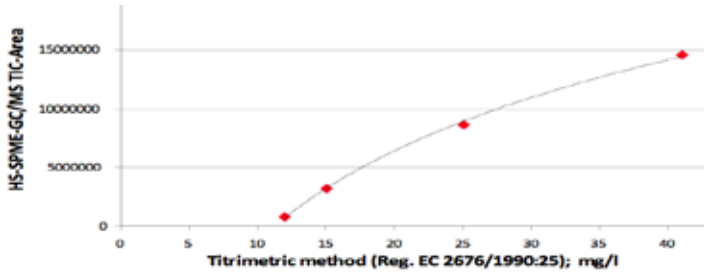


Figure 8 shows an example of using SPME for the quantitative analysis of a gaseous compound, sulphur dioxide in liquid food samples. The same samples were also analysed using a titrimetric reference method. When studying the correlation of two analytical methods, as in Figure 8, or a typical standard curve in quantitative analysis, one often expects to find a linear correlation. By using a ruler one can visualise and estimate, that three of the adjacent points of the above graph could produce an adequate linear correlation, actually calculated for three points R2=0,99 and for four points R2=0,98, but the logarithmic correlation is a much better fit. Now, we will turn back to the theoretical considerations of the SPME methods, and one of the developers of SPME theory, Dr Ai, who has formulated the Equation 4 in a different form (4, 17):

Equation 4: $n = n^{\infty}[1-\exp(-a_h t)]$

Where term ah is a parameter described by the time elapsing to form an equilibrium, i.e. it takes into account how the steady state equilibrium is formed between all the phases existing in a closed system as well the diffusion of the analyte within the coating. Ai (17) demonstrated that the exponential term vanished as time elapsed and the system reached equilibrium, and Equation 4 was developed.

One can postulate the analytical setting producing the graph in Figure 8, whether it is more likely, that a non-steady state exists when the amount of the analyte is small, thus the amount entrapped by the extracting SPME phase is perhaps affecting the equilibrium (thus a non-steady state is present). Similarly, if the affinity of the analyte to partition into the extraction phase is very large, the equilibrium may be disturbed. On the other hand, the term ah is governed by the equilibrium time, and the linearity will be lacking if the steady state is formed more slowly during this particular analytical setting, especially when the amount of the analyte is small and the headspace volume large. An additional reason for the correlation curve might be that the upper or lower limit was reached in one of the two different quantitative methods.

The literature describes several examples of quantitative analysis using SPME. Most of them concern dilute and relatively simple mixtures of organic compounds in water,

especially those published at the beginning of the use of SPME technique. Additionally, one major application area is the analysis of residual solvents in water or in pharmaceuticals. Several papers have evaluated quantitative SPME of volatiles in foods and related materials, and the reader can search from the databases and application books for the individual publications according to one's interest. Here, we have collected a few examples of different kinds of quantitative analysis procedures, and we will focus on the analytical method itself.

Natera Marín et al (19) selected the CAR/PDMS phase to determine volatile compounds found in sherry vinegars. They validated the quantitative method by adding pure chemical compounds identical to those found in qualitative analysis of sherry vinegar to a matrix imitating vinegar composition (i.e. specified concentrations of acetic acid, tartaric acid, ethyl acetate and ethanol in water), but only one internal standard was used. They also studied the recovery of the chemical compounds typical for fermented products by the standard addition method. Their excellent discussion includes other publications describing aspects of quantitative analysis of these compounds.

Boutou & Chatonnet (20) utilized a similar approach in the analysis of off-flavours in red and white wines. For this purpose they used a divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) phase. They also developed a synthetic matrix (ethanol and tartaric acid in water, pH 7) to dissolve 18 standard compounds identical to target analytes to formulate the response curves. In addition, they used seven analyte-identical isotope-labelled internal standards for quantitative analysis. They carried out sample preparation prior to SPME sampling to improve their method. The adjustment of pH of wine sample, dilution of sample with water (1:1) and addition of sodium chloride all reduced the effect of ethanol on the sampling process and enhanced the partition of volatiles from liquid sample into the headspace. The analytes in wines, such as anisoles and fungal metabolites were satisfactorily quantified below their threshold of olfactory perception, and the results were very repeatable. The same cork-taint off-flavour compounds were studied by Bianco et al (7). They found that the DVB/CAR/PDMS fibre phase was not very stable in this analysis system, and the postulated reason for breaking the fibre phase (demonstrated by scanning electron microscopy) was the swelling of the phase by the dissolved ethanol. These workers did not dilute the wine samples prior to analysis, but instead they chose a PDMS fibre phase, which evidently was less susceptible to degradation by high ethanol concentrations.

The quantitative SPME analyses described above involved non-fat samples. Usually, fat-containing samples are analysed at elevated temperatures, i.e. at conditions where the fat is fully liquidified and thus more homogenous sample is obtained. Gonzáles-Córdova & Vallejo-Cordoba (21) studied volatile fatty acids (C4-C12) in milk samples. Their results were calculated using an external standardisation, and pentanoic acid (C5) was also used as an internal standard. Three SPME phases were compared and the method involving Carbowax/divinylbenzene (CW/DVB) fibre phase was found to perform well. They detected free fatty acids up to 18 carbons in their GC-chromatogram, when the sample was equilibrated at 70 °C. However, with the standard addition method they found that the solubility of C12 and longer fatty acids was too inconsistent to facilitate quantitative analysis. Later, Pereira et al (22) used a similar method, but with a polyacrylate (PA) fibre phase to quantitatively determine C4-C10 free fatty acids in goat milk and compare different pasteurisation processes.

In a recent paper, Abilleira et al (23) validated a quantitative SPME-GC-MS analysis method for terpenes. During validation, these workers compared milk oil phase, synthetic oil and methanol as matrix, and found internal standardisation using a single compound to be adequate for the quantitation.

Since there are several papers describing the SPME analysis of rancid vegetable oils, it is difficult to pick only one here. One detailed study was published by Vichi et al (6), who compared four SPME fibre phases for the analysis of volatile composition typical to virgin olive oils, and this work involved more than 100 compounds. As described above, the uptake of compounds into fibre phase is analyte specific (see Figure 5), and Vichi et al (6) reported that it was also fibre phase specific. In addition, the standard deviation of the peak areas of replicate analysis of an individual compound depended on the fibre phase. For standardisation, they used one internal standard and 28 reference compounds identical to analytes found in samples and for the quantitative study deodorised olive oil was used as a blank matrix. Thus a standard curve of area/µg added compound per gram oil was obtained and a good linear regression was obtained for different aldehydes, ketones and alcohols in the concentration range 0.1–2.5 mg/g oil.

The final two examples involve food and drink samples which contain suspended particles. Pohjanheimo & Sandell (24) compared volatile composition and sensory scores of fitness drinks. The responses of volatile compounds in fruit based fitness drinks were analysed using DVB/CAR/PDMS fibre and GC-MS. The quantitation was carried out using two internal standards and an external calibration curve for nine analyte identical reference compounds. Relative responses (STD/ISTD) were used to achieve the the quantification (mg/ml). Due to the fact that the drink samples contained compounds other than those used for external calibration curves, some of the quantitative results were only estimates, but a reliable external calibration was made. One advantage of this method was that the samples were kept at room temperature throughout the analysis procedure.

Jestoi et al (25) studied the furan content of baby foods. Since these samples were semisolid, they were homogenised with water containing sodium chloride prior to analysis to facilitate mixing during SPME sampling. After homogenisation, the internal standard was added. Due to high volatility of deuterated furan (internal standard), these steps were performed at 4 °C, but the equilibration and SPME sampling was performed at 45 °C to ensure the melting of fat. Figure 4 illustrates the chromatographic separation. Since different types of foods were studied, it would have been difficult to find a suitable blank matrix to for the preparation of standard curves for all sample types. Thus the calibration curves were made in water, and the validation studies showed that the results were acceptable. The authors claim this was due to the careful sample preparation, which assured that despite the variations in the sample matrix composition, the furan partitioned effectively into the headspace.

Future aspects and conclusions

Automation of the SPME sampling was introduced already in the early days of SPME applications, however at that time it was not possible to control the temperature of the sample vials prior to or during SPME phase explosure. This shortcoming has nowadays been overcome: sample vials can be cooled and warmed in different zones of automated

multipurpose sample stations such as CTC Combi PAL (CTC Analytics AG). In addition, vibrational systems capable of mixing the sample vial contents and the headspace vapours have definitely increased the attractiveness of headspace SPME in laboratories doing routine analysis, because the equilibrium is reached in a more practical time and it does not require human intervention between every step of sampling.

Throughout the lifetime of the SPME technique new coatings have been introduced by primary inventors and other researchers. Recently, even molecularly imprinted polymers (MIPs) have been introduced to be used as coatings in SPME (26). MIPs, that can confer highly selective sampling are discussed in another chapter of this book. Novel materials science – new mixed polymers and use of nanomaterials, will perhaps create new openings for SPME applications as well. ●

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6
Derivatization

Derivatization in GC analysis
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Derivatization in GC analysis

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Introduction

Good chromatographic behaviour and detectability of samples, but also their sufficient volatility and thermal stability, are crucial factors in gas chromatographic analysis (1). Different derivatization techniques have been developed to address these issues in order to make successful separation of different analytes possible. In GC derivatization, replacement of active hydrogen in functional groups, such as -COOH, -OH, -NH, and -SH, is the primary concern and it is typically accomplished through esterification, silylation, acylation, alkylation or chiral derivatization reactions (2).

The best way to choose a suitable approach is to first look at the functional groups present in the compounds of interest, and then read through textbooks (like (1) and (2)) and different articles to find a suitable derivative or derivatives for those groups. Once a derivative has been chosen, it is advisable to spend some time optimizing the derivatization procedure. Different workers often describe slightly different conditions for preparing the same derivative, and thus there may be a need to do some analyte-specific "fine-tuning" to the original method before embarking on the analysis of the samples. This need arises from the differences in the reactive groupings and their positions within the

molecules (e.g. steric factors) as well as from the differences in sample matrices. These factors potentially affect the reactivity of an analyte with the derivatization reagent.

Acid- or base-catalysed transesterification is a common way to prepare derivatives for the gas chromatographic analysis of fatty acids esterified to glycerol (glycerolipids) or sterol (sterol esters) moieties. The lipid esters may be solvolysed by methanol and, when methanol is present in excess, the original ester group may be replaced by an ester of a fatty acid and methanol (3). In a less common application, transesterification that results in the formation of methyl esters is used in the preparation of monomerized cutin and suberin samples for gas chromatographic analysis. These polymers are present in the aerial and underground parts of plants, respectively, and are potential fragments of dietary fibres.

Silylation is the most widely used derivatization procedure for gas chromatographic sample analysis. The popularity of silylation reagents is enhanced by their ease of use. In silylation, an active hydrogen is replaced by an alkylsilyl group such as trimethylsilyl (TMS) or *t*-butyldimethylsilyl (*t*-BDMS). Various reagents with different characteristics (e.g. basicity) are used in different applications (4). Silyl ethers and esters are commonly prepared from compounds containing hydroxyl and carboxyl groups, respectively. In food chemistry, trimethylsilylation of sugars, organic acids and lipids is a typical sample preparation step before their gas chromatographic analysis.

The purpose of this chapter is to describe in detail some typical derivatization techniques that are used in our laboratory for the analysis of different lipids, sugars, sugar alcohols, organic acids as well as cutin and suberin monomers. All the techniques presented are based on the formation of methyl esters or silyl ethers and esters from the analytes of interest.

Preparation of fatty acid methyl esters

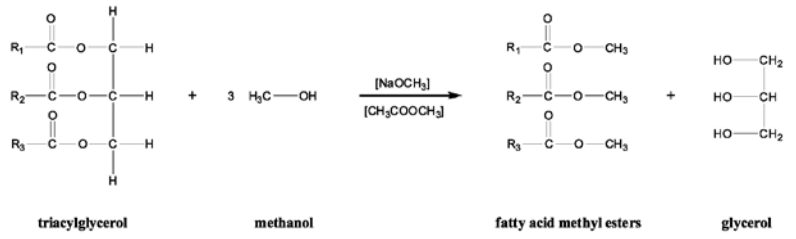
The fatty acid composition of a sample can usually be easily determined by GC after volatile fatty acid methyl esters have been prepared. In our laboratory, both sodium methoxide- and boron trifluoride-catalysed esterification reactions have been successfully used over the years for the derivatization of fatty acids in different types of food-derived and clinical lipid samples.

Formation of fatty acid methyl esters by sodium methoxide-catalysed transesterification

During the 1960's and 1970's, derivatization techniques based on sodium methoxide-catalysed transesterification of fatty acids were reported (5–9). For example milk fat triacylglycerols in hexane solution (19 volumes) were shown to be transesterified rapidly by means of sodium methoxide in methanol (1 volume) (6). In 1982, Christie (10) published a sodium methoxide-based approach that was suitable for a wide range of lipid classes. In his method, diethyl ether is used to solubilize polar lipids, methyl acetate is added to minimize competing irreversible hydrolysis reactions, and the mixtures are neutralized with oxalic acid when the reaction is complete. Small volumes of reagents are needed, and the method has a limited number of work-up steps. This work is the basis for one of the primary methods used in our laboratory for the glycerolipid (triacylglycerols and glycerophospholipids) fatty acid transesterification before their gas chromatographic analysis. In the method, oxalic acid is replaced by acetic acid as the neutralizing agent (Figure. 1).

Figure 1.

Sodium methoxide -catalysed transesterification of fatty acids in a triacylglycerol molecule.



Glycerolipids (up to 2 mg) are dissolved in sodium-dried diethyl ether (1 ml) and methyl acetate (25 µl). After a brief agitation, 1 M sodium methoxide in dry methanol (25 µl) is added, and the solution is agitated again to ensure thorough mixing. The solution immediately becomes cloudy as sodium-glycerol derivatives are precipitated. After 5 min at room temperature, the reaction is stopped by adding 6 µl of glacial acetic acid with brief agitation. The mixture is centrifuged at about 1500 × g for 5 min, and the supernatant is collected into an autosampler bottle. The solvent is evaporated gently under nitrogen (taking care not to blow out the fatty acid methyl esters). Hexane (1 ml or less) is added, and an aliquot of the sample is taken directly for GC analysis after thorough mixing.

Note that a longer reaction time (1 hour) would be needed for cholesteryl esters. If shorter-chain (C12 and below) fatty acids are present, the solvent-evaporation step should be avoided.

Formation of fatty acid methyl esters by boron trifluoride-catalysed (trans) esterification

Boron trifluoride is a Lewis acid that is a commonly used as a catalyst in esterification reactions. Early reports on its usage in the methylation of fatty acids in fats and oils are based on the work by Duron and Nowotny (11), Morrison and Smith (12), Metcalfe et al. (13, 14) and Gunstone and Ismael (15). Boron trifluoride-methanol can be used both in the esterification of free fatty acids and in the transesterification of fatty acids in different lipid classes. It is important to bear in mind that the shelf life of the reagent is limited and that isomerization of conjugated double bonds may be observed (16). In our laboratory, the approach based on the work published by Ågren et al. (17) has been regularly used in the formation of fatty acid methyl esters. The method does not include any purification or solvent evaporation steps during or after the derivatization. We have used the technique especially when total fatty acids have been determined or when sterol esters have been present in the samples.

To a tube containing a total amount of up to a few hundred micrograms of fatty acid-containing lipids and/or free fatty acids, 0.1 ml of toluene and 0.5 ml of BF_3-methanol (Fluka, Buchs, Switzerland, ~10 % BF_3) are added. The tubes are tightly capped and placed into a block heater (90–92 °C) for 60 min. If only free fatty acids are present, the heating time can be shorter. After cooling, 0.8 ml of distilled water and 1.0 ml of hexane are added and the tubes are shaken vigorously for 10 sec. Hexane phase containing the fatty acid methyl esters is directly taken for gas chromatographic analysis. If necessary, centrifugation can be performed to enhance the separation of the liquid phases.

Silylation of sterols

The general reaction for the formation of trimethylsilyl derivatives is:

$$CH_3Si–X + R'–H \rightarrow CH_3Si–R' + HX$$

It must be taken into consideration that silylation reagents and TMS derivatives are sensitive to moisture. Therefore, the reaction is carried out in a sealed reaction vessel resistant to heat (2). Trimethylchlorosilane (TMCS) is one of the first silylation reagents prepared (18). TMCS is rarely used alone in analytical applications, but rather in mixtures, e.g. hexamethyldisilazane (HMDS)/TMCS/pyridine or N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA)/TMCS. Like TMCS, HMDS was one of the earliest silylation reagents (19), and its analytical uses have been extensively reviewed (20). HMDS is a weaker silyl donor than many other silylating reagents (1, 4). BSTFA is probably the most widely used reagent for trimethylsilylation. The reagent was first prepared by Stalling et al. (21), and is available both as the pure compound and mixed with catalysts, commonly TMCS (4). In our laboratory, BSTFA/TMCS (Figure. 2) has been used in the analysis of total, free and esterified sterols of berry pulp/peel and seeds of sea buckthorn berries (22, 23) as well as in the analysis of free sterols and sterol esters in the seeds of bilberry and lingonberry (24). Plant sterols are an important group of bioactive compounds that lower the plasma cholesterol level and may regulate immune function and have anticancer effects (25).

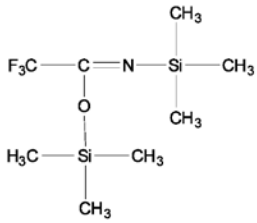
Analysis of saponified sterols

Saponification of lipids. After the addition of cholesteryl palmitate (1.6 mg) as an internal standard, sea buckthorn lipids (400 mg) are saponified by refluxing in 20 ml of a 1 M KOH ethanol/water (8:2, v/v) solution for 1 h. After 20 ml of water is added, the refluxed mixture is allowed to cool down. Thereafter, it is transferred into a separatory funnel. The reflux bottle is washed with 20 ml of water which is also transferred into the separatory funnel. The unsaponifiables in the combined solution are then extracted three times with 40 ml of diethyl ether. The ether phases are combined, washed three times with 20 ml of water, and dried with sodium sulfate overnight.

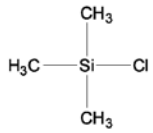
Isolation of sterols and preparation of TMS derivatives. The unsaponifiables (up to 30 mg) dissolved in petroleum ether (bp = 60–80 °C) are applied into a Sep-Pak® Classic/Plus Silica cartridge (Waters Corp., Milford, MA) preconditioned with 5 ml of petroleum ether. After elution of hydrocarbons with 10 ml of petroleum ether and carotenoids with

Figure. 2.

Structures of A) N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA), and B) trimethylchlorosilane (TMCS).



A
N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA)



B
trimethylchlorosilane (TMCS)

10 ml of petroleum ether/diethyl ether (96:4, v/v), the tocopherols and tocotrienols are eluted with 10 ml of petroleum ether/diethyl ether (91:9, v/v) and sterols with 20 ml of petroleum ether/diethyl ether (1:1, v/v). The sterol fraction is dissolved in 1 ml of chloroform. An aliquot of the sample (180 µl) is transferred into a glass tube and evaporated to total dryness under nitrogen. BSTFA (40 µl) and TMCS (20 µl) are added, and the tubes are tightly capped and incubated at 60° C for 2 h before GC analysis.

Analysis of free sterols and sterol esters

Isolation of sterols and preparation of TMS-derivatives. After adding cholesteryl heptadecanoate and free cholesterol as internal standards, the lipids (up to 30 mg) extracted from berry seeds are fractionated into different classes using a Sep-Pak® Classic/Plus Silica cartridge (Waters, Milford, MA) using a method based on the work of Hamilton and Komai (26). First, wax esters and sterol esters are eluted with 10 ml hexane:methyl *tert*-butyl ether (MTBE) (200:1, v/v). After that, triacylglycerols are eluted with 20 ml hexane:MTBE (96:4, v/v) and free sterols with 15 ml MTBE:acetic acid (100:0.2, v/v). The sterol esters are saponified and the sterols are extracted from the saponification mixture with diethyl ether as described above. TMS derivatives of the sterols are prepared in tightly capped glass tubes by incubating all of the free sterol fraction or diethyl ether extract, after solvent evaporation under nitrogen, in a mixture of BSTFA (80 µl) and TMCS (40 µl) at 60° C for 2 h.

Derivatization of sugars and organic acids

Sugars and many organic acids are polar, hydrophilic and poorly volatile compounds. Sugars can not be analysed as such by GC but need to be derivatized, and although many monocarboxylic acids are sufficiently volatile for gas chromatographic analysis, their derivatization yields improved peak shape (1). Sugars are polyhydroxy compounds that commonly occur in nature in many different molecular structures and anomeric forms. The main sugars present in fruits and berries are glucose, fructose and sucrose, and the main organic acids are citric, malic and tartaric acids (27, 28). Most of the dry weight of berries is composed of carbohydrates (sugars and fibres) and organic acids. Sugars and acids are often significant quality factors and they contribute strongly to the flavour of berries and fruits (27, 29–32). The hydroxyl groups of sugars and polyalcohols are easily silylated under mild conditions. Ideally, silyl derivatives are produced quantitatively under soft reaction conditions so that all anomers are eluted as separate silylated products which should be detectable in thechromatogram. The interpretation of the chromatogram with multiple peaks due to the formation of α- and β-anomers of furanose and pyranose can, however, be complicated. One solution for this is that reducing saccharides can be transformed into their oximes prior to silylation process (33). In the same way as for hydroxy compounds, TMS derivatives are also formed – although not as readily – from carboxylic acids. TMS derivatives of carboxylic acids are, however, somewhat less stable toward hydrolysis (1).

In our laboratory, sugars, sugar alcohols and organic acids of different berries have been successfully determined as their TMS derivatives by using HMDS and TMCS in pyridine as the derivatization reagents (29, 31, 34, 35).

Berries (30 g) are gently thawed in a microwave oven. They are crushed and filtered through a cheesecloth in a funnel. If necessary, the filtrate is centrifuged to avoid solid particles. An aliquot of 0.25 ml of the filtrate is diluted 1:20 by adding water (4.25 ml) and solutions of internal standards (0.25 ml of 0.5 g/100 ml sorbitol standard solution for the sugars and 0.25 ml of 0.5 g/100 ml tartaric acid standard solution for the acids, Merck, Darmstadt, Germany). Test samples without the internal samples are also prepared. The dilutions, about 1 ml each, are filtered through a 0.45 μm syringe filter. An aliquot of 0.3 ml of the filtrate is taken into a glass vial and evaporated to dryness under nitrogen flow (40–50° C). The samples are stored in a desiccator until derivatization. Tri-Sil HTP reagent (up to 600 μl, HMDS and TMCS in pyridine (2:1:10, by vol), Pierce Chemicals Co., Rockford, IL) is added to samples and the vials are shaken vigorously for 5 min. The samples are incubated at 60° C for 30 min (heat block) and held at room temperature for a minimum of 30 min before analysis.

Note that the reagents used do not silylate the oxo groups of dehydroascorbic acid. Thus the derivative will not be volatile enough for GC analysis (34). Dual solid-phase extraction (29, 31, 34) can be used for the fractionation of samples to sugars and acids but this step is not necessary.

Derivatization of cutin and suberin monomers

Plants require a watertight layer on their surface to protect them from desiccation, pollutants and other harmful substances. The water resistance is usually acquired by a wax layer, but this wax layer needs a polymer network to support its structure that it also plays important role in controlling the plant morphology. In the aerial parts of the plant, this polymer is cutin, present in extracellular cuticle, and in bark and the underground parts of the plant, such as roots and tubers, this polymer is intracellular suberin. The biosynthesis, structure, and function of cutin and suberin are the least understood of those of the major plant polymers (36–39).

Cutin is a polyester polymer composed of a complex mixture of interesterified and cross-linked long-chain ω-hydroxy fatty acids with typically a 16- or 18-carbon skeleton. The fatty acids are commonly substituted with secondary functional groups such as hydroxyl and epoxy groups. Minor proportions of long-chain fatty acids, diacids, alcohols, aromatic compounds and glycerol increase the extreme diversity of cutin (36, 40, 41). Cuticle is attached to the outer epidermal cells via a pectinaceous layer; cutin plays an important role as a structural component in the plant cuticle and as a defence barrier toward pathogens and the uncontrolled loss of water, as well as in transporting substances across the plant tissues. The wide variation in the structure of cuticle and in the composition of cutin between plant species, age and organ, resulting from the chemical diversity, may explain the different functions of cuticle (36, 39, 41, 42). Suberin is a polyaromatic-polyaliphatic biopolymer that is covalently bound to cell wall carbohydrates present inside the outer cell walls of periderm. Its polyaliphatic domain is an ester polymer whose monomers are similar to cutin monomers, but usually predominated by hydroxy substituted α,ω-dicarboxylic acids instead of ω-hydroxyadics (37–39).

Ester-linked cutin and suberin monomers may be depolymerized with ester-break-

ing reactions for analytical purposes. Some of the functional groups, like epoxy and aldehyde groups of cutin and suberin aliphatic monomers may not be stable in the depolymerization conditions and valuable information may be lost if the methods are not carefully monitored. Gas chromatographic separation of the complex mixture of cutin and suberin monomers obtained from the depolymerization reaction, followed by electron ionization (EI)-mass spectrometric (MS) detection, is necessary for analysis and identification, as reference compounds are commercially available only for some monomers. The most widely used derivatives, methyl ester TMS ether and TMS ester TMS ether derivatives of cutin monomers provide diagnostic spectra that make interpretation relatively unambiguous (43–46).

The method that has been used in our laboratory for the analysis of cutin and suberin e.g. in berries and potatoes, respectively, is transesterification with NaOMe in dry methanol (Figure. 3) followed by chloroform extraction and trimethylsilylation of the monomers (47–49). This method yields methyl ester TMS ether derivatives of cutin and suberin hydroxy acids and diacids, which have been bound via ester linkages in the polymer before the depolymerization by transesterfication. Moisture needs to be carefully excluded from the methanolysis reaction to avoid the formation of free acids.

Transesterification with methanolysis transforms the epoxides present in cutin and suberin monomers into the corresponding methoxyhydrin derivatives, which leads to the formation of vicinal methoxy and hydroxyl groups to the carbons bearing the epoxy group (Figure. 4). As the -OMe ion can attach to both of the carbons bearing the epoxy group, the result is an isomeric mixture of methoxyhydrin compounds. These elute as a single peak with non-polar GC column and can be easily identified with the aid of their mass spectral cleavages (50). Determination of epoxy-substituted monomers is very important in research related to berry cuticle, as they are main monomers for example in sea buckthorn (*Hippophaë rhamnoides*) berry cutin (~70 % of total) (48) and cranberry (*Vaccinium oxycoccos*) (~60 % of total) (49).

Figure. 3.

Cleavage of cutin polymer by transesterification.

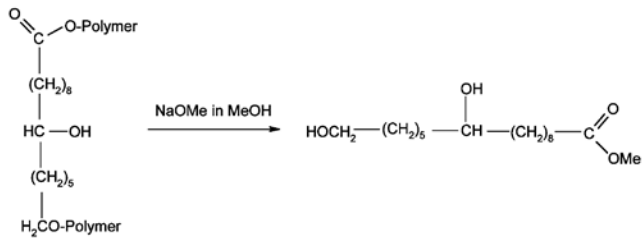
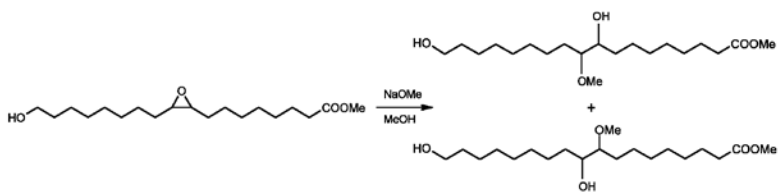


Figure. 4.

Conversion of 9,10-epoxy-18-hydroxyoktadecanoic acid methyl ester to an isomeric mixture of 9(10), 18-dihydroxy-10(9)-methoxyoctadecanoic acid methyl esters before trimethylsilylation.



Isolation. The cuticular and suberized membranes are isolated using the method previously described (47). Briefly, the procedure is a combination of enzymatic treatments with cellulase and pectinase, followed by exhaustive extraction using a Soxhlet apparatus with $CHCl_3$ and MeOH, yielding extractive-free cuticular/suberized membranes.

Depolymeration and derivatization. Dried samples of extractive-free cuticular or suberized membrane (20 mg) are treated with 3 ml of freshly prepared 1.0 M NaOMe in dry MeOH in a sealed test tube (48, 49). Methanolysis is carried out in a shaker (at approx. 500 rpm) overnight at room temperature. The reaction mixture is acidified with 2 M H_2SO_4 in MeOH and the supernatant is separated by centrifugation (1315 × g, 10 min). Then, 10 ml H_2O is added and the cutin/suberin monomers are extracted with $CHCl_3$ (2 × 10 ml). The mixture of monomeric methyl esters in $CHCl_3$ is dried with anhydrous Na_2SO_4, filtered and an aliquot of the monomers is dried under a stream of nitrogen and kept in a desiccator overnight. Trimethylsilylation is performed by adding Tri-Sil HTP reagent (HMDS and TMCS in pyridine (2:1:10, by vol), Pierce Chemicals Co., Rockford, IL) and by keeping the sample at 60° C for 15 min after being vigorously shaken at room temperature for 5 min.

Summary

Derivatization of analytes is essential in many GC applications. When suitable derivatization reagents have been chosen, it is worthwhile to exert some effort in the optimization of the reaction conditions and the amounts of different reagents to be used. In the analysis of samples containing lipids, sugars, sugar alcohols, organic acids or cutin/suberin monomers, approaches based on basic (trans)esterification or silylation reactions are often used. Several types of reagents and catalysts have been developed that can be used in these types of derivatization reactions. In our laboratory, sodium methoxide has been used as the catalyst in the formation of fatty acids and hydroxy acid methyl esters by transesterification, and boron trifluoride has been used in the formation of fatty acid methyl esters by transesterification as well as by the direct esterification of free fatty acids. Sterols have been derivatized by BSTFA and TMCS, and HMDS and TMCS in pyridine have been used in the derivatization of sugars, sugar alcohols, organic acids and cutin/suberin monomers. ●

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Derivatization in HPLC analyses

Pekka Lehtonen

Introduction

Derivatization prior to liquid chromatographic analysis, can be used to improve detectability, to change the molecular structure or polarity of the analyte in order to achieve better chromatography, to change the matrix for better separation and to stabilize a sensitive analyte in the sample preparation (1, 2). Fluorescent derivatives can be prepared to render the substances specifically detectable at high sensitivity. Sometimes, the polarity of a solute needs to be drastically reduced in order to improve its chromatographic behaviour and to reduce tailing.

Ideally, a derivatization reaction should be rapid, quantitative, and produce minimal by-products. Excess reagent should not interfere with the analysis or should be removed easily from the reaction matrix (1). If possible, the derivatization procedure should be adaptable to automation. The first consideration in choosing an HPLC derivatization method for detection enhancement is to decide which type of detection is best. In addition, the choice of pre- or post-column detection is needed. The matrix also affects the choose of the appropriate derivatization procedure: there might be compounds which interfere with one derivative type but not with another derivative. HPLC offers a wide

range of separation modes (i.e. normal- and reversed-phase and ion chromatography) and different types of stationary and mobile phases which can be used for the effective separation of derivatized compounds.

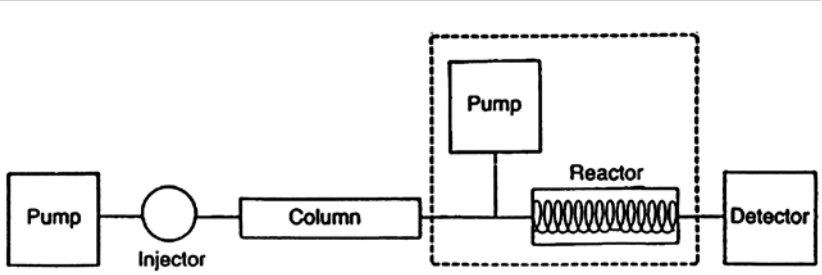
Before chemical derivatization is attempted, one should try to have a good understanding of the reaction mechanism and the factors affecting the ultimate yield. Many reactions that create suitable derivatives with pure standards may be unacceptable when applied to the matrix in question. Therefore, before developing or validating the derivatization process too far, one should conduct some simple experiments with the matrix. If the derivatization occurs effectively in the matrix the procedure can be continued but if this does not happen, it is possible that there is some interference from the matrix and it needs to be resolved whether it is really possible to use the chosen derivative with this matrix (2). Normally an excess of reagent is used to help ensure a high enough yield. The main requirement is that the yield be obtained reproducibly in the presence of the sample material. The sample coextractives can dramatically influence the reaction where no prior chromatographic separation has been employed. Often it is necessary to increase the molar excess of the reagent in a sample compared to a standard reaction in order to maintain reproducible yields of product. Derivatization, in addition to improving the sensitivity, can often be used to identify classes of compounds having specific chemical groups e.g. primary amino group.

Pre- and postcolumn derivatization

Chemical derivatization can be carried out either before chromatographic separation (pre-column derivatization) or after chromatographic separation through the post-column reactions (post-column derivatization). The advantages of the pre-column separation are as follows: there is no time limit for the reaction, reactive reagents may be used, and additional cleanup may be carried out on the derivative if necessary or desired. In the post-column derivatization, the derivatives are formed in a dynamic system. In this case, the LC eluent coming from the column is mixed with the reagents in a mixing coil. The reaction time is dependent on the flow rate and the reaction coil length (2). In particular, this method has been used to separate amino acids, aflatoxins, carbamates and other materials that show no or little UV absorbency. Derivatizing agent is introduced between the column and detector as presented in Figure. 1.

Figure 1.

Post-column derivatization.



The most remarkable difference between pre- and post-chromatographic reactions is that in the former case chromatographic separations are based on the differences in the nature of the product, not of the parent compounds, as in the post chromatographic derivatization. It should be also noted that nowadays also the precolumn derivatization procedure is often automated and can be quite a rapid reaction taking place in the needle of the liquid chromatograph.

Calibration

More accurate results when derivatization is applied are probably obtained using an internal standard due to the fact that derivatization can be considered as a significant sample preparation procedure. However, it can be difficult to find an appropriate substance that will elute in a position on the chromatogram that does not interfere or merge with any of the natural components of the mixture. The internal standard should be chosen so that it mimics the chemistry of the derivatized compounds. Often the internal standard does not mimic the chemistry of all analytes and the choice of the internal standard is based primarily on two criteria. The first is chemical stability. The internal standard must not be labile under the conditions employed. The second is that the internal standard must offer chromatographic resolution. This is not often straightforward, since the overall profile produces a chromatogram that might be cluttered (3). A careful validation process fulfils the suitability of a reagent as an internal standard. Examples of chromatograms of samples with an internal standard in the derivatization procedure are presented in Figures 3, 5, 11 and 14.

Detectors

The modern detectors for liquid chromatography permit highly selective detection of various compounds. A rapid scan feature allows on-the-flow spectral acquisition for UV-Vis as well as both emission and excitation spectra in fluorescence detection without interrupting the chromatographic elution.

UV-Vis absorbance derivatization

The most common LC detector in use for organic compounds is the UV-Vis absorbance detector. Thus most attempts at forming LC derivatives have been aimed at those that are strong absorbents especially of UV and but also of visible light. The chromophore in the derivatizing reagent should have a large molar absorptivity, with an absorption that can be used to maximize detection and minimize background noise. It should be noted that the fluorescent reagents are also strong absorbers of UV light and thus may be used for UV-absorption derivatization as well.

Fluorescence derivatization

Fluorogenic reagents are available for both pre- and post-column derivatization reactions. Most often the derivatization is carried out with reagents which themselves are

not fluorescent but which produce highly fluorescent products with the compounds of interest. Fluorescence derivatization allows more sensitivity and of course also selectivity because most of the possible interfering compounds are not fluorescent. With the correct selection of excitation and emission wavelengths, only the derivatized molecule is detected and those compounds which do not fluoresce under these conditions are not observed.

The solvent can exert a considerable influence on the spectral characterisation, especially where there is a strong interaction between the solvent molecules and the compounds of interest. For example, the effect of solvent on the fluorescence emission of dansyl derivatives of amino acids can be drastically reduced when measured in a polar solvent such as water (4).

Functional groups to be derivatized

The following describes the most common derivatizing procedures for different kinds of chemical compounds taking of the functional group used for derivatization. Phenols are not included because they are analysed most often without derivatization by liquid or gas chromatography or after derivatization by gas chromatography.

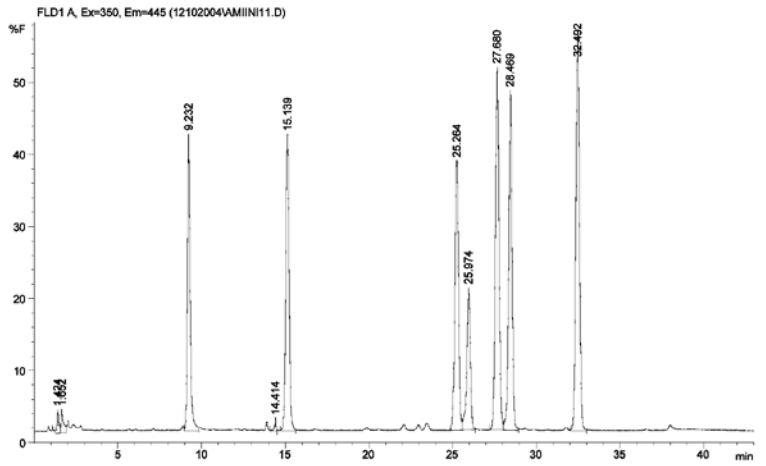
Amines and amino acids

Liquid chromatography is by far the most important technique for determining of amines. Derivatization and a modern fluorescence or UV detector are required. The most popular derivatization reagents for amines and amino acids are orthophthalaldehyde (5–9) and dansyl chloride (10–12). However there are numerous other derivatization reagents that have been used in liquid chromatographic analyses. Some of these reagents are listed in Table 1.

Table 1. The most common reagents that have been employed for the derivatization of amines and amino acids for liquid chromatographic analysis. Abbreviations: p primary amine, s secondary amine, FLD fluorescence detector, UV-Vis ultraviolet-visible detector.

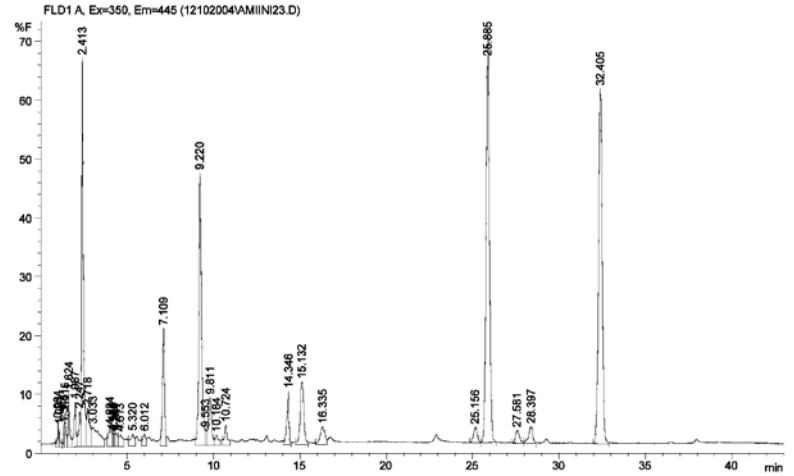
Reagent	*Abbreviation*	*Type Of Amine*	*Detector*
o-Phthalaldehyde	OPA	p	FLD
5-Dimethylamin naphthalene-1- sulfonyl chloride	DNS, dansyl	p, s	FLD, UV-Vis
Dabsyl-Cl	DBS, dabsyl	p, s	FLD, UV-Vis
9-Fluorenyl methyl chloroformate	FMOC	p, s	FLD
4-Chloro-7-nitrobenzo-2-oxa- 1,3-diazole	NBD-Cl	p	FLD
4-Fluoro-7-nitrobenzo-2-oxa- 1,3-diazole	NBD-F	p, s	FLD
4-Dimethylaminoazobenzene-4'-sulfonyl chloride	DABS	p, s	UV-Vis
4-Phenylspiro(furan-2(3H), 1'- phthalan-3,3-dione	Fluorescamine	p, s	FLD
6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate	AQC	p, s	FLD

Figure 2.



Fluorescence chromatogram of reference mixture of o-phthalaldehyde derivatives of amines: 1, histamine; 2, tyramine; 3, putrescine; 4, ß-phenethylamine; 5, cadaverine; 6, isoamylamine; IS (internal standard), hexylamine. Gradient separation with 0.08 M acetic acid – acetonitrile as mobile phase. Column: 125 mm, 4.0 mm LiChrospher RP-18, particle size 5 µm. Fluorescence detector, excitation 350 nm, emission 445 nm.

Figure 3.



Fluorescence chromatogram of a sample of red wine: 1, histamine; 2, tyramine; 3, putrescine; 4, ß-phenethylamine; 5, cadaverine; 6, isoamylamine; IS (internal standard), hexylamine. For chromatographic conditions see Figure. 2.

To prepare fluorescent derivatives of secondary amines, dansyl chloride, 5-dimethyl aminonaphthalene-1-sulphonyl chloride (11, 13), fluorenyl methyl chloroformate, FMOC (13, 14) and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (15) are recommended. Another fluorescent derivative is 4-chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD chloride) which provides highly fluorescent derivatives of primary and secondary amines but aromatic amines only yield weakly or non-fluorescent derivatives.

Often the same derivatizing agents are used for amines and amino acids. Reagents for amino acids include fluorenyl methyl chloroformate (FMOC) (6, 12, 16, 17), 4-chloro-

Figure 4.

Fluorescence chromatogram of a reference mixture of FMOC derivatives of amino acids. Gradient separation using a reversed-phase C-18 column. Fluorescence detection, excitation 254 nm and emission 313 nm.

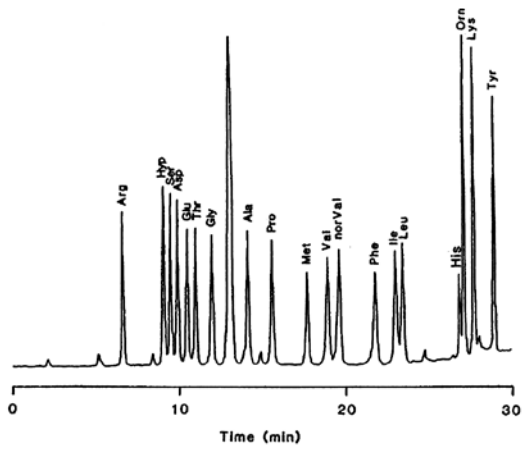
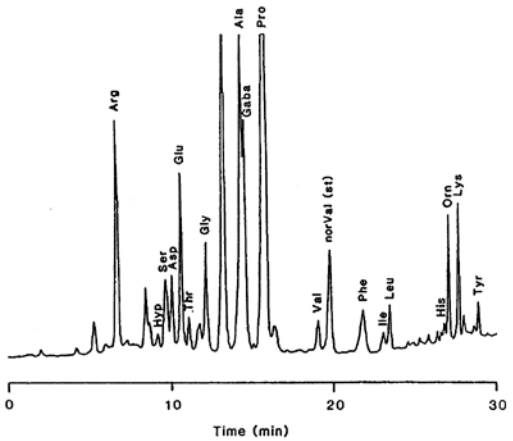


Figure 5.

Fluorescence chromatogram of a red wine sample derivatized with the FMOC reagent. Norvaline (norVal) is the internal standard. For chromatographic conditions see Figure. 4.



3,5-dinitrobenzotrifluoride (CNBF), and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) which can readily react with both primary and secondary amino groups (18), as well as phenyl isothiocyanate (PITC) for primary amino groups (19, 20).

Often the determination of amines is made from a complex matrix. In addition to the amine derivatives, only a few compounds possess absorption when fluorescence detection is applied. However, to avoid the appearance of interfering compounds from the chromatogram and from the column, the amines can be separated by manual or automatic (7) sample pre-treatment. Amines can be absorbed from a solution at pH 7.2 by a cation exchange solid-phase extraction technique, e.g. by using a commercial SCX SPE material (21). The SCX material contains silica with aliphatic sulfonic acid groups that are bonded onto the surface. The sulfonic acid group is strongly acidic (pKa <1), and attracts or exchanges cationic species in a contacting solution. The bonded functional group is charged over the whole pH range. In most

Figure 6.

Standard Chromatogram of aldehydes derivatized with dinitrophenylhydrazine. Peak identification: 1, acetaldehyde; 2, acrolein; 3, acetone; 4, crotonaldehyde; 5, methyl ethyl ketone. Isocratic separation using a 125 mm LiChropher 100 RP-18 column, particle size 5 µm. Isocratic separation with water:acetonitrile 35:65 mobile phase. UV-Vis detector, wavelength 363 nm.

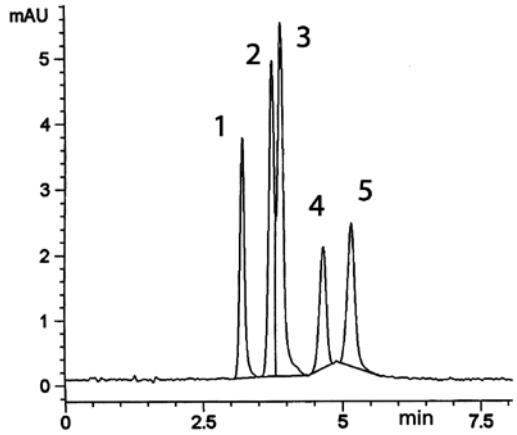


Figure 7.

Determination of acetaldehyde as DNP-derivative (peak no 1) in a black currant fruit wine. The peak corresponds to 27 mg/L content of acetaldehyde in the wine. For chromatographic conditions see Figure. 6.

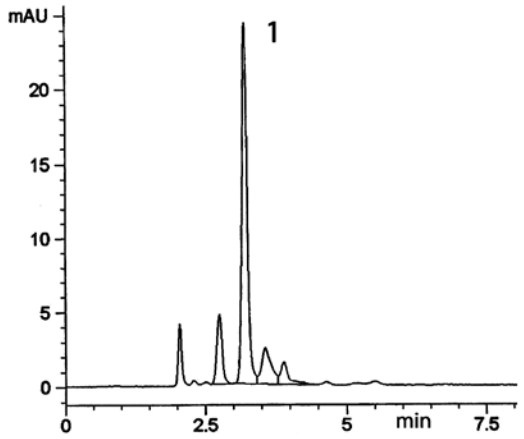
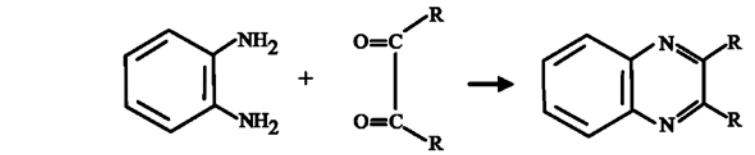


Figure 8.

Reaction schema for the derivatization of dicarbonyl compounds with 1,2-diaminobenzene.



1.2-Diaminobenzene **Di-carbonyl** **Quinoxaline**

Figure 9.

The principal α-dicarbonyl compounds of wine (24).

Glyoxal: OCH−CHO (ethanedial)

Methylglyoxal: CH3−CO−CHO (2-oxopropanal)

Diacetyl: CH3−CO−CO−CH3 (butane-2,3-dione)

Pentane-2,3-dione: CH3−CH2−CO−CO−CH3

cases, the compounds of interest are strong or weak bases. Elution is achieved with a solution at 2 pH units above the cation's pKa, or by adding a different cation that displaces the analyte.

As an example, the determination of amines in wine will be described (5, 22). At present, the amines in wines have been determined mainly by liquid chromatography, although other chromatographic techniques and capillary electrophoretic techniques can also be utilized in amine analysis. Histamine can also be determined by an ELISA method. We undertook a pretreatment of the wine sample by using a cation exchange solid-phase extraction tube (20). The wine was diluted 1:5 with water and applied into the SCX column. The SCX column was washed with a Sörensen buffer pH 7.2 and the amines were thereafter eluted with 3 % sodium chloride: pH 7.2 buffer : methanol in the ratio of 1:1:2 (v:v:v). The automated precolumn derivatization was performed (5). The chromatograms are shown in Figures 2–3.

Examples of the separation of amino acids after derivatization with fluorenyl methyl chloroformate (FMOC) are presented in Figures 4–5 (12). After the addition of norvaline, which was the internal standard, the wine was diluted 1:10 with the derivatization buffer, followed by automated precolumn derivatization with FMOC. The derivatized amino acids were detected with a fluorescence detector. Excitation occurred at 254 nm and emission at 313 nm.

Carbonyl compounds

If one wishes to introduce UV-Vis chromophores into a solute containing a carbonyl group (aldehydes and ketones) dansyl hydrazine, p-nitrobenzylhydroxylamine hydrochloride and 2,4-dinitrophenylhydrazine are probably the most common and effective reagents for liquid chromatographic analysis. The most widely used derivative for carbonyl compounds is the corresponding 2,4-dinitrophenyl (DNP) hydrazone. These hydrazones are formed by the addition of 2,4-diphenylhydrazine in acidic solution; the resulting precipitate can be removed by filtration, then dissolved in a suitable solvent prior to liquid chromatography (23, 24). Alternatively an organic solvent, e.g. heptane, can be added to the derivatization mixture and the formed derivatives can be extracted with an organic solvent which is then injected into liquid chromatograph. Chromatograms after such a treatment are shown in Figures 6–7.

The identification of DNP-hydrazones is usually based upon their retention times. A photodiode-array detecter can also be employed to identify the compounds by recording the UV-Vis spectra during the analysis. Although the shape of the UV-spectra of the DNP-hydrazone compounds is similar, there are significant differences in the wavelengths of the absorption maxima (23).

α-Dicarbonyl compounds

α-Dicarbonyl compounds can be derivatized with 1,2-diaminobenzene (25, 26). The method is based on the formation of the quinoxaline type derivatives (Figure. 8).

For determination of α-dicarbonyl compounds (glyoxal, methylglyoxal and diacetyl) in coffee, a RP-HPLC with diode array detection method was devised that consists of the elimination of interfering compounds, such as chlorogenic acids, by solid phase extraction (SPE) and their derivatization with 1,2-diaminobenzene to produce quinoxaline derivatives. The method resulted in recoveries of 100 %. The method was selective, precise, accurate and sensitive (25).

Figure. 10.

Standard Chromatogram of dicarbonyl compounds derivatized with 1,2-diaminobenzene. Peak identification: 1, glyoxal; 2, methyl glyoxal; 3, diacetyl; 4, pentane-2,3-dione; 5, hexane-2,3-dione (internal standard). Gradient separation with 0.5 % acetic acid – methanol as mobile phase. Column: a 125 mm LiChropher 100 RP-18 column, particle size5 µm. UV-Vis detector, wavelength 313 nm.

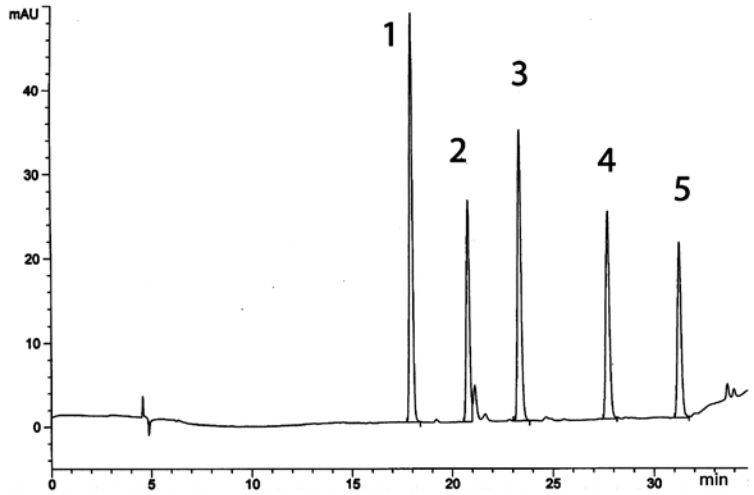


Figure. 11.

Determination of dicarbonyl compounds as quinoxaline derivatives in white wine. For peak identification and chromatographic conditions see Figure. 10.

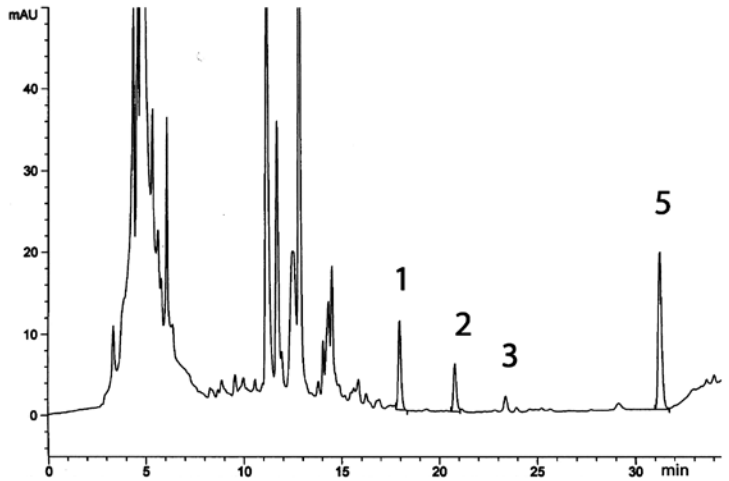
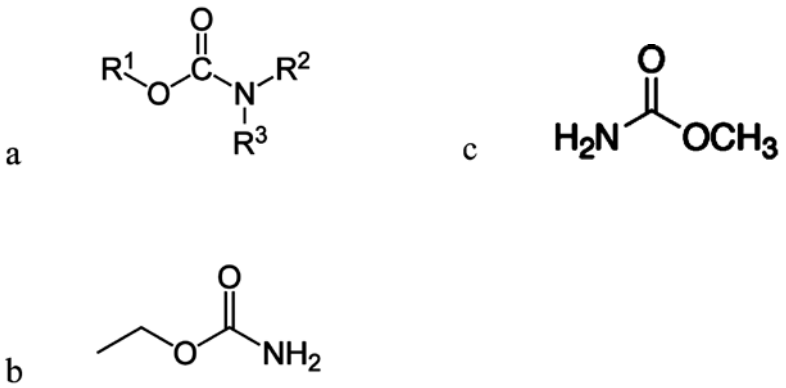


Figure 12.

Chemical formulas of carbamates. a) general structure; b)ethyl carbamate or urethane; c) methyl carbamate. R1, R2 and R3 refer to organic groups.



The principal α-dicarbonyl compounds found in wine (Figure. 9) are: glyoxal, methylglyoxal, diacetyl and pentane-2,3-dione, but only α-diketones are relatively abundant in wine. Dicarbonyl compounds are important in wine for different reasons: their sensory impact, their reactivity with other components of the wine or possible microbiological effects. Carbonylated compounds exist in all types of wines, particularly after malolactic fermentation and in red wines. In addition, sweet white wines produced with botrytized grapes can contain high levels of glyoxal and methylglyoxal (25).

The reaction takes place directly in the wine at pH 8 and after the reaction time of 3 h at 60°C. The analysis of the derivatives is then carried out directly by HPLC with detection at 313 nm. Examples of chromatograms of a standard mixture and of white wine are presented in Figures 10 and 11, respectively.

Carbamates

Carbamates can be determined after the derivatization with 9-xanthydrol under acidic conditions. The chemical formulas of the principal carbamates are shown in Figure 12.

An analytical method based on HPLC-FLD with prior derivatization with 9-xanthydrol for the determination of ethyl carbamate (EC) in cider spirits has been developed (27). The limits of detection and quantification were 1.6 and 3.6 µg/l, respectively. Recoveries ranged between 94 % and 98 %, while the precision of the method was <5 % (RSD).

An HPLC method for determination of urea which used an automated procedure for xanthydrol derivatization has been described (28). Unlike the standard derivatization technique (involving precipitation of dixanthylurea (N,N'-di-9H-xanthen-9-ylurea), the proposed xanthydrol procedure produced N-9H-xanthen-9-ylurea which remains in solution to allow subsequent fluorescence detection (excitation 213 nm, emission 308 nm) following chromatographic removal of interferences. The limit of detection for urea was 0.003 mg/l.

Xanthydrol derivatization procedure has been used for the determination of urea in packaging materials (29). A method based on a xanthydrol derivatization was developed to determine ethyl carbamate in wine, fortified wine and wine brandy (30). The procedure used did not require any prior sample extraction or concentration. The average recovery was 96 % among the samples studied, the detection limit was 4.2 µg/l, and an average intermediate precision was 6.3 %. Chromatograms of the determination of ethyl carbamate in whisky are shown in Figures 13–14.

Methyl carbamates can be determined after purification steps and postcolumn derivatization by RP-HPLC with fluorescence detection. A rapid multiresidue method was developed for the analysis of N-methylcarbamate insecticides (oxamyl, methomyl, propoxur, carbofuran, carbaryl and methiocarb) in fruit and vegetable juices (31). The method was based on the adsorption of the N-methyl carbamates in Florisil and the subsequent extraction of pesticides with acetone. The separation of carbamates was performed on a C8 column with water-methanol as the mobile phase. The method was linear from 10 to 1000 ng/ml, and the detection limits for carbamates varied from 0.8 to 1.9 ng/mL.

Trace levels of N-methyl carbamate pesticides and some of their main metabolites in fruits and vegetables were determined using postcolumn derivatization (32). The residues were extracted with CH_2Cl_2, purified by gel permeation chromatography, and determined by reversed-phase HPLC using fluorescence detection.

Figure 13.

Standard chromatogram of carbamates as xanthdydrol derivatives. Peak identification: 1) ethyl carbamate; 2) propyl carbamate (internal standard). Gradient separation with 20 mM sodium acetate, pH 7.2 – acetonitrile as mobile phase. Column: a 100 mm, 2.1. mm Hypersil ODS column, particle size 5 µm. fluorescence detector, excitation 233 nm, emission 600 nm.

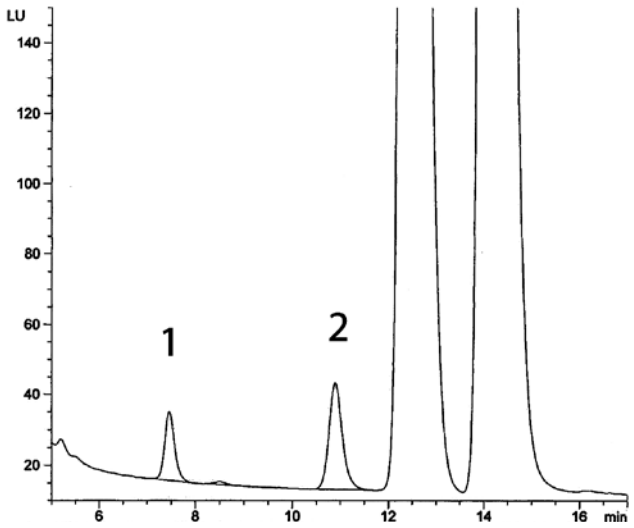
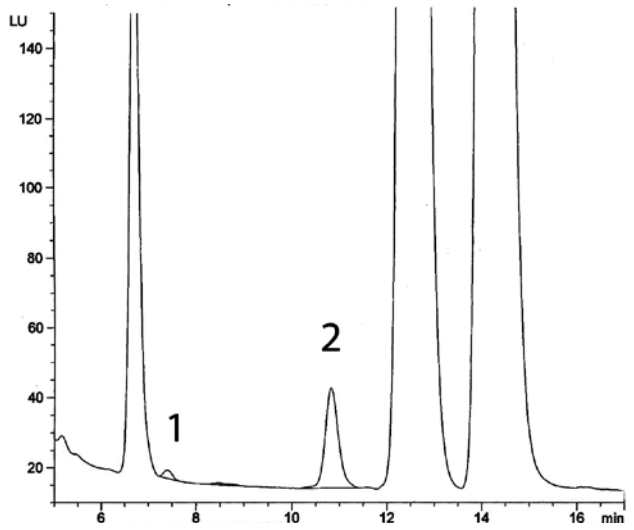


Figure 14.

Determination of ethyl carbamate as xanthydrol derivative in whisky. For peak identification and chromatographic conditions see Figure. 13.



Post-column derivatization was used for the determination of N-methyl carbamates in water. An aliquot of the water sample was extracted with dichloromethane. The raw extract was concentrated and analyzed in an HPLC equipped with a post column derivatization unit and fluorescence detector, or alternatively by LC/MS or LC/MS/MS. Carbamates were separated on a reversed-phase liquid chromatography column, using a methanol-water gradient mobile phase and derivatized with o-phthalaldehyde (33).

Conclusion

Derivatization in liquid chromatography has become extremely important in quantitative analyses of compounds which do not have a suitable chromophore for the most common detectors. Although sensitivity is of prime importance when employing chemical derivatization for LC, selectivity must also be considered. This can be achieved through the reaction itself. The proper choice of the detector, e.g. UV-Vis or fluorescence, can increase selectivity considerably. Reagents which react specifically with a certain compound type, e.g. primary amines, can be considered to be very selective. Derivatization is equally important as sample selection, preprocessing and chromatography as an important part of the overall analytical technique. ●

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7
Quality

Quality assurance and sample preparation

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Quality assurance and sample preparation

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Quality is a relative concept, i.e. it is neither high nor low in an absolute sense. Instead it is rather adequate or inadequate, depending on the requirements for the analyzing method specified beforehand by different factors, like standards, legislation and the customer (1). A formal recognition of quality can be achieved through accreditation or certification based on international quality standards and guidelines (2–4). Essential criteria for the quality of produced analytical information received from using analyzing method are the utility and the reliability, which are closely related to the method uncertainty in the measurement result regarding both the identity and the concentration of the target components. Validation of analytical methods, including validation of sample preparation steps, is an essential part in the process of Quality Assurance (QA) and quality control (QC) of chemical measurements.

Introduction

The sample preparation strongly depends on the analytical techniques to be employed and their capabilities. For instance, only a few microliters can be injected into a gas chromatography. Sampling, sample preservation, and sample preparation are all aimed at producing those few microliters that represent what was present in the native sample. All measurements are

accompanied by a certain amount of error, and an estimate is necessary to validate the results. It is obvious that an error in the first steps like homogenization, size reduction, extraction, concentration and clean-up, before the sample is ready to be injected, cannot be rectified by even the most sophisticated analytical instrument. The importance of the sample preparation cannot be underestimated when the measurement uncertainty is estimated (5). Typically the relative contribution of solid phase extraction step in sample preparation accounts for 75 % of the uncertainty, the rest from the analytical procedure, in this case from the liquid chromatographic mass spectrometric analyzing method. Consequently, both random and systematic errors are higher during sample preparation that during analysis.

QA consists of processes needed to fulfill the requirements specified for the analytical result. The difference to the term QC is that QA can be thought of as being related to process quality, whereas QC is related to the quality of the product, in this case, the analytical information about the chemical composition, usually in terms of identity and/or quantity of one or more relevant components in samples taken from analyzed matrices. Thus the QA provides the frames e.g. calibrations of instruments and instructions to follow and QC produces information e.g. results of control samples and recovery of internal standard during the analyses against the original specifications that were beforehand created. Identifying the quality issues of sample preparation and separating it the whole analyzing method (from sampling to calculation the results) is not quite simple but during method optimization and validation, the important control steps in terms of QA as well as QC can be specified. Validation of sample preparation should include an evaluation of the attributes such as the trueness by recovery studies, precision by repeated determinations and specificity for separate analytes.

Recovery studies

Trueness of the sample preparation techniques is specified by recovery studies. Recovery is the proportion of the amount of analyte, present in or added to the analytical portion of the test material, which is extracted and present for measurement. The distance between the mean value and the true value is a systematic error or bias.

Trueness = (mean – true value / true value)

The recovery of the analyte is especially important if one wishes to estimate in those types of sample preparation techniques where the loss of analyte during the analytical procedure is inevitable. Validation of sample preparation should also include the recovery trials in different concentration range of analytes, while recoveries may not be proportional to the concentration (6).

When appropriate certified reference material is available, the recovery value can be used to estimate the standard uncertainty of the recovery, evaluate it in the whole uncertainty budget and also include the standard uncertainty of recovery into the calculation of the method uncertainty of the test result (3). However, the range of appropriate matrix reference material is limited and especially in food analyses, reference materials usually differ from the same kind of original fresh food samples.. Reference materials are often finely powdered and dried to ensure homogeneity and stability and this kind of treatment is likely to affect the recovery compared to the sample preparation of fresh food. This matrix mismatch is a general problem

in the application of recovery information, even in the case when the matrices are reasonably well matched. This can be avoided in princible only by conducting a recovery experiment for each separate test material analyzed.

The recovery of analyte can also be estimated by studying the recovery of an added compound or element that is regarded as a surrogate for the native analyte. This procedure permits the loss of analyte to be corrected by using the recovery factor in correcting the estimated bias. In order to be valid, the surrogate must behave quantitatively similarly as the native analyte in the matrix, especially with regard to its partition between the sample preparation steps. The best type of surrogate is an isotopically-modified version of the analyte which is used in the isotope dilution approach, but limited by the availability of isotopically enriched analytes.

One common way to estimate the recovery of the analyte is addition as a spike. Spiking can be made to blank matrix or to an ordinary test portion of the sample that is analysed together with an unspiked sample. Problems may be caused by uneffective equilibrium of the supplemental analyte, if the added analyte is not so firmly bound to the matrix as the native analyte, then the surrogate recovery may be higher compared to that of native analyte, thus leading to a negative bias in a corrected analytical result.

The recovery can also be estimated by additions of internal standards before sample preparations steps. This is often the case when multiple analytes are analyzed from the same sample matrix. However biases in both directions could result from the use of recovery estimate of internal standard, which is not chemically identical to analytes.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision is expressed in terms of standard deviation and it is generally dependent on the concentration of an analyte, while it is well known that precision of an analyses decreases disproportionately with decreasing concentration (5). Precision is particularly important when sample preparation is involved. Generally speaking, analytical instruments have become quite sophisticated and provide high levels of precision. On the other hand, sample preparation often remains a complicated process that accounts for the majority of the variability, which can also affect accuracy. Validation aims at specifying the minimum number of samples to meet a certain level of statistical certainty .A simple approach to estimating the number of samples (n) is to repeat the sample preparation and analysis to calculate on overall standard deviation (s) and use Student's t distribution value for a given confidence level and choose the acceptable level of error (e):

$$n = (ts/e)^2$$

The spread of the results would in most case form a normal distribution between the mean values caused by random errors. Precision consists of three components: repeatability, intermediate precision and reproducibility. Repeatability studies consist of multiple measurement of a sample under the same analytical conditions. Intermediate precision, also known as within-laboratory reproducibility estimates the reliability of the method in a different environment (e.g. multiple days, analyst, instruments etc.) than that used during the

development of the method. Reproducibility is precision between different laboratories.

The same quality control samples can be used as in recovery studies: reference material (certified or laboratory made) and spiked samples, The standard deviation, relative standard deviation and confidence interval can be reported as precision values. One common method used in laboratories is to calculate the standard deviation of duplicate determinations as the square root of the sum of differences between duplicate results, divided by 2 times the number of duplicate determinations.

Selectivity and specificity

Specificity is a quantitative indication of the extent to which a method can distinguish between the analyte of interest and interfering substances on the basis of signals produced under actual experimental conditions (4). Random interferences should be determined using a representative blank sample. Thus, a blank sample and one or more samples to which a known amount of the analyte has been added, are analyzed to check for possible interfering compounds in the sample, from degradation products, metabolites or known additives. In some cases, a more concentrated extract of a blank sample may be analyzed to confirm that no signals occur. The specificity is also checked against other methods based on different principles of sample preparation technique. Low specificity of sample preparation method is not a problem if high specific techniques, like mass spectrometric detection are used. Also, screening methods may be un-specific, while positive results are usually reanalyzed and confirmed with another method.

Sensitivity and limits of detection, quantitation and linearity

Sensitivity of a method is a measure its ability to distinguish between small differences in analyte concentrations at a desired confidence level (5). The simplest measure of sensitivity is the slope of calibration curve. Recovery of the sample preparation steps has to be factored in: the higher the recovery, the higher the sensitivity. Since the precision decreases at low concentrations, the ability to distinguish between small concentration differences also decreases, therefore sensitivity is a function of concentration:

$$a = mr / s$$

Where mr is the slope with extraction efficiency r and
s standard deviation of sample preparation.

The detection limit (LOD) is defined as the lowest concentration or weight of analyte that can be measured at a specific confidence level. Usually the signal generated approaches that observed in the blank and the detection limit is defined as the concentration where the signal/noise ratio reaches an accepted value, 2–4 usually. Again, a low recovery increases the detection limit and thus affects also the limit of quantitation (LOQ 10 times signal/noise ratio), while LOQ of the overall method increases by 1/r. The upper LOQ is the point where the calibration curve becomes nonlinear and it is called the limit of linearity. The linear dynamic range, the working area of analytical methods are expected to have at least two orders of magnitude and this area needs to be determined by serial dilution of a sample.

Stability

The sample must be representative of the object under investigation. The physical processes that may degrade the sample are volatilization, diffusion, and adsorption on surfaces. Possible chemical/biochemical changes include enzymatic, biodegradation and photochemical reactions as well as precipitation (5). The stability of an analyte in a given matrix under specific conditions for given intervals of time should be included in the validation of the sample preparation procedure. Stability studies should include data to support the sample solution stability under normal laboratory conditions for the duration of the test procedure. Possible long term storage, for example freezing temperatures, and stability after thawing, need to be tested if this will be a part of sample preparation.

Summary

A typical QA scheme for sample preparation involves validation processes for the following steps:

- trueness and precision are determined in the concentration range where the method is to be used
- detection limit is established for each analyte
- the linear dynamic range is established
- the calibration sensitivity is measured

Once the analytical method including sample preparation has been validated for routine use, its trueness and precision needs to be monitored regularly to ensure that the method continues to perform satisfactorily. The results of QC samples provide the basis foraccepting or rejecting the analyses.

Approaches to reduce uncertainty during sample preparation are available. It is important to have information on the effects of different steps and, if possible, to eliminate the steps causing the most error, e.g. multiple extraction and cleanup methods and choose more selective extraction procedures to improve both trueness and precision. The goal should be to choose a combination of sample preparation and analytical instrument that reduces both the number of sample preparative steps and the deviation. Automated techniques with less manual handling tend to have higher precision. ●

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8
Extraction

Overview of extraction techniques and the theory behind
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Overview of extraction techniques and the theory behind

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Introduction

The separation of desired ingredients from cereals, fatty oil-bearing seeds and fruits, grapes and aromatic plants was already known in ancient times. In the brewing process, cooked and/or uncooked malt were suspended in water and fermentation occurred in the rinsed sugar- and starch-rich liquid (1). There is also evidence of the use of fatty oils for cooking, illumination and medicinal purposes (2), as well as the production of fermented beverages of rice, honey and hawthorn or grapefruits (3). Plant volatiles were of particular importance for flavoring foods and beverages, and preparing perfumes (4,5). A simple distillation device, presumably for the separation of odoriferous principles, was even illustrated by Alexandrian alchemists already in the first century (6).

Analyses of volatile fatty acids and volatile oils were also the first applications of the newly developed gas chromatographic (GC) technique in the 1950's. Within the wide research areas of fats, GC data on human tissue fatty acids was soon published, and the basic features of fatty acid metabolism, which had been studied earlier in animal tissues, could be verified also in humans (7). Since then, the rapid developments in capillary columns, liquid phases, injection techniques and selective detectors have achieved much

higher sensitivity, precision and accuracy in the analyses. The considerable improvements in resolution enabled detailed analyses of highly complex volatile oils containing hundreds of compounds. However, it was also found that the composition of oil did not always represent exactly that present in the intact plant (4, 5, 8).

The next breakthroughs emerged with the improvements made in the instrumentation of liquid chromatography (LC), e.g. better column packing, control of fluid flow, column size, packing material separation capacity and detection, and thus since the late 1960s, high performance LC (HPLC) has become the preferred analytical technique in the separation and quantitative analysis of a wide range of samples (9). The early applications included analyses of antibiotics, analgesics, pharmaceuticals and vitamins, pesticide residues in fruits and vegetables, food toxins and contaminants etc. However, biomedical applications remain one of the most rapidly expanding growth areas of LC. The HPLC coupling with mass spectrometry with new ionization methods has enabled high-precision analysis of biomolecules of high molecular weights. Since the detection limit can reach the low picogram level or even less (10, 11) this has also set special purity requirements for solvents, chemicals and equipment used in the sample preparation.

This chapter will focus on conventional extraction techniques and extraction performance of new instrumentation and their combined use in sample preparation. Selected applications dealing with more detailed optimization of extraction methods from various matrices are illustrated. Theoretical aspects such as mass transfer between immiscible phases are described for different extraction techniques. The physicochemical processes underpinning the extraction technique, parameters of solvents, their classification and solvent selection with applications are explained.

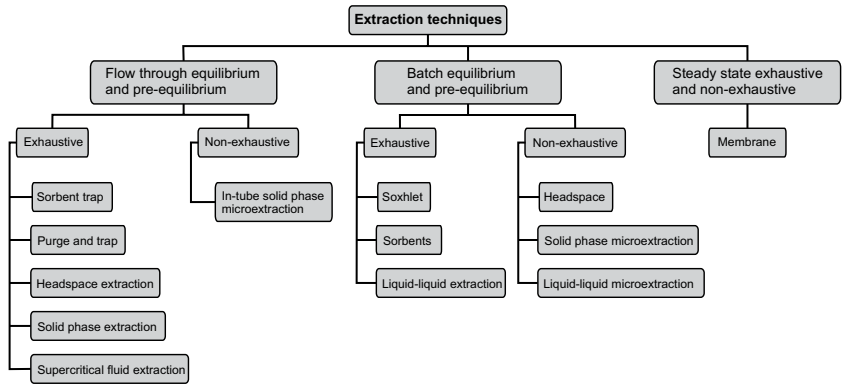
Extraction techniques

The isolation of the desired compounds is a critical step in sample preparation. Often, conventional separation methods such as maceration or Soxhlet extractions are reasonably efficient though the extract may be obtained in large amounts of solvent with unwanted constituents which may interfere in instrumental analysis. This would require time-consuming solvent removal and sample enrichment to a suitable concentration, and a further clean-up. The separation of aroma compounds from volatiles of foods by direct solvent extraction can be problematic because of co-extracted matrix components. Thus, if the true composition present in the plant material has to be determined, the isolation system must be carefully selected. One promising method is headspace solid-phase micro-extraction (SPME) (12). It has also to be noted that even cutting and grinding of plant materials can result in newly-formed volatiles derived from enzymatic cleavage of non-volatile precursors (4, 5).

The aim of an exhaustive extraction method is to completely remove all the analytes from a sample matrix and to transfer them to the extraction phase (13). Based on the fundamental similarities of all extraction techniques i.e. the extraction phase is in contact with the sample matrix, and analytes are transported between phases, a classification between exhaustive and non-exhaustive methods has been made (Figure 1). The extraction procedure can be accelerated by using high temperatures and analytes can be enriched quantitatively without thermal decomposition, and with much smaller vvolumes of organic solvents as in hot solvent (14) and microwave extraction (MAE) techniques (15).

Figure 1 .

Classification of sample preparation techniques according to (13).



Maceration, percolation, digestion

In conventional extraction methods described in many pharmacopoeias, maceration is defined as follows: powdered vegetable drug is soaked at room temperature with a solvent or solvent mixture in a close container by mixing or shaking frequently. The drug is allowed to stand for an appropriate time, after which the mixture is filtered and the residue is pressed out. In digestion, powdered vegetable drug is soaked for an appropriate time at 35–45° C.

In the European Pharmacopoeia (16), the extracts such as tinctures are defined as liquid preparations that are usually obtained using either 1 part of herbal drug or animal materials and 10 parts of extraction solvent, or 1 part of material and 5 parts of extraction solvent. Tinctures are prepared by maceration or percolation using only ethanol for extraction, or by dissolving a soft or dry extract in ethanol. In maceration, the herbal drug or animal material to be extracted is reduced to pieces of suitable size and mixed with the extraction solvent and allowed to stand for an appropriate time in a closed container. The residue is separated from the extraction solvent and, if necessary, pressed out. The liquids obtained are combined. In the production of tinctures by percolation, the material is first treated for an appropriate time, as above. Then, it is transferred to a percolator and the percolate is allowed to flow slowly, making sure that the material is always covered with solvent.

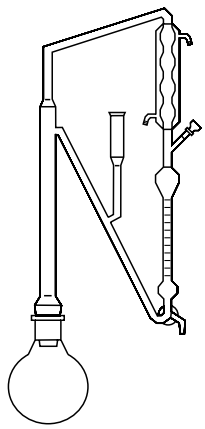
A study investigating the effects of maceration on the transfer of flavanols from grape seed and skin into wine reported increases in the flavanol contents in the wines after a week of post-fermentative maceration (17). The isolation of *Crataegus* flavonoids by maceration and Soxhlet extraction produced lower yields than new instrumental techniques (18), whereas the maceration of alkaloids has given similar yields compared to the MAE procedure. However, the concentration of alkaloids in the final percolation extract was higher than that obtained by other methods (19). In the extraction of mate tea leaves the concentrations of caffeine, fatty acid and terpenes as quantified by GC-MS, showed that the maceration resulted in lower yields than pressurised liquid extraction (PLE), for example (20).

Steam distillation

According to the European Pharmacopoeia, essential oil is produced by the passage of steam through the plant material (steam distillation). The steam may be obtained from an external source or generated by boiling water below the raw material or by boiling

Figure 2 .

Schematic diagram of the distillation apparatus described in the European Pharmacopeia.



water in which the material is immersed (16). A schematic diagram of the distillation apparatus described by the European Pharmacopeia is presented in Figure 2. In general, steam distillation at atmospheric pressure is a convenient method for the isolation of plant volatiles from non-volatile materials. The main advantage is that distilled oil does not contain non-volatiles that could interfere with subsequent analyses. However, some volatiles may be thermolabile or they may form secondary products not found in intact cells, but produced during sample homogenization or via isomerisation, saponification and polymerization processes (4,5). The Likens-Nickerson apparatus and its modifications developed for combined steam distillation-extraction have several advantages: the volatile fraction can be rapidly concentrated and only small amounts of solvents are needed and thermal degradation can be diminished by using reduced pressure (5).

Recently the composition and antioxidant activity of cinnamon oil obtained by hydrodistillation and microwave-assisted extraction was described (21), as well as the use of a hydrodistillation–adsorption device for fractionation, with a column of activated carbon for the adsorption of water-soluble compounds (22), and the duration and yield of hydrodistillation of essential oils of microwave pretreated samples from conifer species (23). Further applications will be described later in this chapter.

Soxhlet extraction

Extraction of plant matter may be performed by simply soaking the material in an Erlenmeyer flask or by using a device useful for many kinds of solvent extractions. A Soxhlet apparatus (Figure 3) consists of solvent vapour reflux condenser, a thimble holder with a siphon device and a side tube (15). The sample, usually approximately 10 g, is loaded into a porous cellulous sample thimble, and about 300 ml solvent with boiling chips is added to the flask and the heating is started. The extraction time can range from few hours up to 24–48 hours (Table 1). The evaporation of a large volume of the solvent in the extract is necessary before extract cleanup and analysis. The Soxhlet method is often the benchmark when comparing other techniques. An automated Soxhlet procedure, instead, is carried out in three stages: boiling, rinsing and solvent recovery (15). The technique is much faster with comparable recoveries but with lower solvent consumption than with the ordinary Soxhlet method.

Figure 3.

Schematic diagram of a Soxhlet apparatus.

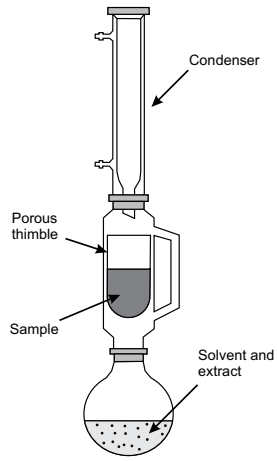


Table 1 .

Advantages and disadvantages of various extraction techniques according to (15).

Techinique	*Advantages*	*Disadvantages*
Soxhlet extraction	Not matrix dependent Very inexpensive equipment Unattended operation Rugged, benchmark method Filtration not required	Slow extraction (up to 24–48 hrs) Large amount of solvent (300–500 ml) Mandatory evaporation of extract
Automated Soxhlet extraction	Not matrix dependent Inexpensive equipment Less solvent (50 ml) Evaporation integrated Filtration not required	Relatively slow extraction (2 hours)
Ultrasonic extraction	Not matrix dependent Relatively inexpensive equipment Fast extraction (10–45 min) Large amount of sample (2–30 g)	Large amount of solvent (100–300 ml) Mandatory evaporation of extract Extraction efficiency not as high Labor intensive Filtration required
Supercritical fluid extraction (SFE)	Fast extraction (30–75 min) Minimal solvent use (5–10 ml) CO_2 is nontoxic, nonflammable, environmentally friendly Controlled selectivity Filtration not required Evaporation not required	Matrix dependent Small sample size (2–10 g) Expensive equipment Limited applicability
Accelerated solvent extraction (ASE)	Fast extraction (12–18 min) Small amount of sample (up to 100 g) Automated Easy to use Filtration not required	Expensive equipment Cleanup necessary
Microwave-assisted extraction (MAE)	Fast extraction (20–30 min) High sample throughput Small amount of solvent (30 ml) Large amount of sample (2–20 g)	Polar solvents needed Cleanup mandatory Filtration required Moderately expensive equipment Degradation and chemical reaction possible

A modified Soxhlet extraction technique, constructed with a column containing glass beads to prevent the cycle of aroma compounds, was among the early applications in the determination of the aroma of dill herb. These studies reported considerably higher aroma contents of dill grown in northern Finland (24), a ten-fold higher aroma concentration of fresh herbs compared to their dried counterparts (25) and evaluated the most important components contributing to the aroma of the dill herb, including their odour threshold values, odour values and sensory evaluation (26).

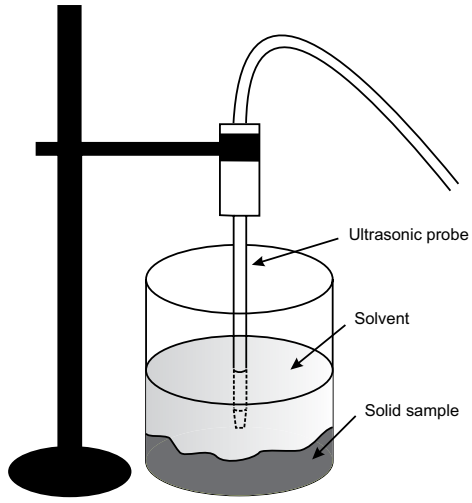
Environmental samples have been the principal applications in the comparison of conventional and new extraction techniques (14,15). In the extraction of polyaromatic hydrocarbons (PAHs) from contaminated soil, the recovery and reproducibility of the Soxhlet method were comparable to pressurised liquid extraction (PLE) (27). The quality of extracts, however, differed greatly from other methods because of the selective extraction of non-target matrix components, which would usually require removal of the matrix organics prior to analysis.

Ultrasonic extraction; Ultrasound-assisted extraction (UAE)

In the ultrasonic extraction technique (sonication), there are ultrasonic vibrations between the sample and the solvent (15). The ultrasonic device consists of a horn-type ultrasonic disruptor and its tip is positioned just below the surface of the solvent (Figure 4). In this method, relatively large amounts of samples and solvents are used, and the extraction process can be completed in about 10–45 minutes (Table 1). However, the extraction may need to be repeated, especially if the sample concentration is low. Evaporation of large volumes of solvent from combined extracts is a major disadvantage, and often further cleanup procedures are required before analysis. However, the technique is inexpensive and relatively fast, though the extraction efficiency may not be as high as with some other methods.

Figure 4.

Schematic diagram of an ultrasonic extraction device.



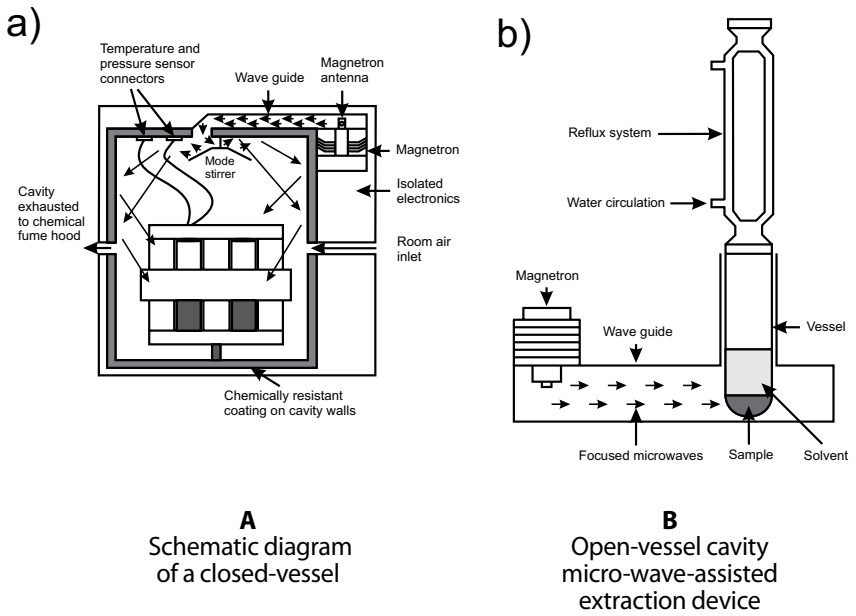
Ultrasound-assisted extraction (UAE) has been used in a number of applications concerning natural products. For example, isoflavones from soybeans have been extracted quantitatively, at 60°C for 20 min, in 50 % ethanol (28). A semiautomatic system for the extraction of strawberry flavonoids by the UAE method, followed by partial evaporation and solid-phase concentration was claimed to be a fast and more efficient technique than traditional maceration/stirring (29). After optimisation of the extraction procedures of polyphenols from the peels of various *Citrus* species, UAE of total phenols, reported to be faster than solvent extraction, was used for sample preparation for antioxidant activity measurements (30, 31). The UAE of salvianolic acid B from *Salvia* was also much more efficient than the traditional reflux method (32).

Microwave-assisted extraction (MAE)

The microwave instrumentation used for the extraction of organic compounds consists of a microwave generator (magnetron), a waveguide for transmission, a resonance cavity and a power supply. A closed-vessel extraction apparatus (Figure 5a), working under elevated pressure (50–150 psi), contains a rotating carousel for multiple sample extractions simultaneously. At elevated pressure, the temperature (usually 100–150°C) in the vessel is higher than the boiling point of the solvent which improves the extraction efficiency (15). Extraction is rapid and sample throughput high (Table 1).

Figure 5 .

a) Schematic diagram of a closed-vessel and b) open-vessel cavity micro-wave-assisted extraction device according to (15) and (33).



A
Schematic diagram of a closed-vessel

B
Open-vessel cavity micro-wave-assisted extraction device

Microwave-assisted extraction in open-vessel apparatus (Soxwave®) shown in Figure 5b works at atmospheric pressure and uses a frequency of 2450 MHz with a programmable heating power (30–300W). The use of microwaves directed to a single-vessel cavity enables homogenous irradiation and reproducible sample treatment (33). The method was applied for the extraction of polycyclic hydrocarbons (PAHs) from solid environmental matrices such as soils and sediments, and compared with the conven-

tional Soxhlet extraction. Several extraction parameters had to be optimised, such as the effect of the type of solvent, the time and the power of microwave irradiation and the water content of the sample. The irradiation time and power did not appear to be important parameters. Instead, the addition of water to the dry matrices clearly increased the recovery of PAHs. The major advantages, when compared to the Soxhlet extraction, were the reduced extraction time (10 min) and solvent volume (10 ml).

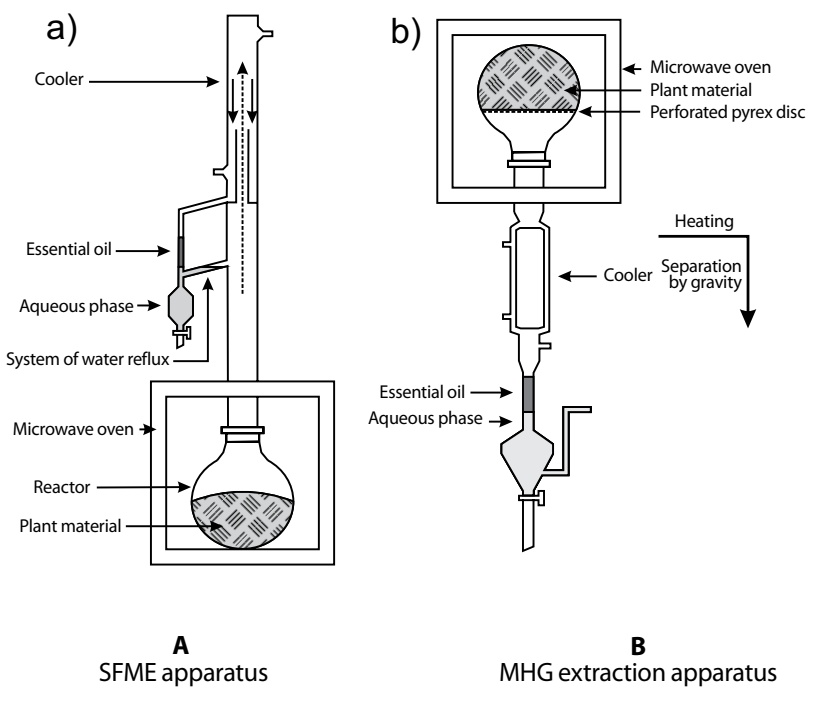
The development of microwave-assisted extraction (MAE) technique has been comprehensively reviewed (34) with a selection of closed vessel applications and comparisons of extraction methods. The early applications of MAE in natural product extraction were performed using the closed or open vessel method and included toxic glycosides, alkaloids, essential oils, terpenes and steroids (35). The rapid development in the area of essential oil separations has been promoted by the fact that MAE treatment causes rapid opening of essential oil glands and a direct release of oil (36,37).

Solvent-free microwave extraction; Microwave hydrodiffusion and gravity technique

Solvent-free microwave extraction (SFME) system (Figure 6a) is a combination of microwave heating and dry distillation, performed at atmospheric pressure without addition of any solvent or water (38). A typical extraction process is carried out at atmospheric pressure, using 250 g of fresh plant material and heating for 30 min at a fixed power of 500W. The extraction can be continued at 100°C until no more essential oil is obtained. The essential oils from the investigated aromatic plants were quantitatively and qualitatively similar to those obtained by a conventional hydrodistillation method. The major advantages of the SFME technique include shorter extraction times, substantial savings in energy and a reduced environmental burden, as compared with conventional extraction methods.

Figure 6 .

a) SFME apparatus b) MHG extraction apparatus. Modified from (38) and (39).



Microwave hydro-diffusion and gravity (MHG) system (Figure 6b), contains a microwave reactor 2.45GHz working with maximum delivered power of 1000W (39). Usually, 500 g of fresh plant material is heated under atmospheric pressure uusing a fixed power density of 1W/g for 15 min, without the addition of solvents or water. The direct interaction of microwaves with steam produced from the water of fresh plant materials favours the release of the essential oils trapped inside the cells of plant tissues. The MHG technique can produce essential oils from aromatic herbs in a concentrated form, free from residual solvents, contaminants, or artefacts. In addition, the method offers many advantages in terms of yield and selectivity, with shorter extraction times and high quality essential oil compositions.

Further applications of the above mentioned techniques in natural product chemistry include the extraction of cinnamon oil for the measurement of its antioxidative activity (21), and the extraction of oils from rosemary leaves and citrus peel by the MHG technique for the determination of antioxidant and antimicrobial activities, and the evaluation of organoleptic properties (40, 41).

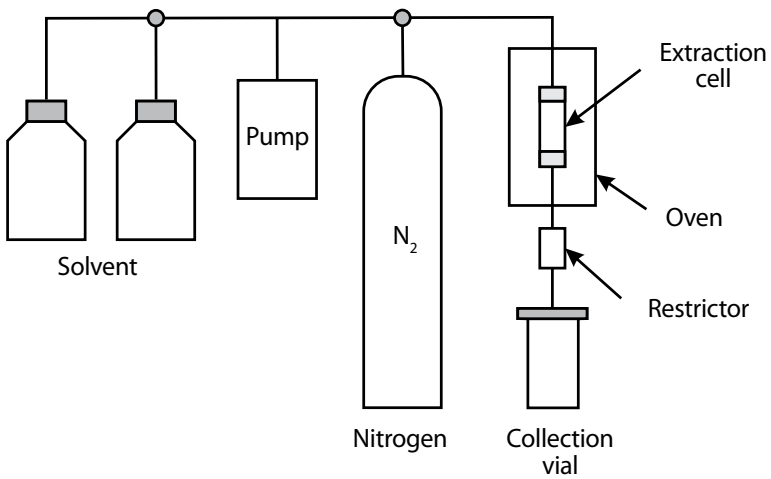
Accelerated solvent extraction (ASE); Pressurised liquid extraction (PLE)

Accelerated solvent extraction (ASE) technique for sample preparation was developed by combining elevated temperatures and pressures with liquid solvents (14). Solvents which are applicable in Soxhlet or sonication methods can be used for the extraction process. A solid sample is enclosed in a sample cartridge with an extraction fluid and subjected to static extraction at elevated temperature (50–200°C) and pressure (500–3000 psi) for 5–10 min. The effects of temperature, pressure, solvent volume, prefill and preheat methodologies have been optimised for the ASE process. The recoveries of PAHs, polychlorinated biphenyls (PCBs), and total petroleum hydrocarbons (TPHs) from reference materials using ASE were found to be quantitative. The extraction time for 1–30 g samples was less than 15 min and the volume of solvent 1.2–1.5 times that of the extraction cell containing the sample. No thermal degradation could be detected during the extraction of temperature-sensitive compounds.

The fully automated instrument (Figure 7) operates at 100–180° C and in a pressure range of 1500–2000 psi. The ASE system that can extract up to 24 samples in one

Figure 7.

Schematic diagram of an ASE system.



operation offers several advantages, i.e. it needs a minimal amount of solvent and a short extraction time, without additional filtration (Table 1). The method has been adopted for the extraction of environmental samples like pesticides, herbicides, PAHs, PCBs, base/neutral/acid compounds, dioxins and furans (15).

Pressurised liquid extraction (PLE) of several drugs containing structurally diverse secondary plant metabolites has been compared with other extraction techniques such as maceration, percolation and Soxhlet extraction with reference extracts prepared according to the monographs in the Pharmacopoeia (42). It was found that the PLE procedures used for quantitive analysis of medicinal herbs were especially useful. For powdered plant materials, the number of extraction cycles required to achieve an exhaustive extraction is mainly dependent on the solubility of the analytes. Further PLE applications include the analyses of terpenes, saponins, flavonolignans, alkaloids and steroids (35). The method is time and solvent saving yet produces equivalent or higher extraction yields than conventional methods. In a study of the flavonoids from black elder (*Sambucus* sp.), optimised ASE resulted in higher yields compared to those obtained by maceration (43).

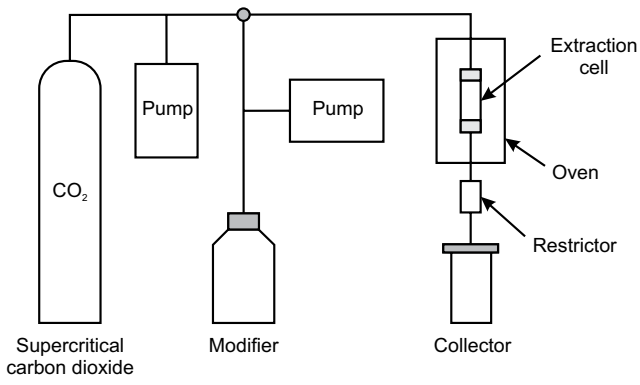
Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) takes advantage of the exceptional properties of supercritical fluids to facilitate the extraction of analytes from solid matrices (15). A supercritical fluid is a substance above its critical temperature and pressure, which has a gas-like viscosity but a density resembling that of a liquid and a diffusion coefficient between these two states. Due to these unique properties, supercritical fluids achieve generally high extraction efficiencies.

The basic components of an SFE instrument consist of a tank of CO_2, a high-pressure pump, an extraction cell, a heating oven and an extract collector (15). In addition, a source for modifier and a pump are often needed (Figure 8). Since the 1970s, SFE has attracted considerable interest in the analytical and preparative extraction of herbal and natural products (44,45,46). The main advantages of the technique include friendliness to the environment and nontoxicity of the most common supercritical fluid CO_2, and the possibility to selectively extract components of interest by changing the extraction conditions. On the other hand, the technique is relatively matrix dependent, necessitating the redevelopment of the method if the sample matrix or target compound has to be changed.

Figure 8.

Schematic diagram of an off-line SFE system.

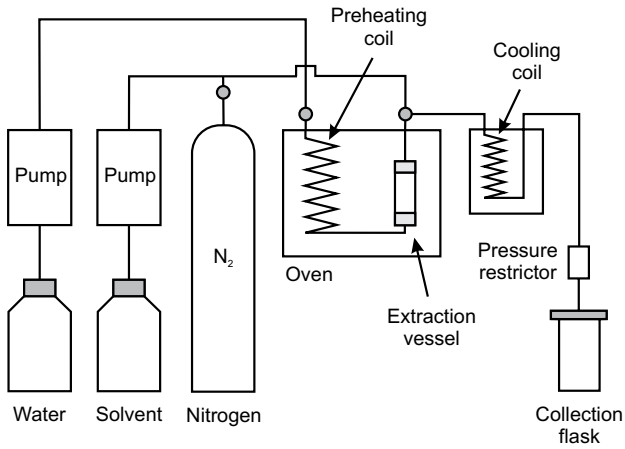


Pressurised hot water extraction (PHWE; Subcritical water e xtraction, SWE)

The term pressurised hot water, also called subcritical water, compressed hot water or superheated water, refers to water that has been heated to a temperature between 100° C and 374° C in an elevated pressure so that it remains in a liquid state (47, 48). Under these conditions, the physicochemical properties of water change and it becomes less polar, thus enabling the extraction not only of polar but also non-polar compounds from the extraction matrix.

Figure 9.

Schematic diagram of a pressurised hot water extraction (PHWE) system according to (49).



The equipment required for PHWE is similar to that used in SFE and ASE and the same instruments can be used in PHWE, with the limitation that temperatures above 150° C and 200° C cannot be reached with the commercial instruments (47). A diagram of a self-constructed apparatus is presented in Figure 9. The technique has found several applications in natural product chemistry (48, 50) and has benefited from the increased awareness in environmental issues. The technique appears to be suitable for the extraction of many polar compounds from a variety of matrices, but may not be the best choice for compounds that are both non-polar and thermolabile (47).

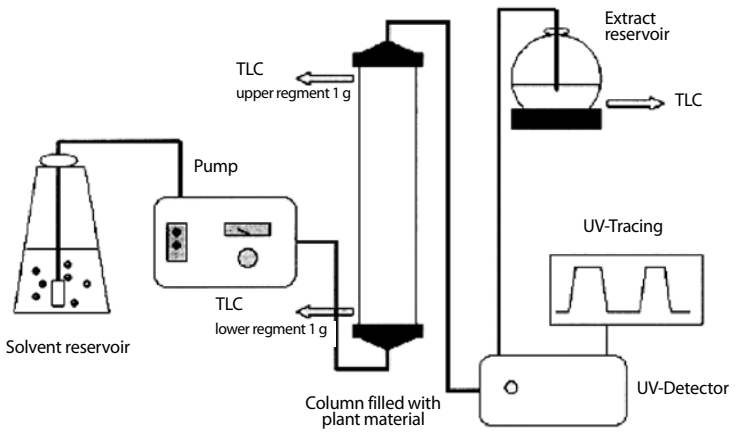
Medium pressure solid-liquid extraction (MPSLE)

Medium pressure solid–liquid extraction (MPSLE) is an extraction method based on the principles of the diffusion-dissolving processes of parametric pumping that have been well characterised (51). The method involves creating periodic changes in the equilibrium of the process, as well as in the flow directions, in order to separate the components in the treated fluid or gas mixture. A change in the intensive parameter of the system, e.g., temperature, pressure, pH, or electric field, results in a reversible differential alteration of the distribution of the components between the solid and liquid phases.

Medium pressure solid–liquid extraction can be performed in a medium pressure liquid chromatography (MPLC) system (Figure 10). The same principal factors are valid in MPSLE as in column chromatography, i.e., the decisive parameters are the geometry of the column, the physicochemical properties of the solvent, the flow rate and amount of solvent, pressure, equilibrium time, particle size, compactness,

Figure 10.

Schematic diagram of the MPSLE system. Reproduced from (53) by permission of Taylor & Francis.



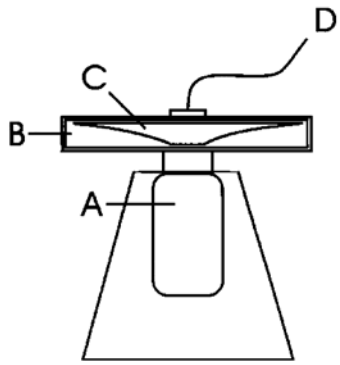
and amount of plant material. The effects of these parameters on the extraction yield and the quality of the extracts have been studied in the extraction of onion (52) and *Ficus* leaves (53).

Rotation planar extraction (RPE)

Rotation planar extraction (RPE) is an extraction method in which accelerated flow of the extractant is achieved by the action of centrifugal force (54). A novel multi-functional separation instrument, prototype ExtraChrom®, even enables the rotation planar extraction of complex matrices, because a planar column can easily be attached to it and then filled with the material to be extracted (Figure 11). The factors affecting the RPE process are basically the same as in MPSLE, with the exception that the solvent is driven by centrifugal force instead of a pump and the geometry of the column differs from that of the MPLC columns used in MPSLE. RPE has been successfully applied in the extraction of 2-pyrone derivatives from *Gerbera hybrida* (55) and coumarins from *Ficus* leaves (53).

Figure 11.

Schematic diagram of the ExtraChrom® separation instrument prototype. Key to abbreviations: A, motor; B, chamber; C, extraction chamber/ rotation planar column; D, tubing from solvent reservoir. Reproduced from (55) by permission of John Wiley & Sons, Ltd.



Micelle-mediated extraction

Micelle-mediated extraction, also known as surfactant-mediated extraction, represents a convenient alternative to the conventional extraction systems. It is based on the special properties of the surfactant: its capacity to solubilise solutes of different characters and natures, and its cloud point behaviour. At an elevated temperature, above the cloud point temperature, the solution separates into two phases: a surfactant-rich phase and an aqueous phase. The small volume of the surfactant-rich phase enables the preconcentration of the analytes (56). This methodology offers the advantages of safety, low cost, ability to concentrate solutes, easy disposal of surfactant, and low toxicity compared to classical organic solvents.

The application potential of micelle-mediated extraction and cloud point preconcentration method has been evaluated e.g. for the extraction and concentration of less hydrophobic compounds aesculin and aesculetin from *Cortex fraxini* (57), glycyrrhizic acid and liquiritin from licorice root (58), osthole and imperatorin from *Cnidium monnieri* (59), and anthraquinones from roots of *Morinda citrifolia* (60). The claimed benefits of the technique include simplicity of the procedure, friendliness to the environment, reduced compound degradation due to lower temperature requirement as compared to PHWE, and high extraction efficiency. The technique, however, appears to be more suitable for the extraction of hydrophobic compounds compared to their hydrophilic counterparts.

Enzyme-assisted extraction

Enzyme-assisted extraction makes use of hydrolytic enzymes that can hydrolyze and degrade the plant cell wall constituents, thus improving the release of intracellular contents (61). The constituents of plant cell walls, i.e. cellulose, hemi-cellulose and pectin, can be hydrolyzed using cellulase, β-glucosidase, and pectinase enzymes. Cellulase breaks cellulose chains into glucose, β-glucosidase cleaves β-1,4 linkages in cellulose, and commercial pectinase preparations from *Aspergillus niger* have pectinesterase, polygalacturonase and pectin lyase activity (62). In addition, phenolic hydroxyl groups present in flavonoids combine with cellulose, hemi-cellulose and pectin as complexes due to hydrogen bonding interactions. These hydrogen bonding interactions can be broken by cellulase, β-glucosidase, and pectinase enzymes, thereby increasing the proportion of free flavonoids. In addition, β-glucosidase is able to break the β-1,4 glucosidic linkages in flavonoid glucosides, resulting in an increased amount of free flavonoid aglycones. Enzyme-assisted extraction has been reported for the extraction of luteolin and apigenin from pigeonpea (*Cajanus cajan* (L.) Millsp.) leaves (61), phenolics from apple peel (63), paclitaxel and related taxanes from *Taxus chinensis* (64) and volatile oils from garlic (65) and celery (66). In these studies, the use of enzymes was found to increase extraction yields compared to traditional non-enzymatic extraction techniques.

Mechanical pressing

Mechanical pressing is a term referring to the mechanical pressing of a suitable plant material to obtain essential or fatty oils. No heat or other forms of additional energy is used to aid the process. The essential and fatty oils obtained by this method are usually called "cold-pressed" or "virgin oils". Good examples of such oils are citrus and olive oils that are expressed from the pericarp of lemon and ripe drupes of olives, respectively, followed by subsequent separation by physical means (16).

Extraction process

As Pawliszyn has demonstrated (13), the sample preparation step in an analytical process typically consists of extraction of components of interest from a sample matrix. This procedure can vary in the degree of selectivity, speed, and convenience depending on the approach and conditions used as well as on geometric configurations of the extraction phase and conditions. Optimisation of this process permits enhancement of the performance of the overall analysis. The optimized design of the extraction devices and procedures facilitates rapid and convenient on-site implementation, coupling to separation-quantification, and/or automation. The key to rational choice, optimisation, and design is an understanding of fundamental principles governing mass transfer of analytes in multiphase systems. There is a tendency to divide extraction techniques according to random criteria. Common principles among different extraction techniques are shown and a unified approach based on convolution of mathematical functions describing the individual steps is presented. This approach considers gas, solvent, liquid polymer, and solid surfaces as extraction phases and air, water, and solids as sample matrices.

The fundamental thermodynamic principle common to all chemical extraction techniques involves the distribution of analyte between the sample matrix and the extraction phase. When liquid is used as the extraction medium, then the distribution constant (K_{es})

Equation 1: $K_{es} = a_e/a_s = C_e/C_s$

defines the equilibrium conditions and ultimate enrichment factors achievable in the technique, where a_e and a_s are the activities of analytes in the extraction phase and matrix, respectively, which can be approximated by the appropriate concentrations. For solid extraction phase adsorption, equilibria can be explained by the following equation:

Equation 2: $K_{es} = S_e/C_s$

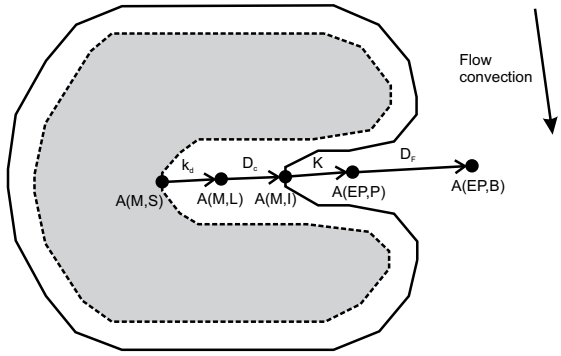
Where Se is solid extraction phase surface concentration of the adsorbed analytes. The above relationship is similar to eq. (1), with the exception that the extraction phase concentration is replaced with the surface concentration. The S_e term in the numerator indicates that the sorbent surface area available for adsorption must also be considered. This complicates the calibration at equilibrium conditions because of displacement effects and the nonlinear adsorption isotherm (67). The above equations can be used to calculate the amount of analyte in the extraction phase under equilibrium conditions (68). For example, for equilibrium liquid-microextraction techniques and a large sample, including direct extraction from the investigated system, the appropriate expression is very simple

Equation 3: $n = K_{es}V_eC_s$

Where K_{es} is the extraction phase -sample matrix distribution constant, V_e is the volume of the extraction phase, and C_s is the concentration of the sample.

Figure 12 .

Processes involved in extraction of heterogeneous samples containing porous solid particles according to (13). The terms in the figure are discussed in the text.



The most challenging extractions occur when a solid is present as a part of the sample matrix. This case will be considered as the most general example of extraction since it involves a number of fundamental processes occurring during the extraction. If we assume that a matrix particle consists of an organic layer on an impermeable but porous core and the analyte is adsorbed onto the pore surface, the extraction process can be modelled by considering several basic steps as shown in Figure 12. To remove the analyte from the extraction vessel, the compound must first be desorbed from the surface (Fig. 12, A(M,S)), then it must diffuse through the organic part of the matrix (A(M,I)) to reach the matrix-fluid interface (A(M,L)). At this point, the analyte must be solvated by the extraction phase (A(EP,P)) and then it must diffuse through the static extraction phase present inside the pore to reach the portion of the extraction phase which can be influenced by convection, in order to be transported through the interstitial pores of the matrix and eventually to reach the bulk of the extraction phase (A(EP,B)) (69, 70).

The relationship between various matrix parameters and flow conditions on the elution profile can be described mathematically, and this can be verified experimentally. In chromatography, this relationship is usually described as contributions from each of the mass transfer steps to the height equivalent to a theoretical plate (HETP). The overall performance of the system can be defined as the sum of the relevant individual components judiciously selected to reflect the most significant individual steps present in the elution process. The effect of slow desorption kinetics of analytes from the matrix on the elution profile, can be described as the contribution to the HETP (70).

Chromatographic and interstitial linear velocities are identical if matrix particles have low porosities. This analysis can be extended to elution through a matrix having multiple adsorption sites characterized by different dissociation rate constants by using the approach described by Giddings (71). The diffusion of the analyte in the liquid or swollen solid part of the matrix is important when polymeric materials are being extracted or the matrix has a substantial organic content.

The analytes migrate in and out of a pore structure of the matrix during the elution. This can be described as resistance to mass transfer in the fluid associated with the porous nature of the environmental matrices. This contribution can be quite important in view of the relatively large particle size (approximately 1 mm) of environmental

matrices and it becomes particularly important when the pores are filled with dense organic material, such as humic matter rather than the extraction phase.

In addition, one needs also to consider analyte diffusion along the axis of the vessel (longitudinal diffusion). This applies only to the situation when the analytes are initially present in a fluid phase, which in flow-through techniques corresponds to the elution of uniform spikes from the extraction vessel or when weakly adsorbed native analytes are removed from an organic-poor matrix such as sand. The above relationships are suitable for systems in which the partitioning equilibrium between the matrix and extraction fluid is reached quickly as compared to the fluid flow. They are also suitable for modeling of static -dynamic extractions, under good solubility conditions ($k = 0$), in which the sample is initially exposed to the static extraction phase (vessel is capped) for the time required to achieve an equilibrium condition prior to elution by fluid flow. If dynamic extraction is performed from the beginning of extraction, then in the majority of practical cases, the system is not expected to achieve the initial equilibrium conditions. This is due to the slow mass transport between the matrix and the fluid (for example, slow desorption kinetics or slow diffusion in the matrix). The expected relationship between the amount of analyte removed from the vessel versus time can be obtained in this case by convoluting the function describing the rate of mass transfer between the phases with the elution time profile.

The outcome is a process where elution and mass transfer between the phases occur simultaneously. This function which is the extraction time profile emphasizes the point that in the majority of extraction cases, these two processes are expected to be combined in descriptions of the kinetics of the process. This defines the release rate of the analyte from the sample matrix and it can include the matrix-analyte complex dissociation rate constant, the diffusion coefficient, the time constant that describes swelling of the matrix thus facilitating the removal of the analyte, or a combination of the above parameters (72, 73).

The above conclusion can be stated in a more general way. Convolution among functions describing individual processes occurring during the extraction, describes the overall extraction process and represents a unified way to describe the kinetics of these processes.

In heterogeneous samples, the release of solid-bound analytes from a sample matrix, through a reversal of chemisorption or inclusion, frequently controls the extraction rate. By recognising this fact, extraction parameters can be changed to increase the extraction rates. For example, dissociation of the chemisorbed analytes can be accomplished either by using high temperature or by application of catalysts. This has lead to the development of high-temperature supercritical fluid extraction (74), followed by the evolution of the hot-solvent extraction (75) and microwave extraction approaches, with more selective energy focusing on the sample matrix -extraction phase interface (76). There is also the possibility that milder conditions can be applied by taking advantage of the catalytic properties of the extraction phase or additives (77).

If the extraction rate is controlled by the mass transport of analytes in the pores of the matrix, then the process can be successfully enhanced by application of sonic and microwave energy, which induces convection even in the small dimensions of the pore. Frequently, diffusion through the whole or a portion of the sample matrix

containing natural or synthetic polymeric material controls the extraction rate (78). In this case, swelling of the matrix and increasing temperature results in increased diffusion coefficients and therefore increased extraction rates.

Coupling equations for systems involving convection caused by flow through a tube are frequently not available for other means of agitation and other geometric configurations. In these cases, the most successful approach is to consider the boundary layer formed at the interface between the sample matrix and the extraction phase. Independent of the agitation level, fluid contacting the surface of a particle is always stationary, and as the distance from the particle surface increases, the fluid movement gradually increases until it corresponds to the bulk flow in the sample. To model mass transport, the gradation in fluid motion and convection of molecules in the space surrounding the particle surface can be simplified as a zone of a defined thickness in which no convection occurs and there is perfect agitation in the bulk of the fluid everywhere else (79).

It is useful to have a precise understanding of the definition and thickness of the boundary layer. The thickness of the boundary layer (70) is determined by both the rate of convection (agitation) in the sample and the diffusion coefficient of the analyte. Thus, in the same extraction process, the boundary layer thickness will not be the same for different analytes. In many cases when the extraction phase is well-dispersed forming a thin coating, the diffusion of analytes through the boundary layer controls the extraction rate. The equilibration time (t_e) can be estimated as the time required to extract 95 % of the equilibrium amount and it can be calculated for these cases from equation (4):

Equation 4: $te = B\,\delta\,a\,K_{es} / D_s$

Where a is the extraction phase thickness, D_s is the diffusion coefficient of the analyte in the sample matrix, and K_{es} is the distribution constant of the analyte between the extraction phase and the sample matrix. B is a geometric factor referring to the geometry of the supporting material upon which the extraction phase is dispersed. The boundary layer thickness (δ) can be calculated for given convection conditions using engineering principles. Equation (4) can be used to predict equilibration times when the extraction rate is controlled by diffusion in the boundary layer, which is valid for thin extraction phase coatings (a < 200 microns) and high distribution constants (K_{es} > 100).

The analyte concentration in the bulk of the matrix can be considered to be constant when a short sampling time is used and there is a constant supply of the analyte via convection. These assumptions are true for most cases of sampling, where the volume of the sample is much greater than the volume of the interface and the extraction process does not affect the bulk sample concentration. In addition, the solid coating can be treated as a perfect sink. The adsorption binding is instantaneous and essentially irreversible. The analyte concentration on the coating surface is far from saturation and can be assumed to be negligible for short sampling times and relatively low analyte concentrations in a typical sample.

Consideration of different arrangements of the extraction phase is always beneficial. For example, extension of the boundary layer by a protective shield that restricts

convection would result in a time weighted average (TWA) measurement of the analyte concentration. Various diffusive samplers have been developed based on this principle. For example, when the extracting phase in a solid phase micro-extraction (SPME) device is not exposed directly to the sample, but is contained in a protective tubing (needle) without any flow of the sample through it, the diffusive transfer of analytes occurs through the static sample (gas phase or other matrix), trapped in the needle. The system can consist either of an externally coated fibre with the extraction phase withdrawn into the needle or an internally coated needle.

The geometric arrangements represent a very powerful method, capable of generating a response proportional to the integral of the analyte concentration over time and space (when the needle is moved through space) (80). In these cases, the only type of analyte transport to the extracting phase is diffusion through the matrix contained in the needle. During this process, a linear concentration profile is established in the tubing between the small needle opening, and it is characterized by the surface area and the distance between the needle opening and the position of the extracting phase.

The extracted amount of analyte is proportional to the integral of the sample concentration over time, the diffusion coefficient of analyte in the matrix filling the needle, in the area of the needle opening, and inversely proportional to the distance of the coating position with respect to the needle opening.

Reliance on physicochemical constants in the calibration of the extraction process might appear unconventional or even discomfort some researchers. However, as the theory indicates, these constants define the extraction process and there is the opportunity to take advantage of this fact. Physicochemical constants can be frequently estimated from simple experiments or calculated by considering the molecular structures of analytes, extraction phase, and matrix, which adds to the attractiveness of this approach (13). For equilibrium microextraction techniques, the extraction phase -sample matrix distribution constant is used to quantify the concentration of analytes in the sample matrix. For extraction approaches controlled by mass transfer in the boundary layer, the calibration can be based on the diffusion coefficient in the sample matrix at constant extraction time under well-defined agitation conditions. In some cases, as in membrane extraction, a combination of constants defines the extraction rate and can be used for calibration. The major argument against using this approach is that physicochemical constants are affected by many experimental parameters, such as temperature and matrix conditions. However, the impact of temperature change can be compensated for and therefore direct calibration for simple matrices is possible (81). For more complex matrices, internal standard or standard addition calibration, which are applied routinely in exhaustive techniques to monitor recoveries, can be used to compensate for matrix variations (82). Correlations between distribution constants and simple measurements such as turbidity and pH may be found to account for matrix variations and therefore eliminate the need for internal calibration. The advantages of nonexhaustive extraction are its fundamental simplicity and fewer geometric restrictions, which permit a number of interesting on-site implementations involving integrating the sampling and sample preparation steps. Additionally, more information can be obtained about the system being investigated. For example, it is possible to specify and determine the distribution of an analyte in multiphase systems since the extraction process does not disturb the

equilibria naturally present in the system. Therefore, different forms of the analyte are extracted and quantified according to their corresponding distribution constants and/or diffusion coefficients.

A better understanding of the fundamentals of the extraction processes facilitates exploration of new opportunities, which makes sample preparation a more interesting and scientifically exciting part of the analytical process (13).

Extraction processes may evoke changes in the physical properties and chemical structure of substances. The behaviour of substances will demonstrate reactivity depending on the molecular structures, and therefore substances are likely to interact with their close environment. The behaviour of plant material in different extraction techniques is influenced by the same physico-chemical processes involving chemical substances in matrices generally. The physico-chemical phenomena affecting compound compositions in extractions include oxidation, evaporation, stabilisation, osmosis, transfer of components, aeration, bubble formation, dispersion, flotation, precipitation, temperature, gravitation, fluids, and pH. Certain plant components such as enzymes, pectins, gelatins, sugars or tannins, when not the targets, often disturb the outcome of the extraction process.

The concentration of the components may decrease or increase due to the extraction procedure. To monitor this parameter, a combination of physical methods such as measurement of concentration, particle size, surface and settling velocity of suspended particles, and chemical analysis of the concentration and partition coefficients of selected components, as well as biological methods for structure and composition can be utilized to characterise the particulate extraction phases. However, the physico-chemical processes involved in different extraction techniques with plant material e.g. for drug discovery purposes, have not been explored extensively.

Properties of solvents, solvent selection in solid-liquid and liquid-liquid extractions

In the extraction of compounds of interest from relevant sources e.g. plant material, several factors affect the extraction yield and the chemical composition of the resulting extract. In general, the compounds of interest reside inside a matrix composed of fairly inert, insoluble and often polymeric material, and their concentration in most cases is very low, compared to the amount of cell wall structures and other unwanted matrix components. The physical characteristics of the matrix may differ from soft and mucilagineous to hard and wood-like, and the desired constituents may vary considerably in their chemical and physical properties e.g. in terms of volatility, solubility, acid/base properties, polarity, reactivity and stability etc.

Though the extraction technique may affect (in some case substantially affect) the extraction efficiency and extract quality, the judicious choice of extraction solvent or solvents provides the most obvious means of achieving the desired outcome in terms of quantity and quality of the extract (83). An initial washing with low-polarity solvents extracts the more lipophilic components, while alcohols isolate a broader spectrum of apolar and polar compounds from the material. If a more polar solvent is used for the first extraction step, subsequent solvent partition can allow for a finer division into different polarity fractions.

Properties of solvents

A substantial number of physico-chemical parameters for pharmaceutical solvents have been characterized over the years in an effort to describe and predict optimum solvent composition e.g. in polymorph screening to improve crystallisation yield and purity, in synthetic chemistry to facilitate chemical reaction rate and completion, and in liquid chromatography to influence analytical or preparative separations (84), as well as optimisation of solid-liquid extraction procedures and liquid-liquid purification processes (85). Some solvent properties which are important in the classification of solvents are listed in Table 2.

In addition to the solvent properties included in the studies referred to in Table 1, a myriad of other parameters have also been applied to the description and classification of organic solvents (see e.g. 90, 91). Some of these properties can be characterised as solvent property parameters, i.e. molecular descriptors such as hydrogen bond donor and acceptor propensities, whereas others are better depicted as bulk property parameters such as viscosity (89). Another possible division of these parameters would be to separate the more commonly used physico-chemical experimentally derived properties from theoretical molecular descriptors calculated using computational software packages (90). Despite the overwhelming number of different solvent parameters published in the literature and the fact that their values may differ to some extent depending on the reference cited, it should be noted that many of them are interrelated and describe the same fundamental phenomena. The interested reader is referred to the original publications cited in this chapter for a more detailed review of this topic.

Solvent classification schemes

The fundamental rationale behind the attempts to construct a universal classification scheme for solvents can be traced to the need to be able to facilitate the selection of the appropriate solvent in a judicious and convenient manner. The division of solvents into an adequate number of sets based on physico-chemical and computational similarities may help in the selection of the optimum solvent e.g. for chromatographic analyses and efficient and/or selective extraction of compounds of interest from a matrix.

Perhaps the most widely known solvent classification system, especially within the chromatographic community, has been proposed by Snyder on the basis of an extensive experimental study conducted by Rohrschneider (86). The classification of solvent selectivity is based on the interaction of a particular solvent with three test solutes, namely ethanol, dioxane and nitromethane. Ethanol was considered a model solute possessing acidic properties, dioxane was the model with basic properties, and nitromethane represented a solvent with dipolar properties. According to the experimental results, solvent selectivity was characterised by x_i values; x_e describes the relative ability of the solvent to act as a proton acceptor, x_d is a measure of the relative ability to act as a proton donor, and x_n describes the ability of the solvent to act as a strong dipole. The polarity scale, *P'*, represents the chromatographic strength ("polarity") of the solvent and is composed of the three types of solvent-solute interactions.

The 81 tested solvents could be classified into eight different groups according to their selectivity as shown on Table 3.

Table 2. *Definitions of some solvent properties used in solvent classification in selected studies.*

Parameter	*Definition*	*Reference*
P'	Rohrschneider polarity scale (Chromatographic strength)	86
x_e	Proton acceptor index	86
x_d	Proton donor index	86
x_n	Dipole index	86
K	Kirkwood function	87
MR	Molecular refraction (Molar refractivity)	87
δ	Hildebrand solubility parameter	87
n	Refractive index	87
bp	Boiling point	87
μ	Dipole moment	87
ε_H	HOMO energy (HOMO = Highest Occupied Molecular Orbital)	87
ε_L	LUMO energy (LUMO = Lowest Unoccupied Molecular Orbital)	87
α	Hydrogen bond acidity	88
β	Hydrogen bond basicity	88
π^*	Dipolarity/polarisability	88
π	Polarity/dipolarity	89
$\Sigma\alpha$	Summation of the hydrogen bond donor propensities	89
$\Sigma\beta$	Summation of the hydrogen bond acceptor propensities	89
μ	Dipole moment	89
ε	Dielectric constant (Relative permittivity)	89
E_{coh}	Cohesive energy density	89
η	Viscosity	89
γ	Surface tension	89
SIAEprobe	Surface integral for enthalpy values of interactions between acceptor atoms of a molecule and a donor probe on the surface	84
SIEDprobe	Surface integral for enthalpy values for interactions between donor atoms of a molecule and an acceptor probe on the surface	84
VOL	Intrinsic volume	84
PPSA-1	Type 1 partial positive surface area	84
PNSA-1	Type 1 partial negative surface area	84
CNTA	Simple count of all hydrogen bond acceptor groups	84
PNHS-1	Type 1 hydrophilic surface area	84
n	Refractive index	84
π	Abraham's dipolarity/polarisability	84
γ	Surface tension	84
ε	Dielectric constant (Relative permittivity)	84
μ	Dipole moment	84
Φ	Aromaticity	84
π_2^H	Viscosity	84
δ	Hildebrand solubility parameter	84
PI	Snyder polarity index	84
BP	Boiling point	84

Table 3. *Classification of solvents based on Rohrschneider selectivity values.*

Group	*Solvents*
I	Aliphatic ethers, tetramethylguanidine, hexamethyl phosphoric acid amide, (trialkyl amines)
II	Aliphatic alcohols
III	Pyridine derivatives, tetrahydrofuran, amides (except formamide), glycol ethers, sulfoxides
IV	Glycols, benzyl alcohol, acetic acid, formamide
V	Methylene chloride, ethylene chloride
VI	(a) Tricresyl phosphate, aliphatic ketones and esters, poly-ethers, dioxane (b) Sulfones, nitriles, propylene carbonate
VII	Aromatic hydrocarbons, halo-substituted aromatic hydrocarbons, nitro compounds, aromatic ethers
VIII	Fluoroalkanols, *m*-cresol, water, (chloroform)

Modified from (86).

In response to criticism concerning the reliability of the Snyder-Rohrschneider solvent-selectivity triangle, Snyder has later proposed a qualitatively similar devised model for classifying solvent selectivity (88). In this solvatochromic model, the solvent parameters x_e, x_d and x_n have been replaced by Kamlet-Taft solvent hydrogen bond acidity (α), basicity (β) and dipolarity/polarisability (π^*) values. In contrast to Rohrschneider's work, these parameters were derived from spectroscopic and other measurements that were specifically designed to measure only a single interaction, and thus the values of these parameters were averages of the results obtained with several probe solutes for each parameter. It can thus be concluded that α, β and π^* are inherently more accurate measures of solvent acidity, basicity and dipolarity than values of x_i.

Despite the fact that the original Rohrschneider selectivity values x_e, x_d and x_n all included mixed-interaction tendencies due to the "impure" nature of the test solutes, in general both classifications appear similar with respect to the relative assignment of different solvents according to their acidity, basicity and dipolarity. The application of Kamlet-Taft selectivity values, however, appears to provide a better qualitative classification of solvent selectivity.

Based on the same Kamlet-Taft solvatochromic parameters α, β and π^*, de Juan et al. (92) subsequently proposed the clustering of solvents into five groups instead of eight when they included 42 different solvents in the statistical analysis. However, they did decide to divide one group into two subgroups as shown in Table 4.

Chastrette et al. (87) classified a total of 83 solvents into 9 groups based on a multivariate statistical treatment of eight solvent physico-chemical variables. These variables are listed in Table 2. The eight variables were reduced to three principal components representing the polarisability of the solvent, the polarity of the solvent, and the acceptor aspect of donor/acceptor interactions and electron affinities. The resulting classification is presented in Table 5.

Of the 83 solvents included in the study, a total of 10 were found to have been clustered in an unexpected group, e.g. benzyl alcohol in aromatic polar solvents and tetrahydrofuran in hydrogen bonding solvents, the authors stressed that their classification was quantitative and mathematically rigorously derived and thus may not provide the most useful classification for a specific chemical application. Nevertheless, it has represented the basis of several more recent statistical analyses of solvent properties.

The classification by Chastrette et al. (87) was later modified and improved by Gramatica et al. (90) who used the *k*-nearest neighbour classification method followed by performing counter-propagation artificial neural networks on a data set of 152 solvents. Instead of physico-chemical experimental properties, the model was constructed based on theoretical molecular descriptors. The number of solvent classes was reduced from nine to five, this included four of the classes characterized by Chastrette et al. (87) and one new class. This classification is presented in Table 6.

Chastrette's aprotic dipolar (AP), aprotic highly dipolar (AHD) and aprotic highly dipolar and polarisable (AHDP) classes were combined into a single class (AP), and the aromatic apolar (ARA) and aromatic polar (ARP) classes were also regrouped into one class (AALP). The electron pair donor class (EPD) remained unchanged, but hydrogen bonding (HB) and hydrogen bonding strongly associated (HBSA) classes were combined into one class (HBD). A new aliphatic aprotic apolar (AAA) class was added

Table 4. *Classification of solvents based on Kamlet-Taft solvatochromic parameters.*

Group	*Solvents*
1	Slightly basic solvents with low polarity (electron pair donors): aliphatic ethers, substituted aliphatic amines
2	Aprotic polar solvents: aliphatic cyclic ethers, esters, ketones, nitriles
3	Strongly basic and strongly polar solvents: pyridines, small amides, sulfoxides, ureas, phosphoramides
4	Relatively polar solvents with low tendency to form hydrogen bonds
	A Aromatic compounds and apolar aliphatic halogenated hydrocarbons: ethers, hydrocarbons
	B Polyhalogenated polar aliphatic hydrocarbons: methylene chloride, chloroform
5	Amphiprotic solvents with marked hydrogen-bond properties: alcohols and water

Modified from (92).

Table 5. *Classification of solvents by Chastrette et al. (87).*

Group	*Solvents*
AD	Aprotic dipolar: 14 solvents, e.g. nitromethane, acetonitrile, acetone, dichloromethane
AHD	Aprotic highly dipolar: 9 solvents, e.g. dimethylsulfoxide, *N*-methylpyrrolidone, cyclohexanone, pyridine
AHDP	Aprotic highly dipolar and polarisable: 2 solvents, i.e. sulfonate, hexamethylphosphotriamide
ARA	Aromatic apolar: 8 solvents, e.g. benzene, toluene, *o*-xylene, *p*-xylene
ARP	Aromatic polar: 12 solvents, e.g. chlorobenzene, bromobenzene, acetophenone, ethylbenzoate
EPD	Electron pair donor: 10 solvents, e.g. triethylamine, diethylether, dioxane, aniline
HB	Hydrogen bonding: 19 solvents, e.g. methanol, ethanol, isopropyl alcohol, acetic acid
HBSA	Hydrogen bonding strongly associated: 5 solvents, e.g. formamide, water, glycol, diethylene glycol
MISC	Miscellaneous: 4 solvents, i.e. carbon disulfide, diethyl carbonate, chloroform, aniline

Table 6. *Classification of solvents by Gramatica et al. (90).*

Group	*Solvents*
AP	Aprotic polar, 24 solvents (includes classes AD, AHD, and AHDP by Chastrette et al. 1985)
AALP	Aromatic apolar or lightly polar, 16 solvents (includes classes ARA and ARP by Chastrette et al. 1985)
EPD	Electron pair donors, 9 solvents (same class as in Chastrette et al. 1985)
HBD	Hydrogen bonding donors, 25 solvents (includes classes HB and HBSA by Chastrette et al. 1985)
AAA	Aliphatic aprotic apolar, 8 solvents (a new class, consists of alkanes)

Table 7. *Classification of solvents by Katritzky et al. (93).*

Group	*Solvents*
i	1 solvent: formamide
ii	9 solvents: hydroxylic solvents, e.g. water, methanol, acetic acid, ethanol
iii	12 solvents: dipolar aprotic, e.g. nitromethane, acetonitrile, acetone, pyridine
iv	15 solvents: ethers, esters, amine, alkyl halides, aromatic ring solvents
v	3 solvents: *n*-hexane, cyclohexane, carbon tetrachloride

and consisting of alkanes. The advantages of this classification over Chastrette's include simplified classification, the elimination of the miscellaneous (MISC) solvent class, and the reassignment of the previously incorrectly classified solvents, e.g. benzyl alcohol, tetrahydrofuran and *n*-hexane, into more reasonable classes.

A pioneer in the field of solvent classification, Alan Katritzky, has classified 40 solvents and the same number of solvent scales by principal component analysis (93). Based on his analysis, the solvents showed clear clustering into five groups as shown in Table 7.

It should be noted that water, which was included in group "ii" in the classification, can be distinguished from the alcohols and acetic acid by the very high first and second principal component scores and could as well be considered a group on its own.

Katritzky et al. have later extended their classification work to include 703 solvents and 100 different solvent scales (94). This analysis supported the classification of solvents into 11 classes according to the presence of various functional groups as shown in Table 8.

A cluster analysis of eight solvent parameters enabled the division of 96 solvents into 15 separate classes (89). The parameters used are listed in Table 2. The results confirmed yet again that solvents with the same functional groups, e.g. hydrocarbons or alcohols or ketones, generally tend to reside within the same class. Additionally, Euclidean distances of all the solvents to the respective cluster centre were calculated, thus making it possible to assess which solvent could be characterised as being the most typical solvent of that particular class.

The work of Gu et al. (89) did not include many pharmaceutically relevant solvents and this prompted Xu et al. (84) to carry out a statistical evaluation of all 57 solvents belonging to classes 2 and 3 recognised by the International Conference on Harmonisation (ICH). Based on correlation analysis, the number of solvent descriptors could be narrowed from 40 descriptors down to 17, which are listed in Table 2. By applying principal component and cluster analyses, the solvents could be divided into 20 clusters. The authors explained in detail the reasons behind their decision to group the solvents into 20 clusters, but it is possible for the reader to reduce or increase the number of groups based on case-specific needs because the entire tree diagram and statistical design were published.

Table 8. Classification of solvents by Katritzky et al. (94).

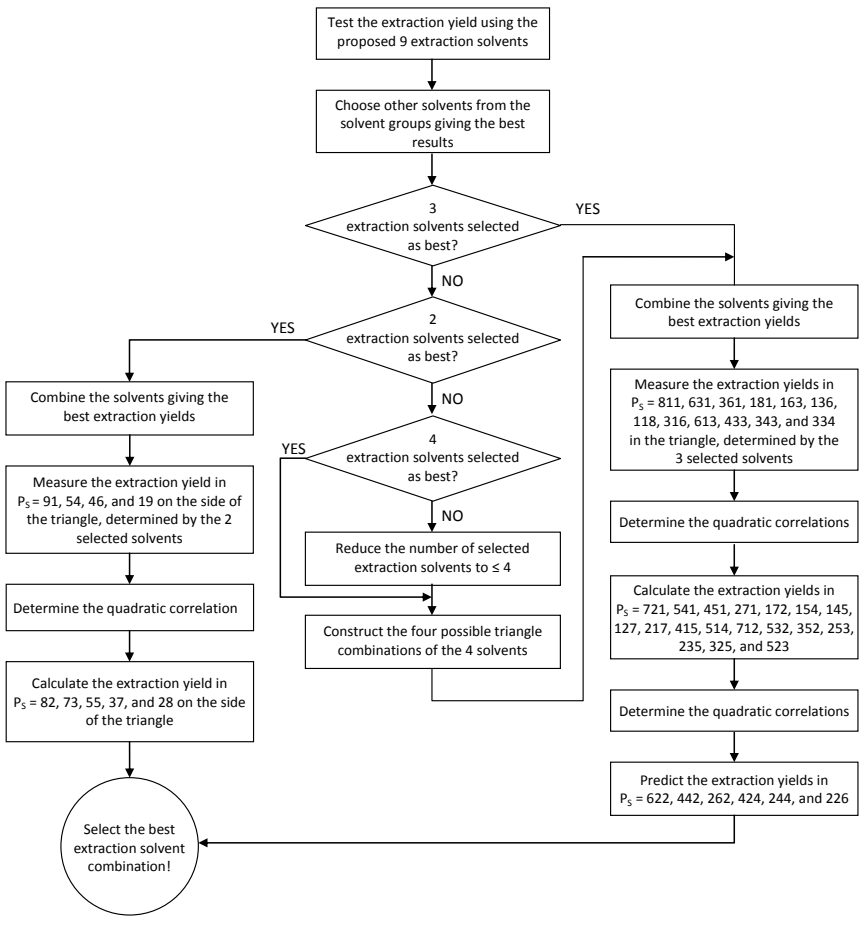
Group	*Solvents*
I	Hydrocarbons, saturated and unsaturated, e.g. *n*-hexane, cyclohexane, 1-hexene, toluene
II	Halo hydrocarbons, e.g. dichloromethane, bromobenzene, iodomethane, trichlorofluoromethane
III	Saturated, unsaturated and cyclic ethers, e.g. diethyl ether, diphenyl ether, anisole, tetrahydrofuran
IV	Esters and polyesters, e.g. ethyl acetate, vinyl acetate, methyl propionate, diethyl phtalate
V	Aldehydes, ketones, amides, e.g. benzaldehyde, acetone, acetophenone, formamide
VI	Nitriles and nitro compounds, e.g. acetonitrile, benzonitrile, nitromethane, nitrobenzene
VII	Hydroxylic compounds, e.g. methanol, diethylene glycol, water, acetic acid
VIII	Amines and pyridines, e.g. triethylamine, aniline, pyridine, 2-bromopyridine
IX	Sulfuro compounds, e.g. ethanethiol, dimethyl sulfide, dimethyl sulfoxide, ethyl isothiocyanate
X	Phosphorus compounds, e.g. triethyl phosphate, triethyl phosphite, phosphoric acid hexamethyltriamide
XI	Miscellaneous, e.g. triethylphosphine oxide, tetramethylsilane, acetic anhydride, tert-butyl hydroperoxide

Solvent selection strategies

In most cases solvent selection in the extraction of natural products has been based on trial and error and/or arbitrary choice by the researcher. However, some more or less structured strategies have been proposed. Barwick (95) has compared the Rohrschneider-Snyder solvent classification scheme and the solvatochromic classification scheme placing an emphasis on solvent extraction and liquid chromatography. Some practical advice and examples are given concerning maximising solubility for a given solute, selective extraction of one solute from a mixture, aspects of liquid-liquid partition and selecting an alternative solvent for a specific extraction task.

The solvent classification of Snyder (86) formed the basis for the solid-liquid extraction strategy delineated by Nyiredy (96). Of the solvents in Snyder's eight selectivity groups, 13 are considered to be commonly used in solid-liquid extraction and therefore were examined in the initial experiments. Based on initial experiments with the pure solvents, two to four solvents were selected for the optimisation procedure, which is illustrated by the optimisation triangle, a representation similar to the "PRISMA" model proposed for liquid chromatography solvent optimisation (97,98). Depending on the number of solvents selected for further optimisation, four or twelve solvent combinations of different composition and selectivity were tested. Based on these experiments the extraction efficiency of all other selectivity points could be calculated and predicted which allows selection of the most suitable solvent combination. The flowchart of this solid-liquid extraction strategy is illustrated in Figure 13.

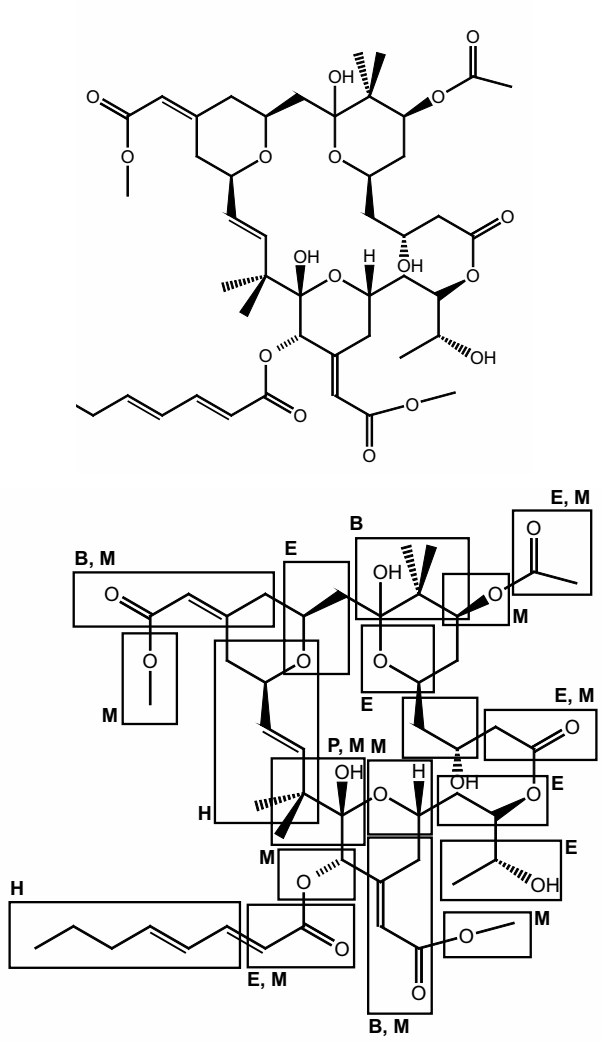
Figure 13. Flowchart for the proposed solid-liquid extraction strategy as delineated in (96).



The strategy proposed by Nyiredy (96) is actually a structured trial and error solvent selection method based on a limited amount of experimental work and it does not require the compounds of interest to be known prior to method optimisation. Fitzpatrick et al. (99) have outlined a method for the prediction of a suitable solvent for the extraction of pesticides, which differs fundamentally from that suggested by Nyiredy, i.e. the analyte or analytes to be extracted need to be known beforehand in order to calculate the suitability of the possible extraction solvents. This procedure is based on the Hildebrand solubility parameter (δ_t), which is a measure of the internal energy of cohesion in the solvent/solute; it can be divided into three components, namely hydrogen-bonding ability (δ_h), dispersion coefficient (δ_d), and polarity contributions (δ_p). The individual group contributions are calculated both for selected solvents and analyte(s) of interest, and a suitable solvent or solvent combination is chosen based on the overall similarity of the contributions between the solvent and the analyte.

Figure 14.

Bryostatin partitioned in carbon fragments between one and six carbon atoms long. Each fragment is matched to a solvent with a similar D/V value. For details, see (101).



A somewhat similar, yet less accurate solvent selection method, was proposed by Harjo et al. (100). The procedure begins with the characterisation of the plant material to be extracted and the compounds of interest in this material. The chemical properties of the constituents to be extracted are sought from the literature or estimated on the basis of the properties of similar compound classes or, if a more rigorous approach is required, predicted using a suitable software package. The most appropriate solvent is then selected based on the solubility of the compounds to be extracted. If considered necessary, preliminary extraction tests using water, methanol, methylene chloride and hexane may be performed.

An efficient extraction of large organic molecules containing both polar and nonpolar substructures is often difficult to achieve using a single solvent or two solvents of similar polarities. An algorithm was recently proposed by Manning (101) to predict the optimum solvent mixture for the extraction of marine natural products, typically large organic molecules composed of polar and nonpolar subcomponents, based on dipole moments and molecular volume values. In this model, the target compound is divided into subcomponents with carbon fragments ranging from one to six carbons according to ten simple rules. Each subcomponent is then matched by its dipole moment and molecular volume to a common solvent. In Figure 14, the division of the bryostatin molecule into 18 fragments is presented resulting in an optimum extraction solvent mixture of 2 parts hexane, 3 parts butanol, 1 part propanol, 7 parts ethanol and 11 parts methanol. This resulting multi-component mixture may be simplified by applying a general equation taking into account the ratios of dipole moment and molecular volume of the solvent components and their proportions in the solvent mixture. Based on the equation, it is possible to calculate a slightly less effective but simpler solvent mixture e.g. composed of only propanol and methanol or two other compatible solvents.

Criteria for solvent selection – practical examples

The definition of the desired outcome is of fundamental importance in the selection of optimum solvent for a particular extraction task, i.e. what constitute the most important criteria for the extraction to be considered as the best possible. The most common criterion used in defining a successful extraction is naturally the extraction yield, either total yield or the yield of a particular compound or compound class. However, in some cases, e.g. taxonomic studies and general screening purposes, the qualitatively representative composition of the extract may be the decisive factor in preference to yield in selecting the solvent of choice. Another equally important criterion, especially in drug discovery, is the biological activity of the extract (102). Quite often all of these parameters, i.e. yield, chemical composition, and biological activity, are included in the study and the optimum extraction solvent and conditions are determined based on the overall analysis of the results.

Härmälä et al. (103) screened a total of 20 different solvents in an attempt to find the optimum solvent for the extraction of coumarin-structured compounds with calcium-antagonistic effect from the roots of *Angelica archangelica*. In addition, the relationships between some selected physical and chemical properties of the solvents and the extraction efficiency were investigated. The extraction yield, the biological activity of the extract, and the amount of nonpolar compounds in the extract were the criteria used in that work. In the final selection of the most suitable extraction solvent, special

focus was directed on the feasibility of the solvent in the extraction procedure, i.e. on the possible chemical interactions between the solvent and the solutes and the ease of removal of the solvent. In this case, chloroform was selected as the solvent of choice for the extraction in preference to two other solvents, methanol and acetic acid, that received higher E_{an} values indicating better extraction efficiency with reference to the three previously determined extraction parameters. The primary reason for the rejection of methanol and acetic acid as extraction solvents was the potential risk of artefact formation and decomposition of the target compounds, and for acetic acid, also the difficulties in achieving removal of the solvent.

Indeed, not only the ability of the solvent to dissolve the compounds of interest, but also other general properties of the solvent need to be considered when selecting the most appropriate extraction solvent e.g. the miscibility of solvents (Table 9), ease of removal, inertness, toxicity, flammability, and cost (95, 104). Especially in screening studies and pilot and industrial scale extractions, the ease of subsequent treatment of the extracts may become a significant factor. As an example, solvents such as dimethyl sulfoxide and acetophenone have very high boiling points, making the evaporation of the solvent a tedious and inconvenient task. The fact that many compounds of interest may be thermolabile further complicates the evaporation process, as temperatures closer to the boiling point of the solvent cannot be used.

The extraction solvent should not be able to evoke any decomposition, dehydration or isomerisation of the compounds of interest nor should it cause artefact formation. In addition, the solvent should be of sufficient purity to limit the possibility of solvent-derived impurities interfering with the subsequent analysis of the extract (104). In order to avoid artefact formation and degradation of the solutes, some basic knowledge of the target compounds and their properties is required. While in many cases the exact chemical composition of the extracted material may not be known, it is nevertheless important to pay attention to this phenomenon this failure to do so may lead to the incorrect identification of the extracted compounds. For example, methanol, a very widely used and efficient extraction solvent, has recently been shown to promote degradation of a natural pyranonaphthoquinone, pentalongin (106) and there was a report of artefact formation in the isolation of indole alkaloids from the mushroom Cortinarius infractus (107).

During recent years, the toxicity of solvents has received ever increasing attention both from the viewpoint of their possible detrimental long-term on health effects and their impact on the environment. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has classified the most commonly used pharmaceutical solvents into three classes, namely Class 1 solvents: Solvents to be avoided, Class 2 solvents: Solvents to be limited, and Class 3 solvents: Solvents with low toxic potential (108). Class 1 solvents should not be employed in the manufacture of drug substances, excipients, and drug products because of their unacceptable toxicity or their deleterious environmental effect; this class includes solvents such as benzene and carbon tetrachloride. The use of Class 2 solvents, e.g. chloroform and methanol, should be limited because of their inherent toxicity. Solvents in Class 3 may be regarded as less toxic and of lower risk to human health e.g. acetone, ethanol and heptane have been placed in this class.

Table 9. Solubility of solvents in water and solubility of water in solvents. Modified from (105).

Solubility in water		*Solubility of water in solvents*	
Solvent	Solubility (%)*	Solvent	Solubility (%)*
Isooctane	0.0002 (25 °C)	Isooctane	0.006
Heptane	0.0003 (25 °C)	Pentane	0.009
1,2,4-Trichlorobenzene	0.0025	Cyclohexane	0.01
Cyclohexane	0.006 (25 °C)	Cyclopentane	0.01
Cyclopentane	0.01	Heptane	0.01 (25 °C)
Hexane	0.014	Hexane	0.01
o-Dichlorobenzene	0.016 (25 °C)	1,1,2-Trichlorotrifluoroethane	0.011 (25 °C)
1,1,2-Trichlorotrifluoroethane	0.017 (25 °C)	1,2,4-Trichlorobenzene	0.020
o-Xylene	0.018 (25 °C)	Toluene	0.033 (25 °C)
Pentane	0.04	Chlorobenzene	0.04
Chlorobenzene	0.05	Chloroform	0.056
Toluene	0.052 (25 °C)	n-Butyl chloride	0.08
n-Butyl chloride	0.11	Ethylene dichloride	0.15
Methyl isoamyl ketone	0.54	Dichloromethane	0.24
n-Butyl acetate	0.68	o-Dichlorobenzene	0.31 (25 °C)
Ethylene dichloride	0.81	n-Butyl acetate	1.2
Chloroform	0.815	Ethyl ether	1.26
Dichloromethane	1.60	Methyl isoamyl ketone	1.3
Methyl isobutyl ketone	1.7	Methyl t-butyl ether	1.5
Methyl t-butyl ether	4.8	Methyl isobutyl ketone	1.9 (25 °C)
Triethylamine	5.5	Ethyl acetate	3.3
Methyl n-propyl ketone	5.95	Methyl n-propyl ketone	3.3
Ethyl ether	6.89	Triethylamine	4.6
n-Butyl alcohol	7.81	Propylene carbonate	8.3 (25 °C)
Isobutyl alcohol	8.5	Methyl ethyl ketone	10.0
Ethyl acetate	8.7	Isobutyl alcohol	16.4
Propylene carbonate	17.5 (25 °C)	n-Butyl alcohol	20.07
Methyl ethyl ketone	24.0		

The extraction of phenolic compounds from natural products serves as a practical example of a specific research area that has received considerable attention during the last years. There is epidemiological data indicating that consumption of foods rich in flavonoids and polyphenols may reduce the risk of cardiovascular diseases (109). Furthermore, flavonoids and polyphenols may have a protective role against different types of cancers (110), and they may have protecting effects against neurodegenerative diseases (111). Finally, they offer an interesting alternative to some widely used synthetic food antioxidants (112).

Proanthocyanidins are polyphenolic compounds consisting of two or more flavan-3-ol units. Extraction by organic solvents releases mainly monomers and low molecular mass compounds, but most of the oligomeric and polymeric proanthocyanidins are partially insoluble in water (113). Flavonoid glycosides, on the other hand, are soluble in water. Due to the contrasting polarity characteristics of these plant

Table 10. *Recent examples of solvent selection in the extraction of phenolics from natural products.*

Extracted material	*Measured parameters*	*Tested solvents*	*Optimum extraction solvent*	*Reference*
Black tea and mate tea	• Assay of total phenolic content by two methods • Antioxidative activity • EtOH • MeOH	• Water • Acetone • DMSO	**Black tea** • 50 % DMSO **Mate tea** • 50 % Acetone	114
Mulberry leaves	• Assay of total phenolic content • Antioxidative activity • Antityrosinase activity	• MeOH • EtOH • n-PrOH • i-PrOH • Water	• 37–70 % MeOH • 31–59 % EtOH • 29–56 % n-PrOH • 28–52 % i-PrOH	115
Stems and aerial parts of *Phyllanthus niruri*	• Extraction yield • Assay of gallic acid, corilagin and ellagic acid by HPLC	• *n*-Hexane • Petroleum ether • CH_2Cl_2 • $CHCl_3$ • 70–100 % Acetone • MeOH • 20–100 % EtOH • Water	• Water	116
Lychee flowers	• Extraction yield • Assay of total phenolic content • Assay of total flavonoid content • Assay of condensed tannins • Antioxidant activity	• Acetone • MeOH • Water	• Acetone	117
Malt rootlets	• Antioxidant activity	• 20–80 % EtOH • 20–80 % MeOH • 20–80 % Acetone	• 60 % EtOH	118
Fruits of *Euterpe oleracea*	• Assay of total anthocyanin content • Assay of total phenolic content • Antioxidant activity	• 16.4–83.6 % EtOH	• 70–80 % EtOH	119
Fruits of *Morinda citrifolia*	• Assay of total phenolic content • Assay of total flavonoid content • Antioxidant activity	• 20–100 % EtOH • Water	• 40 % EtOH	120
Grape marc and elder berries	• Assay of total phenolic content • Assay of total monomeric anthocyanins • Assay of catechin, epicatechin and trans-resveratrol by HPLC	**Grape marc** • Water • 50–100 % Acetone • 50–100 % EtOAc • 50–100 % EtOH **Elder berries** • 20–100 % Acetone • 50–96 % EtOH	**Grape marc** • 50 % Acetone or 50 % EtOH **Elder berries** • 50 % EtOH	121
Bark of *Phyllanthus emblica*	• Assay of total phenolic content	• 30–90 % EtOH	• 75 % EtOH	122
Apple pomace	• Assay of total phenolic content • Assay of chlorogenic acid, flavonols and phloretin glycosides by HPLC	• 7.96–92.04 % EtOH • 7.96–92.04 % Acetone	• 56 % EtOH • 65 % Acetone	123

DMSO, dimethyl sulfoxide; EtOH, ethanol; MeOH, methanol; n-PrOH, n-propanol; i-PrOH, i-propanol; CH2Cl2, dichloromethane; CHCl3, chloroform; EtOAc, ethyl acetate.

phenolics, aqueous organic solvents, namely acetone or alcohols, are usually employed in the extraction of phenolics from natural products. Some recent examples of studies involving the optimisation of the extraction solvent in the extraction of plant phenolics are presented on Table 10. As one would expect, the optimum solvent in most cases is either aqueous acetone or aqueous ethanol. It should also be noted that in many cases methanol, although an efficient and economical solvent, has been excluded from the studies due to its toxicity.

Properties of extracts in drug discovery process

Natural products are still an unexplored research area with great potential for drug discovery. Major sources include plants, microorganisms and marine organisms, but also insects and animals, in particular venomous animals important, are but poorly characterized reservoirs (102,124,125,126). Natural products are currently the subject of much research interest. However, their extraction as part of natural product drug discovery poses specific challenges that must be addressed throughout the solvent extraction process. Successful extraction begins with careful preparation of the samples (e.g. plant material), and a thorough review of the appropriate literature on the protocols that are suitable for that particular class of compounds or plant species. During the extraction of plant material, it is important to minimise interference from compounds that may coextract with the target compounds, which are often secondary metabolites. Furthermore, one should strive to avoid contamination of the extract, as well as to prevent decomposition of important metabolites or artefact formation as a result of extraction conditions or solvent impurities. Exogeneous contamination has to be scrupulously avoided during the plant preparation and extraction process, though the contamination of the extract might also be attributable to endotoxins from microbial contaminations/coexistence in the intact plants. Prevention of decomposition or artefact formation of compounds present in the intact plant is highly relevant in chemotaxonomic studies, or if one wishes to prove existence of bioactive compounds in herbal drug plants, as well as in quality control aspects. In contrast, in any discovery programmes based on natural products it is of more importance to find a highly active compound, irrespective of whether it is an exogenous agent or a reproducible artefact. The impact of various stabilisers in pure solvents may also evoke differences in the chromatographic behaviour, interfering with the retention of compounds in thin layer chromatography.

Considerable conceptual and methodological developments have been taking place in the life sciences over the last few years. The need for extraction of biologically significant molecules from complex natural matrices, for the characterization of active principles in medicinal and dietary plants, or for the scientific validation of a traditional medicinal plant or a phytopharmaceutical, and for the quality control of herbal products still remains a challenging task. When one talks about screening for new drugs in plants this actually implies the screening of plant extracts for the presence of novel compounds and an investigation of their biological activities. The examples here will focus on methods based on bioassays in combination with liquid chromatography.

Strategies for bioactivity screening from plant extracts

There are many strategies for screening new compounds for drug discovery from natural extracts. Typically natural extracts are complex mixtures of compounds with different polarities and other qualities affecting their analytical properties. One commonly used approach to identifying active compounds is to investigate crude extracts for bioactivities and to continue with bioassay-guided separation and isolation of bioactive compounds (127, 128). At the beginning of this procedure, the crude plant extract(s) are submitted to different bioassays (or one specific) to achieve a rapid estimation of their bioactivity (e.g. 129). The extracts of interest are then fractionated with the help of different chromatographic methods. The bioassays serve as a guide during the isolation process, and all the fractions continuing to exhibit activity are carried through further isolation and purification until pure active compounds are obtained. Isolation of compounds from complex mixtures in adequate amounts for bioactivity assays and identification is challenging and laborious. It requires high amounts of starting material, multiple separation steps and may still lead to known or impure end product. Thus novel approaches have been developed that reduce time and resources.

Micro-fractionation

HPLC-based activity profiling for natural products compared to traditional fractionation and screening methods, offers direct information about which parts of the chromatogram of a sample are active. Potterat et al. (130) demonstrated how the separation of an active extract was performed on HPLC and fractions were collected for chemical structure and bioassay determinations. They were measuring cyclooxygenase-2 (131) and monoamine oxidase (132) inhibitory activities for Isatis tinctoria L. (Brassicacea) and Salvia miltiorrhiza Bunge (Lamiaceae), respectively, and this procedure located the active compounds of the plant extracts from the activity profiles and HPLC-fingerprints. The procedure combining the HPLC microfractionation with bioassay reported by Wennberg et al. (133) for natural products uses similarly HPLC-fingerprinting, but collects the fractions directly into 96-microwell plates for bioassay determination. Different bioassay types have been evaluated for this procedure, including biochemical/enzyme based or microbial and mammalian cell-based assays. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging effect (133) and acetylcholinesterase inhibitory activity (134) have been used as homogeneous assays. Antimicrobial activity against resistant bacterial strains (133) and the use of ion channels as therapeutic targets have been implemented as whole cell applications (135).

The benefit of determining the fractionated extract, instead of the whole extract, is that with the microfractionation method it is possible not only to demonstrate that the extract is active, but also to demonstrate which components of the extract display activity. When the extract is fractionated, the inactive components of the extract do not interfere readily with the active constituents or with the detection systems, and it is possible to achieve stronger signals. For example, Eloff (136) proposed use of microwell plate method to replace the agar diffusion technique, when studying the antimicrobial properties of whole extracts. However, he was not able to determine the turbidity of the microcultures with a microwell plate reader, partly because of the presence of colored compounds in the concentrated extracts. This problem can

be overcome by using the microfractionation method. Another considerable benefit of this procedure is the need for only a small amount of sample, compared to the bioactivity tests conducted on the whole extract.

With the advent of microwell plate based assays and information-rich HPLC-coupled spectroscopy (102,130), it was shown that it was possible to quickly identify any active substance with nuclear magnetic resonance (NMR) and/or mass spectrometry (MS). In this technique, an extract is separated by analytical gradient HPLC, one part of the effluent is fractionated into microwell plates, while the other part serves for on-line spectroscopic characterization. The microwell plate is used for the bioassay determination, so that ultimately one will end up with activity correlated with the HPLC-chromatogram. Structural information can be used for natural products database searches and tentative structural assignments. Furthermore only if the active principle is of sufficient interest, does a targeted preparative isolation need to be carried out.

One suitable approach to start screening is to pre-screen the extracts on TLC e.g. for an enzyme inhibitory effect, followed by tracking down the active compound by combining HPLC micro-fractionation to an enzyme assay in 96-microwell plate (134). In this case, LC-MS-MS was utilized for identification of the active compound from an identically fractionated 96-well plate. Pre-fractionated, freeze-dried extracts on microwell plates can be easily stored and utilized in a suitable bioassay thus making the off-line bioassaying independent of time. Furthermore, these fracions can be retested when new bioassays become available (137).

Recently Adams et al. (138) described a protocol for the discovery of natural products with antimalarial, antileishmanial and antitrypanosomal activity from extract libraries in 96-well format. Analytical gradient HPLC on a 3 x 150 mm column of 350 µg of extract, and the collection of one-minute fractions into 96 deep-microwell plates, parallel evaporation of the micro-fractions, and a suitable dilution scheme permitted parallel activity profiling against three parasites from a single HPLC injection.

The HPLC-microfractionation approach helps to identify the compound, giving rise to the activity in complex crude extracts without the need for laborious purification steps. There is also clear documentation of the drug discovery trail; this type of approach is part of the natural product drug discovery programme at Novartis (139).

Dialysis

Dialysis is a process of selective diffusion through a membrane. It is usually used to separate low-molecular-weight solutes which diffuse through the membrane from the colloidal and high-molecular-weight solutes which do not. This concept has been utilized in drug discovery receptor binding studies. In equilibrium dialysis, the bound and unbound compounds are separated using dialysis tubes (140). In the ultrafiltration method, the bound and unbound compounds are separated in ultrafiltration cones (141–44). The concentrations of bound and unbound compounds can be determined e.g. by scintillation counting (145). A schematic illustration of binding studies with an ultrafiltration/HPLC system is presented in Figure 15. The advantages of this method include short analysis time, simplicity, lack of dilution effects and low volumes (146). One disadvantage is the loss of compound or protein during the ultrafiltration process.

Figure 15.

Schematic illustration of the ultrafiltration/ HPLC protocol as described in (148).

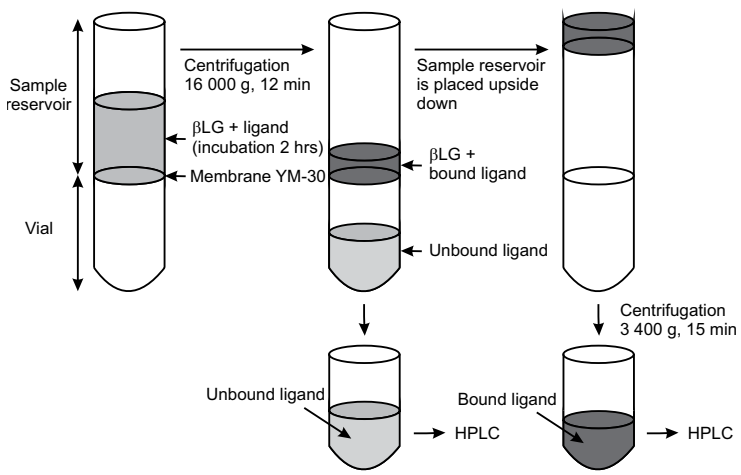
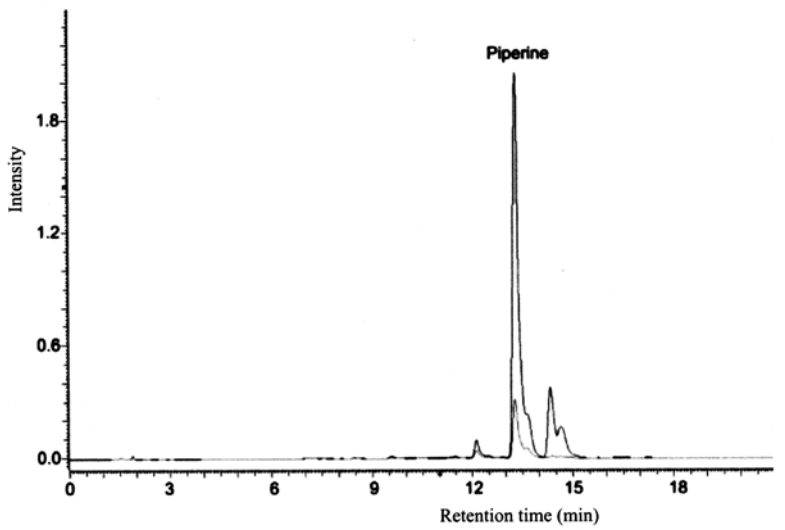


Figure 16.

Overlay RP-HPLC-chromatograms of the microdialysates of Piper nigrum at 340 nm before and after incubation with βLG. Modified with permission from (148).



An ultrafiltration/HPLC system has also recently been described for the investigation of protein binding with plant extracts (147), and the method is also applicable to the 96-microwell plate format. Since the fluorescence quenching method, which is the standard method for *β*LG binding measurements (148), can only be used with pure compounds, a biofingerprinting chromatogram analysis using ultrafiltration sampling combined with HPLC was developed to detect the binding of compounds from plant

extracts. The method was used to study the binding of compounds from the ethanol extract of Piper nigrum (black pepper). The piperine from *P. nigrum* extract was able to bind very tightly to βLG (Figure 16). The binding of pure piperine to βLG was also confirmed with fluorescence quenching, and from the literature (149).

On-line

One line of development has been to seek on-line assay technologies and flow injection systems with biosensors as selective detection devices. In the first type of these systems, an HPLC separation is coupled with an immunochemical or enzymatic detection step. In immunochemical detection, a fluorescent-labeled antibody is added to the HPLC effluent. Antigenic analytes form labeled immunocomplexes, which can be detected after removal of unbound antibodies with the aid of an affinity column. Several variants of this type of system have been described (150, 151). HPLC-based on-line assays for detection of enzyme inhibitory activity have been developed and applied to extracts of natural products, e.g. in the search for acetylcholinesterase inhibitors (152), in the evaluation of radical scavenging abilities (153), and in a generic assay for phosphate-consuming or -releasing enzymes (154, 155). These approaches are often coupled to UV/MS-detection. One important fact of this approach is the ability to correlate with confidence the chromatogram and the bioactivity. The use of flow cytometry for detection provides the possibility to utilize this consept on insoluble targets, such as cells, as well as in multiplexing assays, i.e. performing a number of assays simultaneously. During one run, the same fraction is fractionated into smaller units, each of which is fed into biological reaction coils representing different molecular targets.

Another novel approach is the use of microchips for selective extraction (156, 157) or with separate inlets for the solutes and for the enzyme or even cells as the bioassay (158). In addition, immobilized living cells in columns have been evaluated for screening purposes (159). This level of integrated functionality within one device clearly achieves very rapid compound separation, and immediate bioactivity screening of individual compounds, can be performed with minute samples, and it also save chemical and biological reagents. The miniscule sample-need would increase the possibility of using plant extracts from collections with limited availabilities such as those held in botanical gardens, even endangered species. ●

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Novel accelerated extraction techniques

Kari Hartonen

Introduction

Sample pretreatment, including extraction, is usually the most error-prone, laborious, and time-consuming step of any analytical determination. The increasing desirability of reducing total analysis times, and obtaining routine, robust, more efficient and reliable extraction techniques have been the driving forces for the development of extraction technology. Furthermore, sample pretreatment, extraction in particular, traditionally is the stage where large amounts of harmful organic solvents are consumed. A reduction of solvent consumption has been one additional target in developing and speeding up the extraction.

Solid samples (food, environmental sample, biological tissue, polymeric materials, etc.) are often heterogeneous with a number of interfering matrix components usually present in much larger quantities than the analytes. They are also more challenging and more difficult to extract compared to liquid samples due to various analyte-matrix interactions and poorer accessibility of the analytes for the solvent. Harsh extraction conditions and proper solvent selection are commonly required if one wishes for quantitative extraction. An efficient extraction can sometimes call for matrix modification

to release the analytes out of it or analyte derivatization to make them more soluble towards extraction solvent. Efficient extraction often poses an additional demand for the analysis due to co-eluting matrix components or the necessity of separate cleanup steps. However, sometimes a compromise between efficiency and selectivity might be a good idea to obtain a clean extract for the analysis and to avoid extra cleanup steps where some analytes may be lost thus negating the benefits.

The simplest liquid-solid extraction (LSE) can be achieved by shake and filter technique, where a solid sample is placed in a container and the appropriate extraction solvent is added. After shaking for some time, the mixture is filtered and the solvent (extract) now containing the analytes is subjected to further treatment or analysis. Usually this technique is not so efficient mainly due to inefficient contact and mass transfer between the sample and the solvent. The reason for this can be traced to too high analyte-matrix interactions to be overcome by the solvent, slow initial desorption and diffusion of the analytes from the matrix and low analyte solubility (low distribution coefficient) towards the solvent. The limitation due to low analyte solubility or distribution into solvent can be overcome by continuous (dynamic) extraction with fresh solvent which is superior to the static extraction. The low extraction efficiency is usually compensated for by using with long extraction time (2–24 h). Continuous solvent extraction, Soxhlet, is the technique that has been relied on for decades and it still is the reference technique for extraction of semivolatile compounds in solid samples.

It is only in the last two to three decades that serious work has been done on developing and enhancing extraction techniques. Often the extraction is speeded up by additional energy supplied into the sample which may overcome the above mentioned problems that hinder the extraction process. Traditional solvent extraction techniques like Soxhlet are being challenged by sonication, supercritical fluid extraction (SFE), microwave assisted extraction (MAE), accelerated solvent extraction (ASE), and, more recently, pressurized hot water extraction (PHWE). Some unique combinations of these systems have also been presented and applied for various solid samples.

Acceleration of extraction

Extraction of solid samples can be enhanced in many ways, mainly by moderate (conventional) heating, applying high temperature and pressure, via the use of ultrasound, using microwaves and with the aid of supercritical fluids. Additionally, surfactants, ionic liquids, derivatization reagent and other additives can be used to increase the solubilities of the analytes and to release them from the matrix more efficiently. In some cases, the additives may modify the matrix either physically or chemically.

Moderate heating

Even a relatively small change in extraction temperature may have a positive impact on extraction efficiency due to increased vapor pressures and solubilities of the analytes. Traditional LSE in an open flask at atmospheric pressure can be speeded up by mild heating (with the boiling point of the solvent as the limiting factor). Generally, mechanical stirring or shaking must be arranged to provide good contact between the sample and extraction solvent. However, the increase in efficiency can be relatively small. One of the most important examples of this is the modern improved Soxhlet extraction origi-

nally developed by Randall 1974 (1) where the extraction can approximately be 4 to 10 times faster. Compared to classical Soxhlet, where the solid sample is continuously flushed with condensed fresh solvent, the improvement is made by first immersing sample into the boiling solvent and after a certain period (usually less than one hour), the sample is raised from the solvent and flushed similarly as in the classical Soxhlet. Today commercialized instrumentation is offered by different companies with varying degrees of automation. A fully automated Soxhlet system is presented in Figure 1 with its different operation steps. It is also possible to automatically concentrate the extract and to recover the excess solvent for reuse.

High temperature and pressure

Applying high temperature and pressure at the same time offers advantages, since temperatures higher than the boiling point of the solvent can be employed while still keeping the solvent in a liquid form via pressure (excessive pressures are normally not needed). A relatively large increase in temperature (energy to overcome solute-matrix interactions) will also speed up the thermal desorption even more compared to moderate heating, thus, solvent viscosity and surface tension will also decrease, and diffusivities will be increased. Additionally, high pressure can enhance the solvent penetration into the pores of the sample matrix, increase the solvent contact with the matrix and speed up analyte elution from the extraction vessel.

An interesting paper was published in 1987 by Mangani et al. (2) where hot solvent (toluene at 100°C) was forced to flow through the solid sample that was packed into a 20 cm long and 4 mm i.d. stainless steel tube. The pressure (2.5 kg/cm^2) was generated by nitrogen gas into the solvent reservoir. For PAHs in ash samples they reported the method to be more efficient than Soxhlet and much faster (0.5 h compared to 24 h with Soxhlet). Although they referred to the technique as direct elution extraction or extraction technique based on liquid-solid chromatography, this technique is very close to the modern accelerated solvent extraction, described in chapter 2.2.1., and it has similar benefits.

In addition to accelerated solvent extraction, there are two other techniques that utilize high temperature and pressure. These two techniques are microwave assisted extraction (chapter 2.2.2.) and pressurized hot water extraction (chapter 2.2.3.), in which the former uses microwaves instead of conventional heating. Techniques utilizing high temperature and pressure include also supercritical fluid extraction (SFE). This will be described in more detail in chapter 2.4.

Accelerated solvent extraction

Accelerated solvent extraction (ASE) was introduced in 1995 where it was employed as an exhaustive extraction technique for solid environmental samples (3, 4). As ASE is a registered trade mark of Dionex Corporation (Sunnyvale, CA, USA), other names have been employed such as pressurized fluid extraction (PFE) and pressurized liquid extraction (PLE). A schematic of the commercial ASE instrument is shown in Figure 2. In the normal ASE procedure, the extraction vessel is loaded with the sample and the vessel is filled with organic solvent. The extraction vessel is then heated and pressurized and a short static extraction step (usually 5 min) is employed. After the static extraction, the analytes (extract) are transferred into the collection vial by pumping fresh solvent through the vessel and finally the residual solvent is purged with a

Figure 1.

Different steps of operation with fully automated Soxhlet (Soxtec) system (reproduced with permission from FOSS Analytical, Denmark).

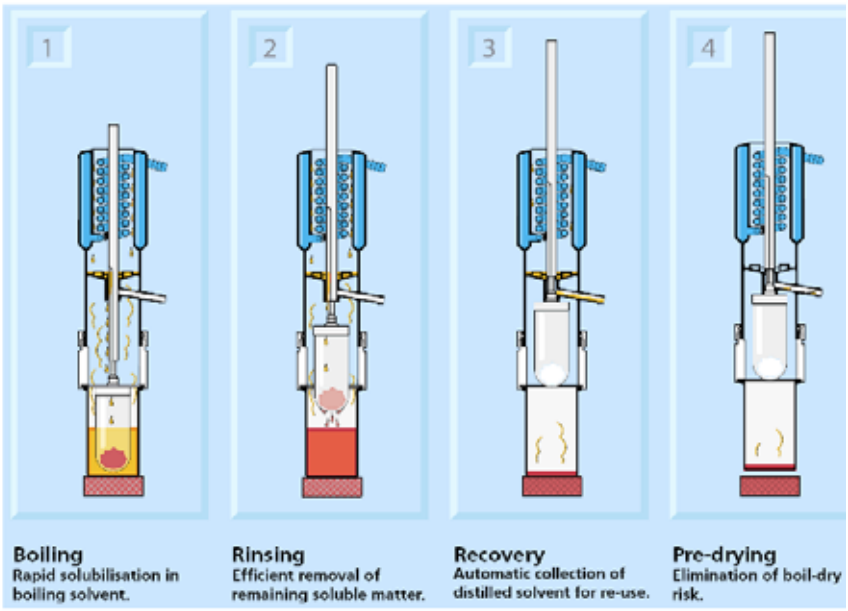
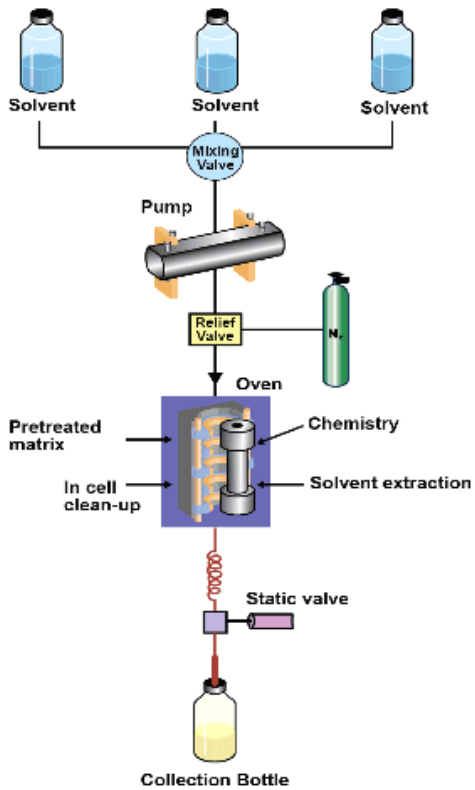


Figure 2.

Accelerated solvent extraction (ASE®) system (Reproduced with permission from Dionex Corporation, USA) (6).



pressurized gas like nitrogen. One static extraction period is often sufficient, but additional static steps (heating/pressurizing cycles) for complex samples can be added. The total extraction time with one static step is generally 10–20 min. Old solvent extraction methods are generally easily transferable to ASE, the only differences being the higher extraction temperature and the much shorter extraction time. Depending on the extraction solvent, temperatures from 50 to 150 °C are usually applied (maximum temperature with the commercial instrument is 200 °C). The extraction temperature needs to be optimized to achieve quantitative recovery with low RSD. A too high temperature will only result in a dirty extract (more matrix components extracted), which can cause a positive error in the response with certain detectors and an increase in the RSD values. Some losses for the volatile compounds can occur if the sample is preheated before pumping solvent into the extraction vessel (5). In this case, the filling of the vessel before heating is preferred (volatiles trapped into the surrounding solvent). Thermal degradation is also more probable when heating the sample without the solvent. The solvent volume needed in ASE is known to be 1.2–1.5 times the volume of the empty extraction vessel used for the soil sample, which applies also for heavily contaminated soil (5).

In the ASE instrument, the cooling of the collection vial seems to have no effect on recovery (5). The memory effect (carry-over from previous sample) can be avoided when using a rinsing solvent volume equal to 60 % of the empty extraction vessel volume (3). ASE has been mostly performed in the static mode since this is the type used in the commercial instrument, but several articles have appeared where the dynamic mode has been utilized (7, 8, 9). The dynamic mode is attractive especially with online connection to other dynamic analytical systems (10). The main drawbacks are the higher consumption of the solvent and dilution of the extract that requires an extra concentration step, usually a solid phase extraction (SPE).

Automated ASE has been successfully used for complex sample matrices such as food and other natural products. Three static steps were needed for the extraction of carotenoids from different food samples (11), for fatty acids from wheat germ (12), for isoflavones from soybean (13) and for anthocyanins and phenolic compounds from grape skin (14). The extraction temperature in the first case was only 40 °C but in the other cases 105 °C, 100 °C and 80–100 °C temperatures were applied. Other applications include isolation of capsaicinoids from peppers with methanol at 200 °C (15), limonoid derivatives from Cortex dictamni with methanol at 150 °C (16), lipids from poultry meat with chloroform-methanol at 120 °C (17), anthaquinones from Rhubarb with methanol at 140 °C (18) and zearalenone and α-zearalenol from cereals and swine feed with methanol-acetonitrile at 50 °C (19). More recent investigations with ASE include extraction of oligosaccharides from Lupinus seeds (20) and anthocyanins from red cabbage (21). Various examples for the extraction of different environmental pollutants from various food and natural product samples can also be found (22–24). Water has been used in many cases as a solvent in commercial ASE systems (antioxidants in rosemary (25) and caffeine in green tea leaves (26), which is actually analogous to pressurized hot water extraction (chapter 2.2.3) though this is done most often with laboratory made instruments and in the dynamic mode.

Microwave assisted extraction (MAE)

Industrial and domestic microwave heaters are required to operate at either 2450 MHz or 900 MHz so as not to interfere with the wavelengths that are generally used for radar and telecommunication transmissions. Heating with microwaves is controlled by the dielectric properties of the material, i.e., the dielectric constant ε' (polarization of the molecule in an electric field) and the dielectric loss ε" (efficiency, how the dielectric energy can be converted to heat). The loss tangent ($tan\delta=\varepsilon''/\varepsilon'$), also called the dissipation factor, is a measure of the material's heating ability (how electromagnetic energy is converted into heat). Usually, the greater the dielectric constant, the higher the degree of microwave absorption (Table 1). Water has the highest dielectric constant of the common solvents. However, the loss tangent (0.123) is relatively lower than with other solvents. Thus the rate at which water absorbs microwave energy is higher than the rate at which the energy is transformed into heat. In addition to the loss tangent, the heating rate depends on the amount of solvent and its heat capacity. Overall, the microwave environment increases the extraction kinetics and diffusion processes via ionic conduction and dipolar polarization. Microwave heating is also much more rapid compared to conventional conductive heating (Figure 3).

Table 1.

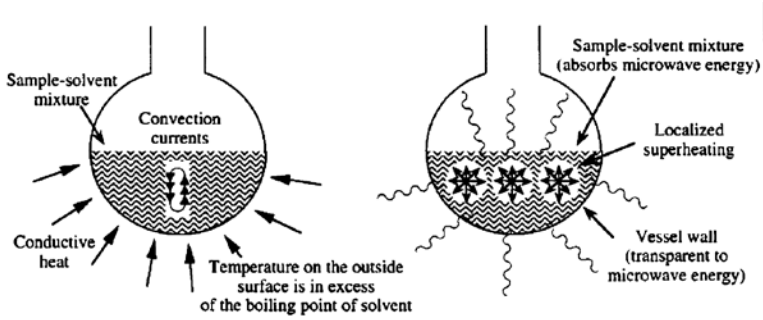
Dielectric constants and loss tangent values for selected solvents. Dielectric constant (27) and loss tangent (28) values at 20 °C temperature.

Solvent	*Dielectric constant*	*Loss tangent*[a]
Acetone	20.6	0.054
Acetonitrile	36	0.062
Dichloromethane	9.1	0.042
Ethanol	24.6	0.941
Ethyl acetate	6.2	0.059
Hexane	1.9	0.020
Methanol	32.7	0.659
Tetrahydrofuran	7.6	0.047
Toluene	–	0.040
Water	80.4	0.123

[a] Determined at 2.45 GHz.

Figure 3.

Microwave heating versus conventional conductive heating (30). (Reproduced with permission from EDP Sciences)



a: Conventional heating b: Microwave heating

Important parameters for MAE include solvent composition, temperature, extraction time and solvent volume. Extraction solvent volumes in MAE are in the range of 30–45 ml per 2–5 g sample. Optimization of MAE parameters for withanolides (steroidal lactones) from air-dried leaves of Iochroma gesnerioides has been reported (29).

For microwave extraction, one must choose a solvent in which the target analyte is suitably soluble. Depending on the matrix and the interactions between the matrix and the analyte, one may need to choose a solvent with a high extracting power. In order to obtain maximal heat distribution through the matrix, it is best to choose a solvent that has a high dielectric constant as well as a high loss tangent. If no single solvent is available with the appropriate microwave absorbing capability, then a solvent mixture with absorbing and non-absorbing components can be used. MAE with non-absorbing solvent can often be possible if the sample matrix is absorbing microwaves. This is often case with food and biological samples where some water is present. One additional solution with microwave transparent solvents is the use of a microwave absorbing stirring bar. These usually consist of a chemically inert fluoropolymer (Teflon) with a few percent of microwave absorbing graphite mixed within it.

Extraction temperatures up to 115–145 ºC are typically used (pressure is 200 psi or lower). A high temperature increases the recovery and is usually needed to obtain quantitative extraction or at least it is the most important parameter to study. The temperature is usually measured inside the reference extraction vessel in commercial systems and the microwave power is regulated to keep the temperature within selected values. Closed vessels are necessary with extraction temperatures higher than the boiling point of the solvent.

The extraction time in MAE is typically 5–20 min, rarely being more than 30 min. Since static extraction (batch) is generally used in commercial systems with the possibility to extract 6–36 samples simultaneously, degradation of thermally labile compounds is more likely and too long extraction times need to be avoided. However, some reports for the dynamic MAE has been published (31).

Additional examples to accelerate extraction process using microwaves include the use of microwave assisted Soxhlet extraction (Figure 4), focusing the microwaves into the sample (Figure 5) and conducting MAE in vacuum (32). Focusing microwaves improves the extraction efficiency further and shortens the extraction time needed. Applying vacuum can be advantageous for volatile and thermally labile compounds by enhancing the volatilization and lowering the required temperature.

The following are some examples of food applications of MAE e.g. determination of isoflavonoid aglycones in soybeans (35), phenolic acids in Radix Salviae Miltiorrhizae (36), triterpenic compounds in olive leaves (37), quercetin in *Psidium guajava* leaves (38) and total fat and trans fatty-acids content in bakery products (33). Focused microwaves have been employed for the extraction of polyphenolic acids in Eucommia ulmodies (39). One interesting use of the MAE of is the extraction of essential oils from dried fruits of *Illicium verum* Hook. f. and *Cuminum cyminum* L. using ionic liquid as the microwave absorption medium (40).

Figure 4.

Microwave assisted Soxhlet extraction system (33). (Reproduced with permission from Elsevier B.V.)

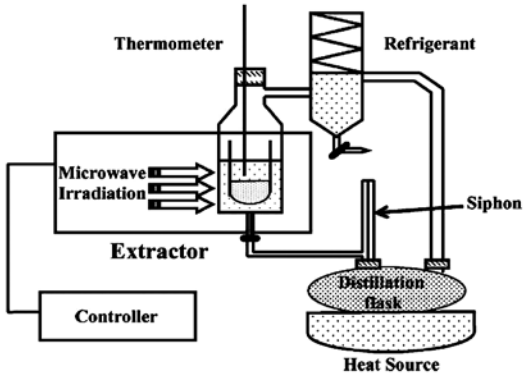
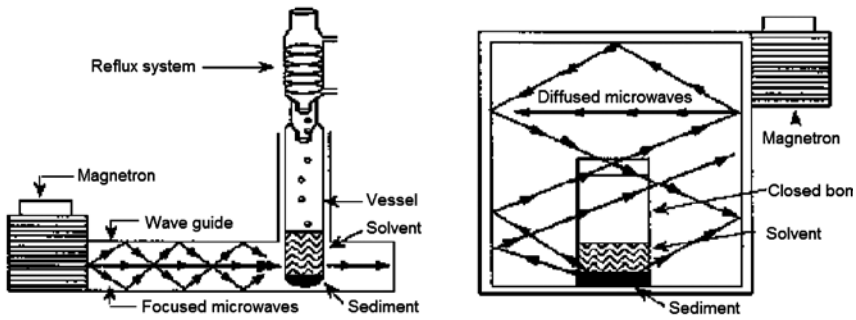


Figure 5.

Two modes of microwave irradiation. a) Focusing microwaves into the sample and b) microwaves reflected inside the oven (34). (Reproduced with permission from Phcog.Net)



Pressurized hot water extraction

Hot water extraction has long been used to extract organics from solid sample matrices (41). Typically, the temperature has ranged from 50 °C to 100 °C (at atmospheric pressure). The extracted compounds usually are relatively polar and thus soluble in water at these temperatures. If volatile, they are recovered by hydrodistillation or some other similar method. It was not until 1994 that Hawthorne and co-workers described fully how to utilize the special solvent properties of water by extracting non-polar semivolatile organic compounds from solid sample matrices with pressurized hot water (subcritical water extraction) (42). Other terms, such as superheated water extraction has been used for this technique. Pressurized hot water extraction (PHWE) can be defined to refer to these extractions where temperatures are higher than 100 °C but lower than T_c. PHWE describes the technique well since it involves both liquid water and steam; at high temperatures (>100 °C), steam, too, needs to be pressurized if it is to be efficiently pumped through the sample. Since this pioneering work, pressurized hot water extraction (PHWE) has been extensively used by the Hawthorne research group (43) and by several other groups (44–47).

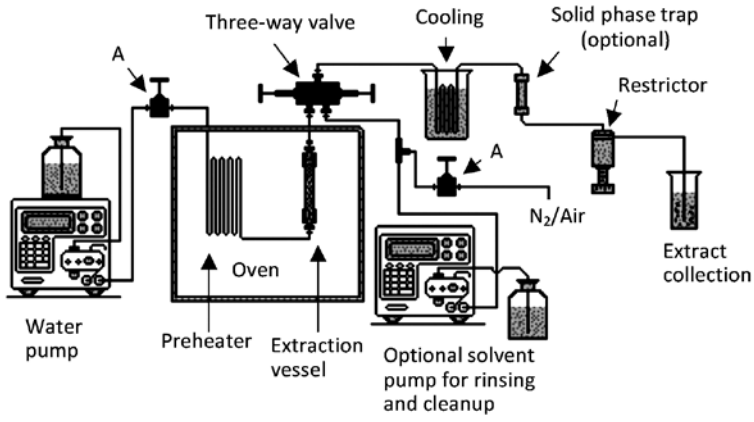
Temperature is the main parameter controlling the physicochemical parameters of water and the compounds to be extracted and thus the extraction-rate, efficiency,

and selectivity in PHWE. Similar physical advantages (faster diffusion, lower viscosity, and lower surface tension) to those obtained with ASE, MAE and especially with supercritical fluids (SCF) are achieved with pressurized hot water. Enhancement of the extraction efficiency may mostly be related to the increased vapor pressures and accelerated thermal desorption of the compounds (48). The effect of these factors is greatest in PHWE than it is in other techniques owing to the higher extraction temperatures often applied. Additionally, the high temperature significantly alters the solvent properties of water. This enhances dramatically the solubility of less polar compounds in water (49). As the temperature of the water increases, the hydrogen bonding, ionic, and dipole–dipole interactions between water molecules decrease. This solvent property (polarity) change of water can be seen in the value of the dielectric constant ε (measure of solvent-solute interactions), which drops from about 80 at room temperature to below ten at above 300 °C. This is in the same range as for medium polar organic solvents. The solvent strength of water and the degree of the thermal effects, both of which affect the extraction efficiency, can thus be adjusted with the temperature. This forms the basis for the selectivity, and class selective extractions according to solute polarity have been demonstrated (43).

In general, pressure has only a minor effect on the dielectric constant and the solvent strength of liquid water. The value of ε is slightly increased with pressure and this is not desirable when non-polar compounds are to be extracted. In practice, the pressure is set high enough to keep the water in a liquid form at all extraction temperatures. Some samples, however, may need higher extraction pressure to efficiently push the water through the sample, i.e. to overcome the back pressure generated by the sample. In the case of steam extraction, the pressure must be lowered to ensure that the water is in the gas phase. Extraction of non-polar compounds (alkanes) is generally more efficient and repeatable with steam than with liquid water at the same temperature (50).

The equipment needed for PHWE (Figure 6) is very similar to that used in SFE and accelerated solvent extraction (ASE). Although, in theory, both SFE and ASE systems are suitable for PHWE, commercial SFE and ASE instruments are limited by their temperature maximums of 150 °C and 200 °C, respectively. Temperature is restricted by the

Figure 6. Pressurised hot water extraction (PHWE) system. A=on/off valve. Nitrogen or air is used for purging the lines and drying of the trap.



polymeric material (PEEK) normally used in seals and extraction vessels. For low polarity compounds, such as alkanes, PAHs, PCBs, and PCDFs, extraction temperatures usually must be higher than 200°C (43, 51, 52). Lower extraction temperature can be applied for more polar analytes (47, 53). Special extraction vessels made only of metal (including soft metal seals) are necessary when work is done at high temperature. Polyimide ferrules can be used to seal the tube connections to the extraction vessel when the operation is conducted at high temperatures and pressures. A second, stainless steel ferrule must be used to keep the tubing in place at high pressure (slip-free connection). Analyte collection is much easier in PHWE than in SFE, because there will be no phase change from gas to liquid. The extract can easily be collected into the empty flask (solvent trap for volatiles can be applied if necessary) and recovered by liquid–liquid extraction (LLE) with several portions of fresh solvent. Alternatively, tedious and time-consuming LLE can be avoided by a solid-phase trap. After drying of the trap, the trapped analytes can easily be eluted with a small amount of organic solvent directly into the chromatographic sample vial (54). Furthermore, PHWE can easily be connected to the chromatographic devices for direct analysis (55, 56).

Careful degassing of the water is recommended to reduce the amount of dissolved oxygen, which otherwise will enhance corrosion of the system at high temperatures. One advantage with PHWE is that wet samples can be extracted as they are. At optimum conditions, the PHWE is very fast technique, majority of the analytes can thus be extracted in a few minutes (50). Additional attempts to enhance the PHWE can be made by adding a few percent of inorganic or organic modifier and by using surfactants. One recent example is the micelle formation to improve PHWE kinetics of PAHs in soil (57).

PHWE has been applied to isolate different natural products from plants, food by-products and algae. Antioxidants have been extracted from rosemary (58) and sage (59). PHWE of essential oils or other components from various plants, like clove (60), laurel (61), fennel (62), kava (63), peppermint (64) and oregano (65, 66), has been the widely studied area. Although PHWE is mainly performed in dynamic mode, the static extraction of antioxidants in microalgae using ASE instrument has been reported by Herrero et al. (67). PHWE has also been employed for the extraction of pesticides in fruits and vegetables (68), strawberries (69), grapes (70) and kidneys (71). Other plant or food applications include the extraction of aroma compounds in lime peel (72) and catechins and proanthocyanidins in winery by-products (73).

Ultrasounds assisted extraction

When sonicating (>20 kHz frequency) liquids at high intensities, the sound waves that propagate into the liquid media result in alternating high-pressure (compression) and low-pressure (rarefaction) cycles. During the low-pressure cycle, intensive ultrasonic waves create small vacuum bubbles in the liquid. When the bubbles reach a volume at which they cannot absorb energy any longer, they collapse during a high-pressure cycle. This phenomenon is called cavitation. During the implosion, very high local temperatures (ca. 5000 K) and pressures (ca. 100 MPa) can be reached. In extraction, this means that a greater penetration of a solvent into a sample matrix and improved mass transfer can be achieved with the aid of ultrasounds. Ultrasonic waves causing cavitation can disrupt cell walls (mechanically breaking them) and

facilitate the release of matrix components. In addition, ultrasound can lead to an increase in permeability of cell membranes to ions which can change the selectivity of the cell membranes. The mechanical activity of the ultrasound supports the diffusion of solvents into sample matrix, which facilitates mixing of the sample and generates additional heating. Furthermore, the particle size reduction by the ultrasonic cavitation increases the surface area between the solid and the liquid phase, leading to enhanced contact between phases. Especially for food processing, the homogenizing and preserving effect of ultrasounds can be easily utilized for fruit juices and purees as well as for vegetable sauces and soups.

Ultrasound assisted extraction can practically be arranged by employing an ultrasonic bath, in which the solid (or liquid) sample is immersed in flask containing the appropriate extraction solvent. Ultrasonic bath usually contains a timer to set the extraction time and temperature control or additional heating system. Although the energy incorporated into a bath is usually not very large (5 W/cm^2 or less), the temperature (an important parameter to be controlled if one wishes to achieve reproducible extraction) would be difficult to maintain without a control device which is not usually included in the common cleaning baths use in the laboratory.

Another option for ultrasound assisted extraction is the use of an ultrasonic probe. While in the bath the sound waves are spread in all directions, the probe can be utilized to direct and focus the ultrasounds directly into the sample. A probe is generally more energetic than a bath and enhances the extraction much more, thus decreasing the time needed. A probe is also more flexible since it can be positioned or directed into a sample more freely. Although the ultrasound assisted extraction (sonication) is more frequently used in the static mode, there are an increasing number of applications where dynamic extraction can be aided by the use of ultrasounds (Figure 7) (74–76).

Volatile compounds in a solid sample can also be extracted via ultrasonic nebulization extraction (UNE) where the purge gas is used to direct volatiles into the gas chromatograph for the analysis (Figure 8). This was successfully applied for the determination of trans-anethole in spices (78).

Basically in ultrasound assisted extraction, the variables that need to be considered (optimized) are the extraction solvent, extraction time, ultrasound energy and temperature. Additionally, selection between static/dynamic and bath/probe needs to be done.

Food applications can be divided according to analytes of interest into metals and organic compounds. For example, ultrasound assisted extraction of essential macro and micronutrient metal elements in animal feeds (77), zinc in meat samples (79), metals in vegetables (80) and manganese in solid seafoods (81). On the other hand, acaricides from honey were recovered with a new and fast ultrasonic-based solid phase micro-extraction (82), PAHs were extracted from food samples (83) and isoflavones from soybeans (84). Additionally, enzymatic probe sonication extraction of selenium in animal-based food samples (85) was found to be an efficient method catalyzing the breakdown of Se containing proteins into selenoamino acids.

Figure 7.

Experimental set-up used for the dynamic ultrasound-assisted leaching of metal elements. LC leaching carrier; PP peristaltic pump; UP ultrasonic probe; EC extraction chamber; WB thermostatic water bath; ER extract reservoir; C extraction coil; FAAS flame atomic absorption spectrometer; PC personal computer (77). (Reproduced with permission from Springer-Verlag)

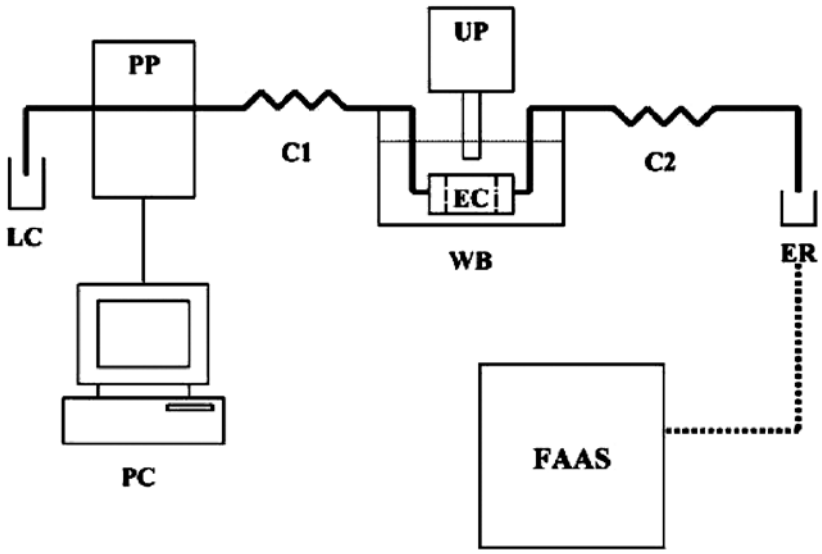
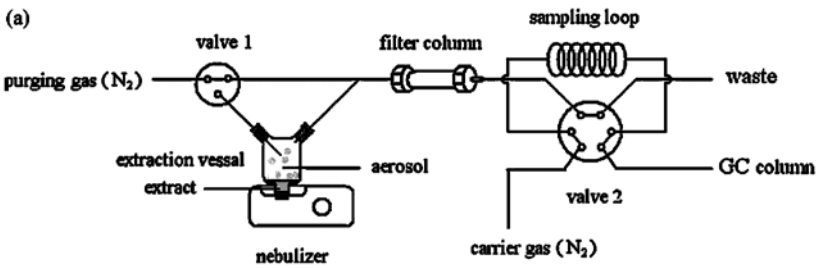


Figure 8.

On-line ultrasonic nebulization extraction (UNE) system (78). (Reproduced with permission from Elsevier B. V.)



Supercritical fluids

IUPAC defines a supercritical fluid (SCF) as any element, substance, or mixture that is heated above its critical temperature and pressurized above its critical pressure (86). SCF exists as a single phase (neither gas nor liquid) and it cannot be liquefied or vaporized by increasing the pressure or temperature. The density and other properties can easily be varied from gas-like to liquid-like by adjusting the temperature or pressure, or both. A supercritical fluid is an intermediate form of gas and liquid with some of the good properties of both states. The liquid-like high density and dissolving power make SCF useful as a solvent in a variety of applications, including extraction. In addition, favorable gas-like properties of SCF, i.e. low viscosity, zero surface tension, and high diffusion rates for analytes, make it an excellent extraction medium for a variety of organic (and inorganic) compounds in solid sample matrices.

Supercritical fluid extraction (SFE) can be simply defined as an extraction technique that uses SCF as an extraction medium (solvent). Since SCF can easily penetrate into porous sample particles, the mass transfer (diffusion) of the compounds from the matrix is faster than with similar organic solvents. In general, SCF results in faster and

more efficient extraction with better selectivity than can be obtained with traditional solvent extraction (87–90). The selectivity relies on the nature of the SCF, the possibility to adjust the properties via temperature and pressure, and the optional selective analyte collection system. This means that SFE extracts will usually be cleaner (fewer disturbing components) than those obtained with organic solvents, and ready for chromatographic analysis without the need for cleanup.

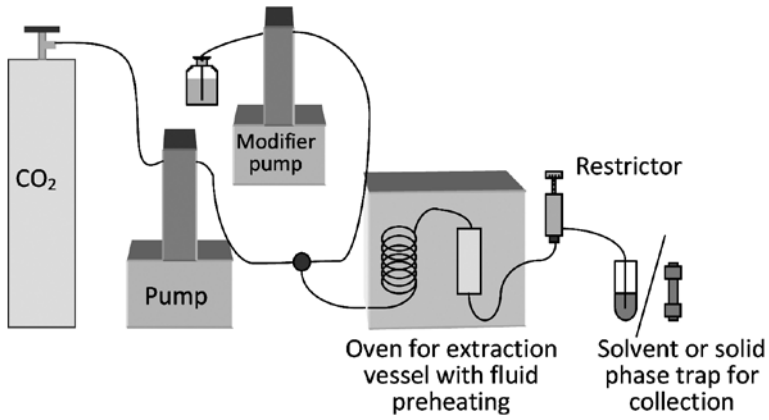
The typical instrumentation used for SFE is presented in Figure 9. A high pressure pump is needed to pressurize the extraction fluid and to deliver it at a constant flow-rate through the sample and the whole system. An optional second pump can be used to add modifier to the main fluid stream. A few percent of polar organic solvent is usually added to non-polar fluids like CO_2 to increase the solvent strength towards more polar analytes or to better overcome matrix effects. A modifier can also be added, directly into the extraction vessel, or premixed fluids can be used. The solid sample is placed inside an extraction vessel, e.g., a metal tube similar to those used in ASE and PHWE with frits and seals at both ends. However, if the pressure inside and outside the vessel is kept the same, the vessel can be made of inert polymeric material like PEEK. The extraction vessel can be heated in an oven or heating block.

The pressure (or flow) restrictor maintains the pressure inside the system and controls the flow-rate of the fluid through the sample. For example, good and repeatable flow control can be achieved with adjustable and computer-controlled needle-valve-type restrictor or mechanically compressed gold-plated disk that can control the flow through a small orifice. At the exit of the restrictor, the fluid expands to the atmospheric pressure and the extracted compounds must be collected. Compounds can be collected by retention on a solid support (adsorbent) that can be cooled to increase the trapping efficiency, through absorption to a few milliliters of organic solvent (bubbling the gaseous fluid through the solvent), or by depressurizing into a cooled empty container. These trapping techniques can also be combined to ensure quantitative recovery (91). SFE can further be combined with chromatographic methods like GC (92) with on-line collection and sample enrichment. When SFE and a solid-phase trap with a detector are used to monitor the analytes to be separated or fractioned, the system is in reality SFE–LC (93). Automated SFE systems include an extraction vessel and collection vial carousels for processing multiple samples.

SFE can be run in either the static or dynamic modes. In the static mode the sample is pressurized with the fluid at a set temperature for a certain period (no flow through the system). After the static period, the extracted compounds are eluted out in a short dynamic period. (Dynamic SFE means that the SCF is constantly flowing through the sample.) The dynamic mode is always the most efficient way to perform SFE; use of static extraction is worthwhile only if modifiers or reagents are added to the extraction vessel with the sample or if fluid consumption needs to be lowered. Pressure, temperature, fluid composition, extraction time, collection technique, sample loading and fluid flow-rate are some of the commonly optimized parameters in SFE. Typical pressures applied are in the range of 20–40 MPa, temperatures 40–150 °C and flow-rates 1–3 ml/min. Most often, carbon dioxide (T_c=31 °C and P_c=72.9 atm) is used as an extraction fluid. Methanol is the most frequently employed modifier. Especially with food samples, adsorbents are often loaded with the sample into the extraction vessel (for example to retain fat).

Figure 9.

Supercritical fluid extraction (SFE) system.



Most widely known industrial food applications of SFE are the selective extraction of caffeine from coffee beans and extraction of hops. However, numerous other SFE applications for food or related samples are being used or developed. These can be separated into groups according to compounds of interest, such as lipids, vitamins, flavor and fragrances, toxic compounds, antioxidants, steroids and drugs. Generally, the samples for the SFE need to be finely ground and dehydrated.

Fat can be extracted successfully from various food matrices and this has become a widely applied technique (94–98). Vitamin K1 has been extracted from soy protein-based and milk-based powdered infant formulas (99) and a higher vitamin A activity was obtained with SFE than with conventional methods (100). Carotenoids have been extracted from vegetables (101) and from leaf protein concentrate (102) with good recoveries. SFE has been widely applied for aroma compounds in different plants, like thyme (103), chamomile (104), eucalyptus (105), anis (106) and rosemary (107), to mention a few. Antioxidants have been extracted from many plants, like coriander (108), ginger (109) and lemon balm (110). A few examples of SFE of steroids (111) and drugs (112), as well as the extraction of toxic compounds like mycotoxins (113), PAHs (114) and pesticides (115) in food samples need to be mentioned.

Further interesting applications are on-line SFE-APCI-ITMS which is a suitable method for a fast extraction and analysis of volatile aroma compounds (116), extraction of rice bran oil that contain oryzanols (117), supercritical fluid extraction of lycopene in tomato skins (118) and extraction of carotenoids and chlorophyll from microalgae (119). ●

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9
Solid phase extraction

Solid-phase extraction as sample preparation technique – Background
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Modern approaches to solid-phase extraction (SPE)
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Solid-phase extraction as sample preparation technique – Background

Heli Sirén

Need for sample preparation

Sample preparation is needed to remove interferences due to matrix from the analysis. In particular, the presence of proteins in the analysis of biological and food samples may disturb chromatographic separation. The matrix effect may complicate quantification and identification of analytes, when analyte resolution is not adequate. Sample preparation is also needed to concentrate the analytes for detection. For example, two herbicides, chloroprop and metaprop, can be detected in drinking water at pg/l or lower levels. Their quantification without supplemental concentration is challenging.

History of solid-phase extraction

Solid-phase extraction (SPE) is today a widely used sample preparation method. Its advantages over traditional liquid-liquid extraction (LLE) are that it is faster and more repeatable. In addition, cleaner extracts are obtained and also consumption of solvents is reduced. Due to the technology used in SPE, smaller sample sizes are needed than in LLE. Nowadays, there is also a large selection of sorbents for polar analytes, which

cannot be extracted well enough into organic solvents with liquid-liquid extraction. The main reason for this is the water solubility of polar compounds.

Solid-phase extraction became commercialized in 1978 with the introduction of packed-bed column cartridges (1). Nowadays, it is a well established technology with thousands of literature references and many application areas. Table 1 lists the history of SPE.

Table 1. Early history of SPE (1).

Year	*Significance*
1906	Term "chromatography" coined by Tswett
1930	Normal-phase applications for liquid chromatography
1941	Partition chromatography
1950	Reversed-phase chromatography; widespread use of charcoal as a sorbent
1960	Bonded sorbents synthesized
1968	Polymer sorbents, XAD resins developed
1973	Gilpin and Burke develop chlorosilane bonded phases for HPLC
1974	XAD resins used for trace organic contaminants in water
1975	C18 reversed phase becomes popular for HPLC
1975	The term "trace enrichment" coined
1978	Sep-Pak introduced by Waters
1980	Automation of SPE begins
1982	The term "SPE" coined by Zief and J.T. Baker
1985	Proliferation of manufacturers and introduction of new SPE phases, such as mixed mode
1989	3M introduces the disk format for SPE
1992	Introduction by Supelco of solid-phase microextraction (SPME)
1993	Proliferation of automation products for SPE
1995	On-line analysis by SPE-HPLC becomes commonplace
1996	On-line analysis by SPE-GC becomes routine, including automated SPME

Solid-phase extraction is an increasingly exploited for preparation of concentrates and for purification of analytes from solution by sorption onto a disposable solid-phase material in a cartridge, followed by elution of the analytes in a solvent which is appropriate for instrumental analysis. The SPE method may be utilized also separating mixtures, when the use of multiple solvents might prove excessively cumbersome and/or expensive. Many of the problems associated with liquid-liquid extraction can be avoided with SPE (Table 2).

Table 2. Reasons to use solid-phase extraction (SPE) methods.

Requirement	*Benefits*
Need to remove specific interferences from the sample being analyzed.	Problems for detection and quantification of analytes of interest are excluded.
Need to increase concentration of analytes.	A large sample volume may be loaded onto an SPE column material if the analyte of interest is strongly retained. Analytes may be eluted with a very small volume or with a very large solvent volume followed by solvent evaporation and dilution to small volume.
Need to remove interferences in matrix that are invisible and suppress signals for the analytes when detected e.g. by mass spectrometry.	Removal of large amounts of biological compounds that cause ion suppression. Clean extract development.

There can be several disadvantages associated with LLE e.g. incomplete phase separations, low quantitative recoveries, and use of expensive chemicals and disposal of large quantities of organic solvents. SPE yields quantitative extractions that are easy to perform and due to its rapidity it can be automated and miniaturized. Furthermore, solvent use and working time are remarkably reduced from that needed in LLE.

Initially, SPE was based on the use of polymeric sorbents, such as XAD resins (hydrophobic crosslinked polystyrene copolymer resin), which were packed into small disposable cartridges for their use in drug analysis. As a reference, early environmental applications involved of both XAD resin and bonded-phase sorbent, such as octadecyl modified silica (abbrev. C18). These pre-columns were used for sample trace enrichment prior to liquid chromatography and were often arranged on-line, which means that sample preparation was conducted at the same time as the liquid chromatography. However, the first steel cartridges used in on-line were quickly replaced with off-line columns made of plastic since these were both inexpensive and disposable (1).

Solid-phase extraction is an attractive approach in sample preparation prior to LC separation. SPE can be used off-line so that the sample preparation is separated from the analysis (Figure 1). It may also be performed in the on-line mode, where sample preparation is directly connected to the chromatographic separation. The coupling of SPE with LC has several advantages e.g. improved sensitivity and selectivity and also better precision. Furthermore, since there is no sample manipulation between the pre-concentration and the analysis steps, the loss of analyte and the risk of contamination are reduced.

SPE is used most often to prepare and concentrate liquid samples and extract semi volatile or nonvolatile analytes, but also it can be used with solids that are pre-extracted into solvents and need extensive clean-up prior to analytical separation.

There are many SPE products that are available for sample extraction, concentration and cleanup. The methodologies are used with a wide variety of adsorbents and sizes. It is possible to select the most suitable chemicals and solvents for each SPE application. SPE treatment may be performed with the sorbents in cartridges and tubes, sorbents in Büchner funnels, sorbents placed to 96-well plates, in discs (Empore disk) (Figure 2), with the sorbent placed into the mini-tips of a pipette, on coated fibers placed in solid-phase micro extraction (SPME) syringe needles or in stir bar sorbent extraction (SBSE) modules. There are many manufacturers of SPE columns for example Supelco, Waters, Phenomenex, IST, Whatman, Baker, Sigma-Aldrich, 3M and SPWare. They all have a different production process for the sorbents, but in practice, (Table 3) the products do resemble each others, with the difference being in particle size, material quality and structure. Solid-phase extraction supports are also sometimes used in the form of filter-type extraction disks that are placed within Büchner type funnels for vacuum-assisted separation of mixtures. The membranes are fabricated in the form of functionalized silica disks although these are often brittle or fragile and for this reason are often strengthened by incorporation of a surrounding outer polymer (e.g. PTFE) support to provide mechanical strength. Functionalized powdered silica is often commonly doped within an inert fibrous polymer support membrane made from PTFE to produce a flexible extraction phase (2).

Figure 1.

Steps needed in the SPE clean-up and concentration before chromatographic analysis.

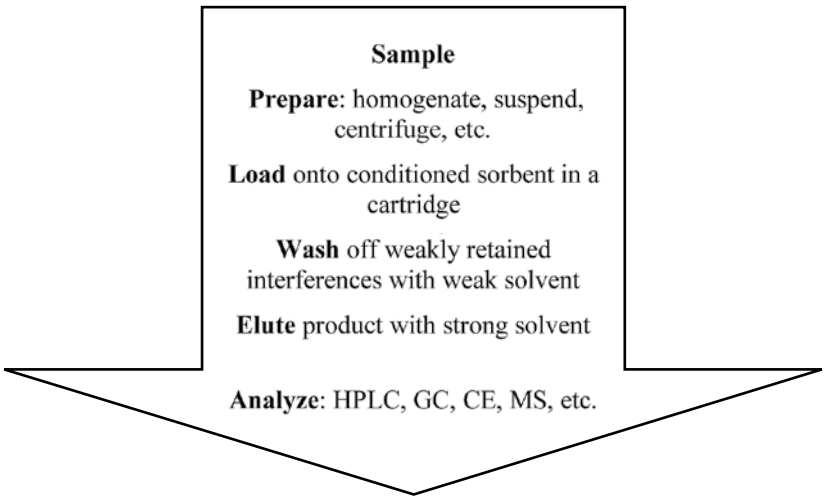


Figure 2.

Solid-phase cartridges.(3).

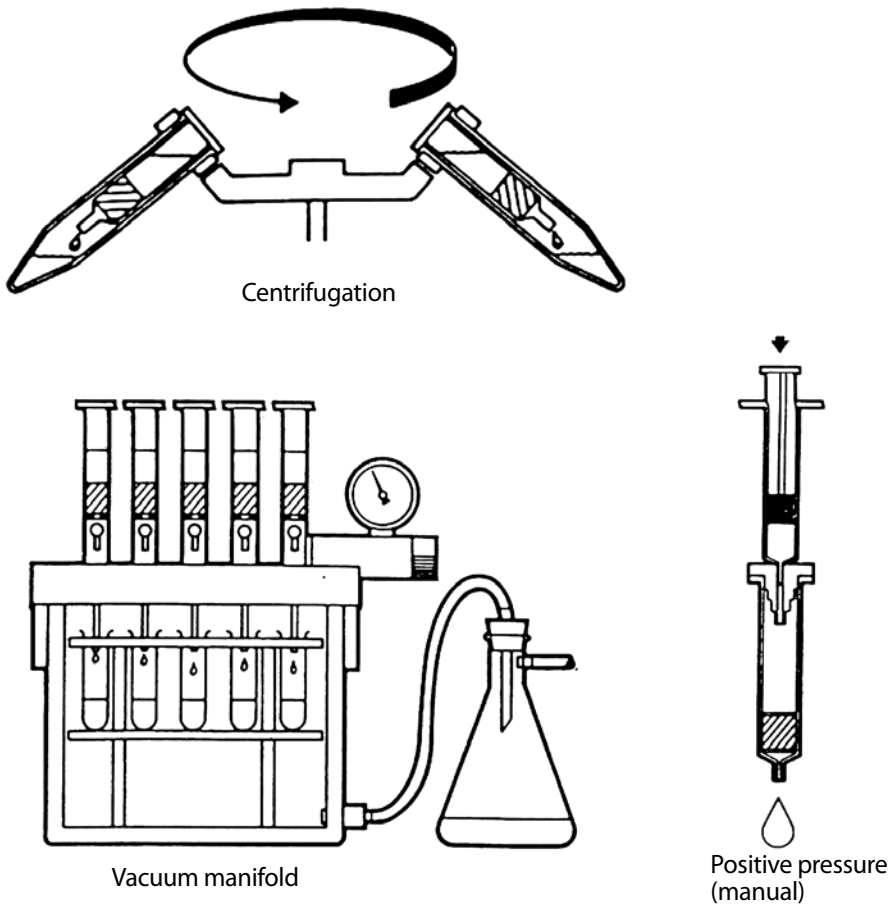
Table 3.

Brand names of selected manufacturers of solid-phase materials.

Manufacturer	*Brand name*
Waters	Sep-Pak, OASIS
Varian	BondElute
Baker	BakerBond
International Sorbent Technology	Isolute
3M	Empore
Supelco	DSC, ENVI, LC, Supel, Discovery
Phenomenex	Strata
Sigma-Aldric	h Supelclean, ENVI

Figure 3.

SPE extraction devices: centrifugation, vacuum manifold and manual pressurized tandem cartridge.



The solid partitioning phase normally takes the form of either powdered silica or a polymer powder support packed into a custom-fabricated cartridge. In some cases the separation may be accelerated by pressure exerted by means of a syringe barrels, of cartridges.

The types of retention agents include reversed phase, normal phase and ion exchange materials. Another popular approach involves using functionalized organic hydrophobic groupings such as C18 moieties bonded to the solid support. Trace hydrophobic organics are pre-concentrated on the sorbents in the cartridges as the sample is introduced and drawn through the catridge. The forces used are gravity, pressure or vacuum (Figure 3).

Solid-phase extraction columns are typically constructed of polypropylene or polyethylene. They can be filled with 40 μm packing material with different functional group. The sizes of the particles vary depending on the manufacturer. Commercial SPE cartridges are prepared in cartridges in volumes from 1 ml up to 160 ml.

Figure 4.

Molecularly imprinted polymers (MIP). EGDMA = Ethylene glycol dimethacrylate.

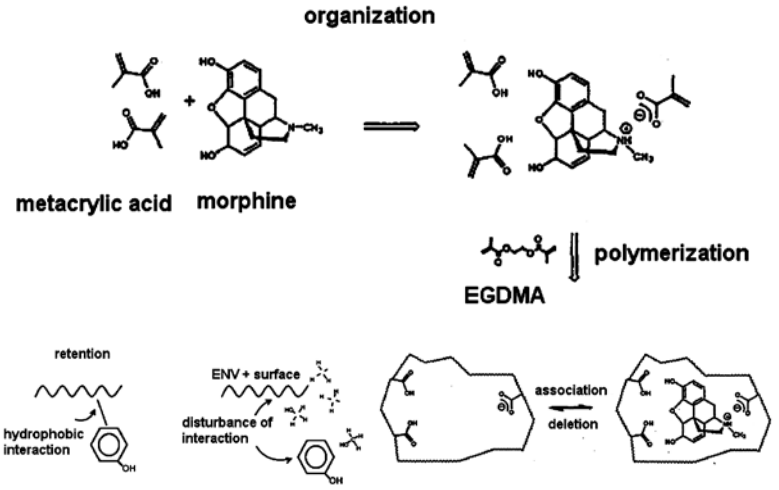


Table 4.

SPE strategies of SPE elution.

Elute the analyte, retain interferences
- k=0 for all analytes
- k high for interferences

Elute interferences, retain analytes
- k=0 for interferences
- k high for analytes

Concentrate analytes
- k large for analytes (large sample volumes are introduced)
- elute concentrated analytes
- enhanced sensitivity

k > 1000 (retention onto the sorbent is maximal)
k < 0.01 (elution of analytes is rapid after addition of extraction solution)

The most widely used SPE sorbents are chemically bonded sorbents based on silica and polymers. The drawback of these frequently used sorbents is their generic selectivity. In the case of non-selective sorbents, the analyte retention is based on hydrophobic interactions and there is co-extraction of analytes and matrix interferences. This may lower the purification efficiency of the method and this may represent a problem when analytes are at a low concentration level. Highly selective sorbents such as immunoaffinity sorbents (IA) and molecularly imprinted polymers (MIP) (Figure 4) can be used to improve the selectivity. In the case of the immunoaffinity sorbents, the analyte retention is based on an antigen-antibody interaction, whereas the high selectivity of molecularly imprinted polymers is achieved with molecular recognition mechanisms, similar to antigen-antibody interactions (4, 5).

As mentioned earlier, SPE represent chromatography, where the retention factor (k value) is the largest ($k_{HPLC} < 200$) in analyte retention and lowest when eluting the analytes for analysis. As in HPLC, polar and non-polar interactions such as those of ion exchange are valid (Table 4). As an example, non-polar compounds in the polar medium are readily retained onto non-polar sorbent (like dissolves like).

Solid-phase sorbents

The basic materials are 1) non-polar, 2) polar, 3) ion exchangers and 4) mixed mode sorbents. Furthermore, the columns filled with various sorbents can be coupled for tandem technique for selective isolation of analytes (Table 5) (1, 2).

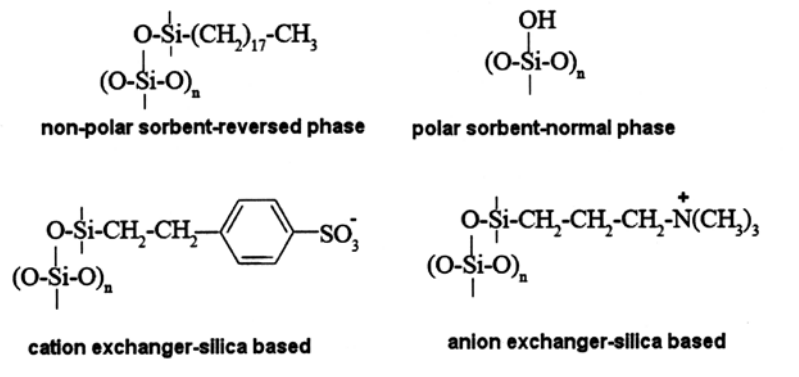
Sorbents have been synthesized from silica or polymers (e.g. polystyrene-divinylbenzene). The most traditional material, at least up till the beginning of the year 2000, was silicon oxide (silica) that has active groups like Si-OH and OH-Si-O-Si-OH. (Figure 5) Those groups have been synthesized to incorporate organic functional groups like alkyls, benzyls, amines and their derivatives. The adsorption properties of silica depend on the functionalized chemical groups and their activity (ionization, polarity), but also on the solvents used to immobilize the analytes onto the sorbents. Therefore, in the simplest case, the analytes are either retained to the sorbent or they elute through it without undergoing any interaction. The situation depends on what type of SPE clean-up is needed.

Table 5. *Sorbents with standard directions.*

Mechanism	*Sorbent*)*	*Solvents in conditioning*	*Solvents in elution*
Non-polar	C18, C8, C2, C1, PH, CH, CN	methanol and purified water	polar solvents (methanol, CH_2Cl_2)
Polar	NH2, 2OH, Si, CN	non-polar solvents	non-polar solvents
Cation exchanger	sorbents with sulphonic acids sorbents with carboxylic acids	methanol, acidic buffers (pH 26)	basic buffers (pH 8–12) high ionic strength
Anion exchanger	sorbents with prim. or sec. amines	methanol, basic buffers (pH 8–12)	high ionic strength

*) Non-polar and polar sorbents synthetized by C18 – octadecyl; C8 – octyl; C2 – ethyl: C1 – methyl; CH – cyclohexyl; PH – phenyl; CN – cyanopropyl; 2OH – diol; Si – silica; NH2 – aminopropyl. Ion exchangers synthetized by PSA – N-propylethyldiamine; SCX – benzenesulphonylpropyl (sulfonic acid bonded silica with Na^+ counterion); WCX (carboxylic acid bonded silica with Na^+ counterion); PRS – sulphonylpropyl (counterion Na^+); CBA – carboxymethyl (Propylcarboxylic acid functionalized silica); DEA – diethylaminopropyl; SAX – triethylaminopropyl (quaternary amine bonded silica with Cl- counterion); WCX (carboxylic acid bonded silica with Na^+ counterion).

Figure 5. *Some of the sorbent materials based on silica. Due to the steric effects the materials are derivatized further with alkylation to protect the analytes from undergoing composite interactions with different functional groups.*



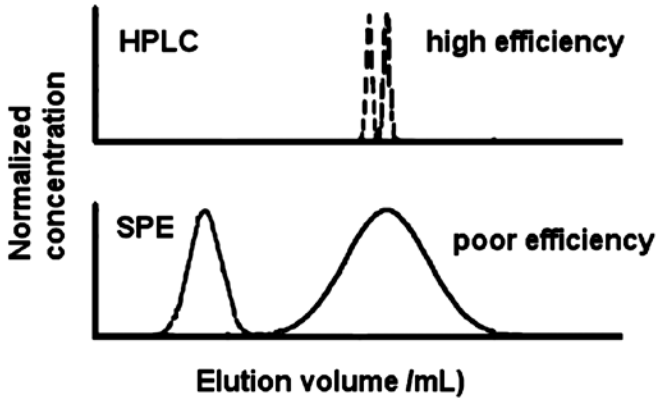
The amount of sorbent in the column is low (depending on the capacity of the sorbent). The retaining capacity is estimated as 1–5 % of the material weight, i.e. 100 mg of sorbent can adsorb 5 mg of analytes and sample matrix. The capacity of ion exchange sorbent is 0.5–1.5 meq/g (milli equivalent is a unit that provides information about the number of charges in solution volume of one liter.).

HPLC can separate similar compounds. SPE requires a significant selectivity difference between the analytes for separation. Compounds not well resolved by HPLC cannot be separated by SPE with a similar retention mechanism.(Table 6, Figure 6).

Table 6. *Differences between HPLC and SPE.*

Variable	*HPLC*	*SPE*
Particle size	~ 5 µm	40–80 µm
Sorbent efficiency	High	Low
Extra column volume	Low	High
Bed length	5–30 cm	~1 cm
Number of plates (N)	~10 000	< 50

Figure 6. *Differences with HPLC and SPE in efficiency.*



Usual structures of commercial sorbents

Due to the steric interactions, most of the functionalized silica materials are still active. To reduce this activity, the materials are derivatized after functionalization with short length alkyl groups that will substitute hydrogen in silanol groups, which have polarity but are independent on pH because of ionization. Thus derivatization can improve the behavior of the material and help the user to choose the optimal the chemicals and solutions for the interactions needed for the clean-up steps. In the case of polymers, the above mentioned active functionality is excluded already in synthesis.

Method development

The schematic of the SPE-method optimization procedure is presented in Figure 7. The main aspect of the method development of SPE is the choice of the sorbent (Figure 8). The adsorption property of sorbent may be understood as the ability of the sorbent surface atoms and molecules to have free valence electrons or other forces that can retain molecules.

Figure 7.

Scheme of the SPE procedure for optimization of the method.

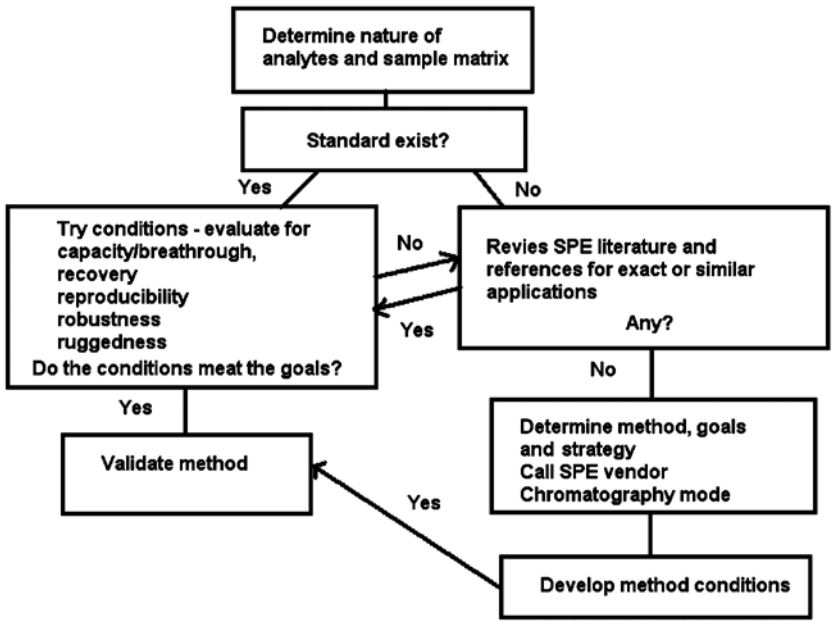
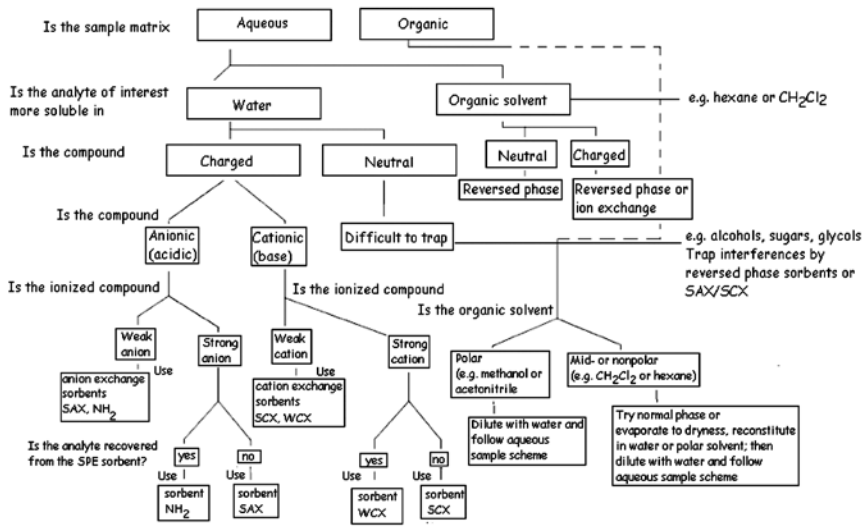


Figure 8.

Solid-phase extraction scheme to find the most suitable sorbent material.



Silica based sorbents need to be treated by washing techniques to allow them to swell before sample introduction onto the material (Figure 9, Figure 10). In addition, the polymer based sorbents also need washing with polar or non-polar solution before use (Figure 11). There are many standards available for treating the materials before they are used. Depending (Figure 8)on the functional groups of the sorbent and the analytes, the possible retaining parameters can be determined by chemicals which are present. In general, hydrophilic phases are washed with suitable solvents (e.g. methanol), water or buffer solution.

Figure 9.

Why does the SPE sorbent need to be conditioned? Untreated sorbent is dry and is not able to retain analytes. Conditioned material is activated so as to undergo physical interactions with the analytes entering the sorbent volume. (Thurman EM, Mills MS, Solid-Phase Extraction, Principles and Practice, Wileye&Sons, New York 1998)

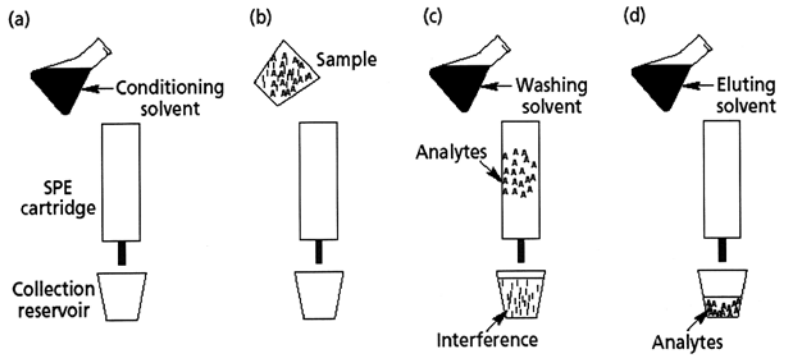


Figure 10.

Conditioning of SPE material. Conditioning of reversed phase with polar solvent (methanol, water).

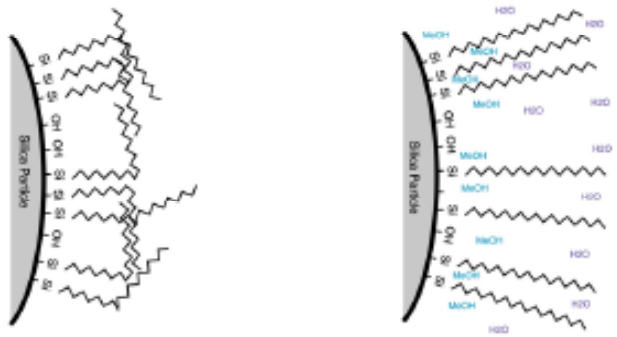
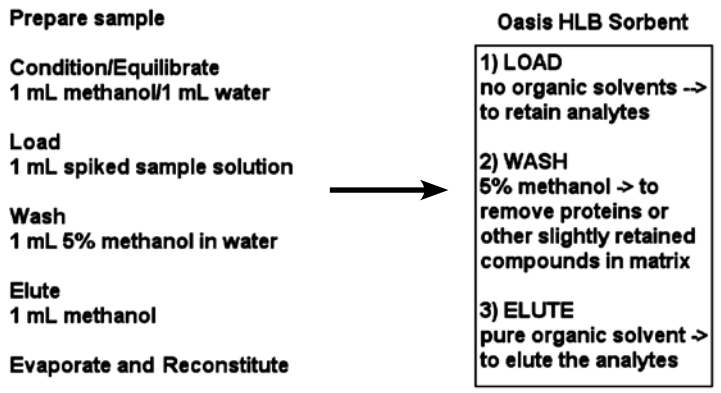


Figure 11.

Scheme of suggested procedure when non-polar OASIS HLB sorbent is used.



Adsorption is a general property of surfaces. Depending on the nature of the compounds and the surface structures, adsorption behavior may vary extensively from case to case. The concentration of analytes absorbed on the surface may be extremely high only when the amount of the sorbent material surface is high. Porous and small sized materials often fulfill these demands. The material can be chosen to elute extremely high sample solvent volumes, with only minor sample analytes being retained. (1–17).

The interactions listed on Table 7 are totally dependent on the functional groups of the sorbent and the analyte. There are molecular attractions that correlate with the dipole moments in the structures. The counter ionic parts of the dipole attract and those with co-ionic parts repel. In SPE treatment it is important to find a suitable solvent and the correct pH for ionization of the analytes (Table 8).

Table 7. Interactions and bond energies in SPE treatment.

Interactions	*Energy (kJ)*
covalent bond	410–1240
ionic bond	205–310
hydrogen bond	20–29
dipole bond	8–13
van der Waals interaction	4

Table 8. Characteristics of solvents used in SPE.

Polarity			*Solvent*	*Solvent type*	*Solubility in water*
Non-polar	Strong reversed phase	Weak normal phase	Hexane	Dipole-dipole	No
			Iso-octane	Dipole-dipole	No
			Chloroform	Dipole-dipole	No
			Dichloromethane	Dipole-dipole	No
			Tetrahydrofuran	Dipole-dipole	No
			Diethyl ether	Dipole-dipole	Slightly
			Ethyl acetate	Dipole-dipole	Yes
			Acetone	Dipole-dipole	Yes
			Acetonitrile	Dipole-dipole	Yes
			Isopropanol	Proton donor	Yes
			Methanol	Proton donor	Yes
			Water	Proton donor	Yes
Polar	Weak reversed phase	Strong normal phase	Acetic acid	Proton donor	Yes

The elution strength of a solvent is measured against n-pentane. The elution strength of n-pentane is set at zero. Although in practice the elution strength can be measured experimentally, it is possible to calculate this value
from the adsorption energy. The higher the adsorption energy, the larger will be the elution strength.
$\varepsilon o = E/Ae$, εo= elution strength, E= adsorption energy elution solvent, Ae= necessary space at the surface

When the analyte is an acid (e.g. carboxylic acid, sulphuric acid, phenol), it is non-ionic at low pH and anionic at high pH. When the analyte is a base (e.g. an amine) it is neutral at high pH and cationic at low pH. Ionization of the analytes may be calculated from the Henderson-Hasselbalch equation. In that case, their pKa values should be known.

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

The analytes are immobilized onto a sorbent surface also chemically, as mentioned earlier, i.e. the analytes can form chemical bonds with the sorbent. This type of bonding is much more efficient than physical adsorption. With respect to adsorption, the forces involved may be hydrogen bonding, dipole forces, dispersive forces, ionic forces, coordination forces or chelate formation (complex formation) (Table 9).

Hydrophobic interactions take place between the adsorbent's C-H –groups and the sample analyte's C-H-structures. Since all organic molecules have these kinds of bonds, hydrophobic interactions, are utilized in SPE extraction e.g. to C18 (Figure 12).

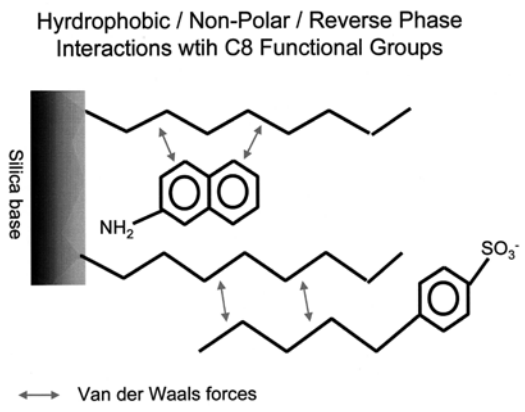
Table 9.

Interactions of analytes with sorbents.

Reversed phase • polar liquid phase • non-polar modified solid phase	*Hydrophobic interactions* • nonpolar interactions • van der Waals or dispersion forces
Normal phase	*Hydrophobic interactions* • polar-polar interactions • π-π interactions • dipole-dipole interactions • dipole induced dipole interactions
Ion exchange	Electrostatic attraction of charged group on analyte to a charged group on the sorbent´s surface
Adsorption	*Interactions of analytes with unmodified sorbents* • hydrophobic and hydrophilic interactions may apply • depends on which solid is used

Figure 12.

Hydrophobic/ non-polar/ reversed phase interactions of C18 functionalized sorbent with analytes.



Polar interactions are formed between the adsorbent's polar functional groups and the sample analyte's polar groups. These interactions are attributable to the positive and negative charges present in the molecule. Groups undergoing these types of bonding are hydroxyl, amino, carboxyl, sulphoxy and other polar functional groups.

Ionic interactions are also utilized in SPE extraction, when the analyte is an ion or the ionic form of the analyte provides better isolation from the matrix. In that case, it can be advantageous that the sorbent has also functional groups that are ionized. When exploiting ionic interactions, the pH of all solutions used needs to be adjusted carefully to the SPE system.

Reversed phase separations involve a polar, usually aqueous, or moderately polar sample matrix and a nonpolar stationary phase. The analyte of interest is typically mid- to nonpolar. Several SPE materials, such as the alkyl- or aryl-bonded silica are included into the reversed phase category. The hydrophilic silanol groups at the surface of the raw silica packing (typically 60 Å pore size, 40 μm particle size) are chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silanes (Figure 13).

In general, it needs to be remembered that it is always worthwhile to test various sorbents and to compare their cleaning properties with each other and then to choose the most efficient option. For example, C18, C8 and phenyl substituted materials are all non-polar phases but their physicochemical behaviors are different. This means that their cleaning properties will also be quite different. The literature contains many applications of use of the material tests and their selectivity for special cases.(1–17).

Octadecyl (C18) functionalized materials are useful for purification of drugs and pharmaceuticals and their metabolites in body fluids (Figure 14). The material is used for removal of peptides in biological samples. It can be used for extraction of organic material from environmental waters and isolation of organic acids from beverages. The material behaves as in reversed phase separation in HPLC. C18 is suitable for reversed phase extraction of non-polar to moderately polar analytes.

As an example, there are also sorbents available for environmental purposes, e.g. ENVI-C18. It has a higher phase coverage and carbon content than C18, greater resistance to extreme pH conditions and a slightly higher capacity for non-polar analytes. Like C18, it is used for reversed phase extraction of non-polar to moderately polar compounds, such as drugs. There are some other commercially available sorbents that have a shorter alkyl group than octadecyl. It should be remembered in those cases that the shorter the alkyl chain length, the less hydrophobic it will be. Therefore, when compared with C18, their extractability will be different. When the alkyl chain is replaced with aromatic substituent, the material is less hydrophobic than octadecyl. ENVI materials are used for extraction of polar aromatic analytes from aqueous samples. It can also be used for non-polar and mid-polar aromatic compounds.

Aminopropyl (NH_2) functionalized material is capable of extracting of phenols, fragmentation of petroleum, isolation of saccharides and can be used in the extraction of drugs and metabolites from biological materials. It is also used to remove excess derivatization chemical from samples to be analyzed with gas chromatography.

Cyanopropyl (CN) material is also useful in cleaning of biological matrices in drug analyses. It has mild and neutral medium polarity and can be used as a normal phase

Figure 13.

Hydrophilic/ polar/ normal phase interactions on silica and cyano functionalized sorbents.

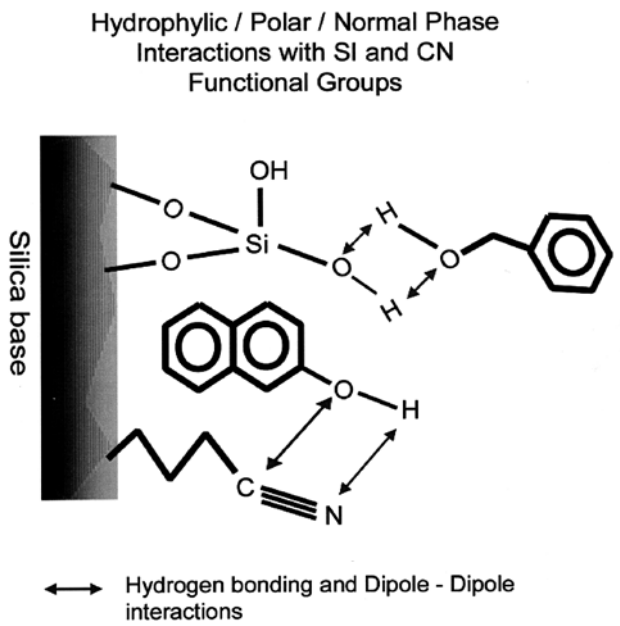


Figure 14.

Preparation of octadecyl (C18) functionalized silica sorbent (substitution reaction).

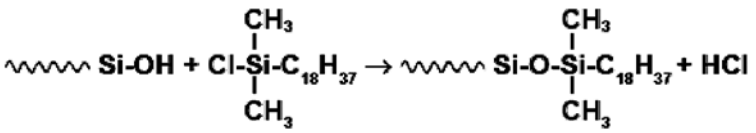
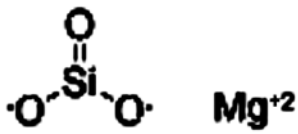


Figure 15.

Functional groups of Florisil (MgO3Si, MW 100.39 g/mol).



extraction sorbent for polar analytes like aflatoxins, herbicides and pesticides. It behaves as weak cation exchange for carbohydrates and cationic analytes.

One special application is the use of *Florisil*. This sorbent often helps, when analytes are unstable on silica sorbent. (Figure 15). It is more expensive than silica gel. With a particle size of 200 mesh it can be effective in straighforward separations. Less than 200 mesh particles are best for purification by filtration. Some compounds stick to Florisil and therefore the analyte-sorbent combination should be tested first before samples are extracted. It is used as a special material in cleaning of pesticides (AOAC and EPA methods). The material is suitable for isolation of polychlorinated biphenyls from petroleum.

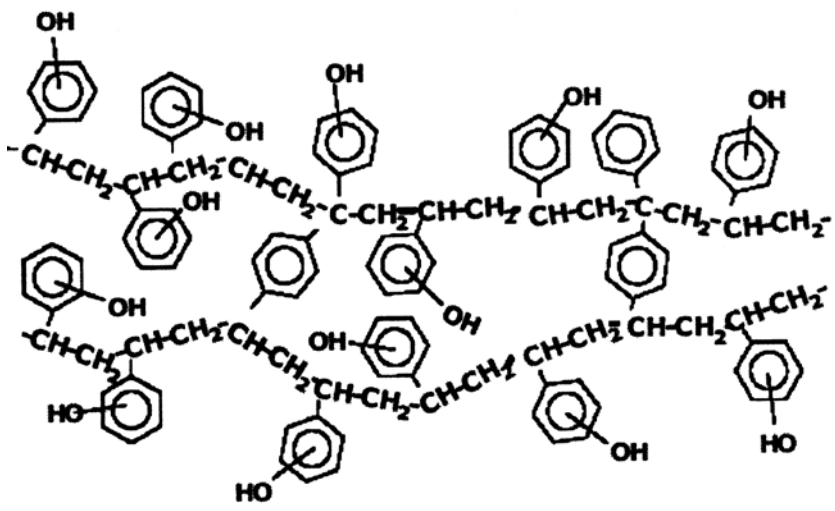
Florisil is used for the extraction of polar analytes, such as alcohols, aldehydes, amines, dyes, organic acids and cyclic organic compounds.

Alumina based packings have been differentiated into three categories based on the changes in the sorbent as a function of pH. The groups are 1) acidic alumina (pH ~ 5), 2) basic alumina (pH ~8.5) and 3) neutral alumina (~ 6.5). Acidic sorbent behaves as anion exchanger and adsorption extraction for polar analytes. Basic sorbent is used for adsorption extraction of polar analytes and as a cation exchanger. Neutral alumina is used for extraction of polar analytes by adsorption, but when needed, with pH adjustment it behaves as either a cation or anion exchanger. Many different analytes can be extracted e.g. essential oils, enzymes, glycosides and vitamins.

Polymeric bonding (Figure 16) is more resistant to pH extremes, and thus is suitable for environmental applications for trapping organic compounds from acidified aqueous samples. All silica based bonded phases have some percentage of residual unreacted silanols that act as secondary interaction sites. These secondary interactions may be useful in the extraction or retention of highly polar analytes or contaminants, but may also irreversibly bind the analytes of interest. Carbonaceous adsorption media, such as the ENV-Carb, consist of graphitic, nonporous carbon; they have a high attraction for organic polar and nonpolar compounds from both polar and nonpolar matrices. The carbon surface consist of atoms in hexagonal ring structures, interconnected and layered in graphitic sheets. The hexagonal ring structure displays strong selectivity for planar aromatic or hexagonal ring-shaped molecules and hydrocarbon chains with potential for multiple surface contact points. The retention of analytes is based primarily on the analyte's structure (size and shape), rather than on interactions of its functional groups with the sorbent surface. Elution is performed with mid- to nonpolar solvents.

Figure 16.

Structure of ENV+ polymer material (ref. Waters, Oasis).



Phenols are sometimes difficult to retain on C18-modified silica under reversed phase conditions, mainly due to their greater solubility in water than in organic sorbent. Polymeric adsorption media based on polystyrene-divinylbenzene can be used for retaining hydrophobic compounds, which contain some hydrophilic functionality, especially aromatic compounds. (1–17).

Ion exchange extraction is needed for very polar analytes that easily dissociate into water and are ionized. Anion or cation exchange extraction may be needed for organic acids, surfactants and metals.(Figure 17, Figure 18).

Figure 17.

Ionic/ ion exchange interactions on SCX and WCX functionalized sorbents.

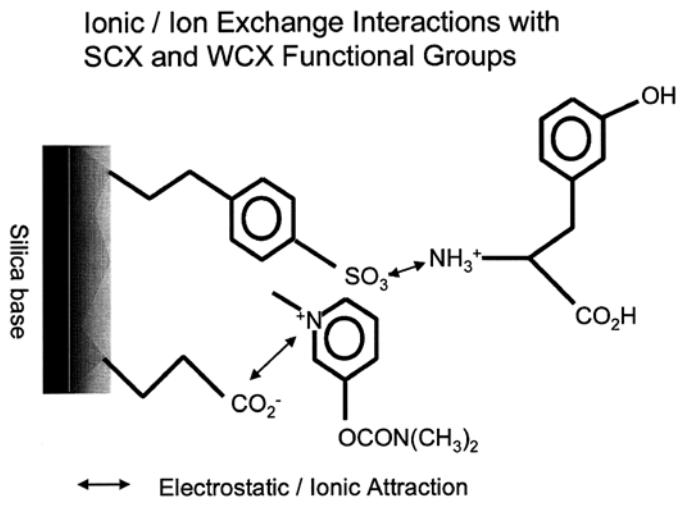
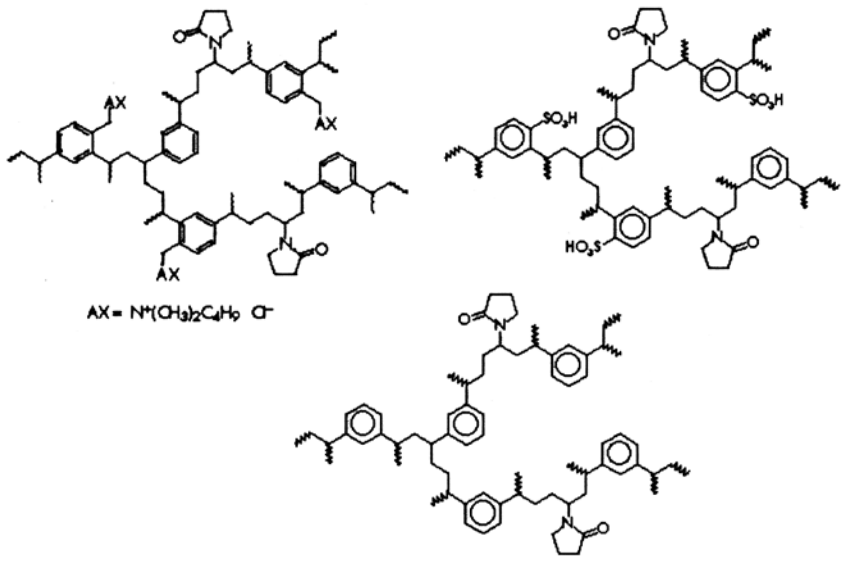


Figure 18.

Divinylbenzene-vinyliryrrolidone copolymer materials (mixed sorbent): (from left to right) anion exchanger, cation exchanger and non-functionalized material.

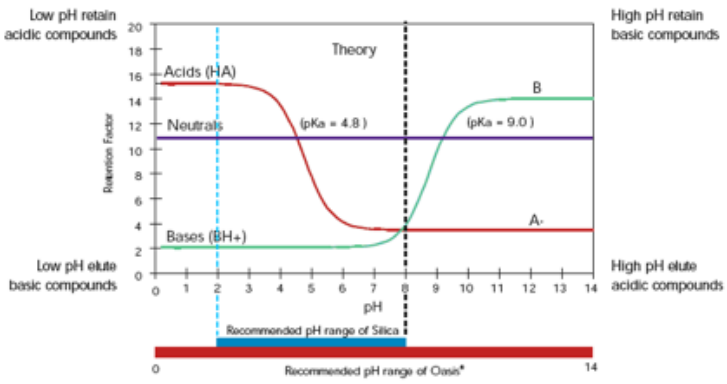


The role of pH in SPE

In SPE, the solutions used for conditioning of sorbent have quite a broad pH range, especially in ion chromatographic extractions. The base material in silica is stable only at pH of 2 to 7.5. At pH levels outside that range the bonded phase may be hydrolyzed and cleaved off from the silica surface. This may lead to reactions where the silica becomes dissolved. In SPE, the solutions usually are in contact with the sorbent for a short time, and thus hydrolyzation may be eliminated. Furthermore, if reactions occur they may not influence the extraction since these cartridges are disposable. If the stability of the SPE sorbent at an extreme pH is crucial, polymeric or carbon based SPE sorbents may be used, since they are stable over a pH range of 1–14 (Figure 19). The pH of the solvent does not influence the retention of neutral analytes. Therefore, it is possible to use pH where the disturbing compounds in the sample are retained, but the analytes of interest pass through the sorbent unretained. The situation is different when the analytes possess charges. (1–17). The pH of the sample and solvents play a significant role in extraction. In that case, the pH of the eluent should be adjusted to the optimal analyte retention and then usually most of the matrix compounds will elute without retention through the sorbent. Since analytes are eluted from the sorbent with organic solvents, the pH does not exhibit any role in the final elution, except in ion exchange elution systems. In that case, elution solvents need to be modified to formulate the analytes in an uncharged form.

Figure 19.

Recommended pH ranges of silica and Oasis materials (ref. Waters Catalogue: Oasis).



Immunoaffinity solid phase extraction

Immunosorbents are highly selective towards the analytes modified. They have been introduced as a selective new material during the last few years. The selectivity is based on antigen-antibody interactions (molecular interactions). They are constructed of specific antibodies, which are immobilized onto a solid support which is packed into a SPE cartridge or pre-column. Initially, immunosorbents were used in the biological field, because of the availability of antibodies for large molecules. Due to difficulties

in synthesizing selective antibodies for small molecules, their application to environmental analysis has been rather limited. (1, 2, 4, 5).

Binding of antigen to antibody is a result of good spatial complementarily and is caused by intermolecular interactions. Therefore, the antibody may bind also other structurally similar analytes, a phenomenon called cross-reactivity. In the biological field, this is usually not considered a problem but in environmental analyses it is valuable, because usually the aim is to isolate a group of analytes of similar structure and to separate them subsequently with the analytical technique.

Novelty special techniques of solid-phase extraction

Column extraction on solvent layer of solid-phase particles

Chromatographic purification techniques utilize adsorption chromatography as the basic mechanism. In the Extrelut technique (Figure 20), which is performed on liquid film on solid-phase sorbent particles, extraction of analytes is based in liquid-liquid partitioning.

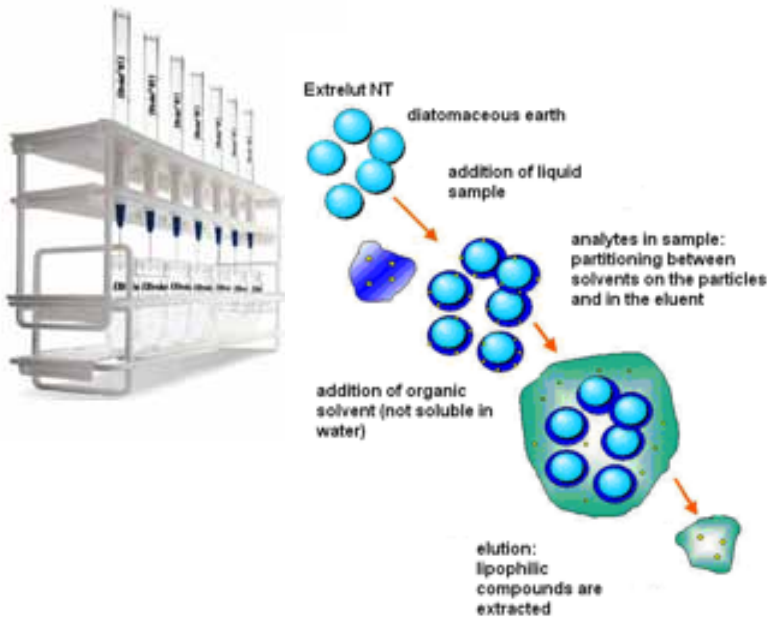
The cartridges and columns contain diatomaceous earth (Kieselguhr, amorphous silicic acid) as the sorbent. The methodological aspects of column extraction on a solvent layer differentiate it from SPE.

The technique can be applied to the separation of analyte groups from sample matrices, purification of matrices and for drying of organic solvents. In particular, the Extrelut technique has been found useful in drug and metabolite extraction from biological fluids.

The trade names of the sorbent materials are Extrelut NT (www.merck.de), Chem Elut, Tox Elut, Hydromatrix (www.varianinc.com), Isolute HM-N (www.arqotech.com) ja Celite 545 AW (incl. 0,1 % CaO, 0,3 %Fe_2O_3, 98,7 % SiO_2) (www.worldminerals.com).

Figure 20.

(A) Extrelut extraction device. (B) Operational principle of diatomaceous earth . 1) Dry sorbent particles in column. 2) Introduction of liquid sample onto the sorbent: liquid is partitioned on to sorbent particles. 3) Addition of organic solvent (the solvent should not ave solubility in water). 4) Elution.



Solid-phase microextraction (SPME)

Solid-phase microextraction was already developed at the end of 1980´s, but reported in 1990. The method was developed by C. Arthur and J. Pawlizyn in Canada.

The method has been become very popular in all kinds of analyses, because it can be on-line coupled with chromatography. It has been commercialized with automation.(17–29).

Solid-phase sorbent and films are used in the SPME technique. The particles are cylinder-shaped polymer fibers (Figure 21). This techniques differs from the traditional SPE, in that amount and volume of the sorbent material are very low. Fibers are attached to the inner walls of a syringe's steel needle. This means that the needle is not totally filled with the sorbent.

Figure 21.

Solid-phase microextraction syringes.

The fiber materials have been synthesized to be analyte group specific. For example, PDMS (polydimethyl ¬siloxane) is used for the extraction of polar compounds, PDMS/DVB (polydimethyl siloxa¬ne/divinyl benzene) for the extraction of polar compound, CAR/PDMS (carboxen/ poly¬dimethyl siloxane) for extraction of gases and very volatile compounds and DVB/CAR/PDMS can be used for the extraction of odor and flavor compounds.

Polar polyacrylate (PA) is a suitable extraction material for rather volatile compounds, like phenols. Polar Carbowax/DVB material (CAR/DVB) extracts alcohols and other polar compounds. In general, if one wishes to maximize the extraction polymer layer on the needle then the walls should be very thin in order to achieve an efficient extraction. It is known that the thickness of the fiber has an influence on the recoveries, because it will modify the equilibrium time and the concentration in analysis. If one uses a thin film, then the transfer of analytes will be faster.

SPME is performed in either the direct or the headspace mode. In the direct mode the fiber is in contact with the sample solution since it is immersed in the solution. However, in the headspace mode, sample is heated and volatile sample analytes will concentrate above the sample, forming a gas phase (headspace). The vapor compounds are sampled into the fiber in the syringe and injected into a gas chromatograph.

Stir Bar Sorptive Extraction (SBSE)

Stir Bar Sorptive Extraction is a technique that can be used for isolation of analytes directly from sample solutions (DI method) or from the headspa¬ce above it (HS). In solution, the bar behaves like a spinning magnet rod by with the rotation promoting; the analytes in the sample to concentrate on the sorbent inside the stir bar. The sorbent is polydimethyl siloxan (PDMS, length 0.3–1 mm). In headspace SBSE, the stir bar in placed on a holder above the sample in the gas phase. (30, 31).

Usually, the volume of PDMS sorbent is 20–350 µl. In general, the stir bars are 2 cm long and contain 55 µl PDMS. SBSE techniques have been used in environmental analyses.

Liquid Phase Membrane Extraction

Liquid Phase Membrane Extraction (LPME) represents the newest SPME technological advance.(32, 33). The LPME technique is an application where the SPME fiber qualities and coating techniques are used (comparable to Extrelut). LPME is appropriate for sample preparation for both gas and liquid chromatographic techniques as well as capillary electrophoretic analyses. The sample matrix is placed in contact with a porous fiber located as a membrane between the solutions in Hollow-Fibre Liquid-Phase Microextraction (HF-LPME).

The LPME membrane consists of porous and hollow polypropylene fiber connected to theheads of two needles. Using one of the needles, the fiber pore is filled with 15–25 µl of extraction solvent, and the solvent is absorbed onto the fiber. Then the fiber is set into the sample solution. The analyte solubility can be enhanced by pH adjustment. The extract is gathered from the fiber with the other needle. ●

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Modern approaches to solid-phase extraction (SPE)

Kati Hakala | Armi Asola | Marika Jestoi

Introduction

Solid-phase extraction (SPE) is a widely used clean-up technique in chromatographic analyses (1) and is presented in more detail in another chapter of this book. However, conventional SPE may have some weaknesses, such as lack of specificity due to its adsorption properties as well as difficulties in developing so-called multicompound-methods for simultaneous determination of chemically diverse compounds.

According to recent national and international laws and regulations (e.g. European Commission regulations for the control of official analysis in food safety), there is, however, a demand for more sensitive and selective analyte detection in complex matrices. To fulfil these requirements and furthermore to obtain multi-analyte applications to meet increasing demands for laboratory efficiency, liquid chromatography-mass spectrometry (LC-MS) has gained more and more popularity as an analytical technique over recent years. Nevertheless, it has been shown that compounds that co-elute with the analyte in LC-MS analyses can affect the analyte signal when compared to pure standard solution – a phenomenon known as matrix effect (2). The consequences of matrix effect include higher detection and quantification limits together with decreased

accuracy and precision. These problems will be minimized when interfering matrix compounds are removed. This means that improved sample clean-up is still needed before the LC-MS analysis.

In this chapter, we describe in detail some modern approaches to resolving the problems inherent in conventional SPE. Immunoaffinity columns (IACs) containing a specific antibody against an analyte have long been used for specific sample clean-up, and nowadays IA-columns recognizing several analytes are also on the market. Interest in using molecularly imprinted polymers (MIPs), referred to as synthetic immunosorbents, in sample preparation is also on the increase. MIPs can be produced to recognize a group of similar compounds and even tailor-made MIPs are commercially available. During the last few years, dispersive-SPE (dSPE) or the so-called QuEChERS (quick, easy, cheap, effective, rugged, and safe) method has entered routine use in multi-residue analysis.

Immunoaffinity columns

Antibodies are a group of globular proteins which consist of monomers or multimers of a four-chain structure containing two light and two heavy chains (3). Antibodies are also known as immunoglobulins named with letters IgA, IgD, IgG, IgE and IgM. They contain both constant and variable regions which are responsible for most of their immunological functions such as their interaction with immunoglobulin receptors and the ability to bind antigen. IgGs are mostly used in immunochemical techniques but IgA and IgM can also be used in some cases. The diversity of immunoglobulins produced is based on so-called hypervariable region sequences. Theoretically, a human being can produce 108 different antibodies.

Antibodies against foreign substances (antigens) are produced by B lymphocytes. Different B cell clones can produce a great variety of antibodies against a single antigen. This is called the polyclonal response. The produced antibodies display differences in their amino acid sequences in the antigen binding area. Monoclonal antibodies (Mabs), instead, are produced by a single B lymphocyte and the amino acid sequence is consequently identical in each antibody. A typical procedure for producing Mabs is fusing B lymphocytes from an immunized animal (mouse, rat and rabbit) with myeloma cells to produce so-called hybridoma cell lines. These cell lines are relatively easy to culture and grow to allow long-term antibody production. Both monoclonal and polyclonal antibodies have been used for immunoaffinity columns but the use of Mabs is increasing (4). Producing monoclonal antibodies is more costly and they are more susceptible to organic solvents. However, the use of Mabs reduces the need for animals in large-scale production.

Since the antibody-antigen binding is extremely specific in its nature, it can be utilised in analytical approaches either in sample preparation or for separation purposes, i.e. as a sorbent in SPE-cartridges (immunoaffinity SPE-columns) or in analytical HPLC-columns (immunoaffinity-HPLC). If one wishes to create immunoaffinity (IA) SPE-columns, the antibodies produced by B-cells are immobilized on different solid-supports (5), which should be chemically and biologically inert, easily activated and hydrophilic. Typical supports are agarose, sepharose, cellulose, polystyrene, poly-

acrylamide, silica gel or glass beads (3, 5, 6). Most commonly antibodies are covalently attached onto the supports but they can also be noncovalently bonded, adsorbed onto the surface or the antibodies can be encapsulated into the pores of the matrix using the sol-gel method (5). Immobilization happens when antibodies bind to an immobilized ligand (3) which is usually an amine, carboxyl or thiol group on the solid support.

The use of IA-columns is in principle similar to conventional SPE. Firstly, a sample extract containing antigens (analytes) is applied onto the column (5, 6). If the chosen conditions are appropriate, antigens are specifically bound to antibodies and the interfering compounds are washed away. Binding of the antigens often involves electrostatic interactions, hydrogen bonding and van der Waals forces (6). The most commonly used solvents for antigen applications are methanol or acetonitrile with different amounts of water (3). The pH-value of the buffer, if used, is quite often in the physiological range (pH 7.0–7.4) (6).

Antigens are eluted from the column by applying conditions that break the antibody-antigen binding. Generally this is achieved by using a high percentage of an organic solvent (methanol, ethanol, acetonitrile) to disrupt hydrophobic interactions, by adding a chaotropic compound or some other displacer agent that has a strong affinity for the antibody, or by changing the pH-value (5, 6). The conditions used must not be excessive so that the immobilized antibodies can be regenerated, if necessary.

As the production of immunosorbents is more expensive than producing a normal SPE column, the regeneration of antibodies is important in many cases (6). It is preferable that antibodies can be regenerated many times after use. One possible way to regenerate antibodies is to wash the column with buffer. However, it must be guaranteed that there will be no carry-over to the following analyses, especially in the case of trace analyses.

Although specific in principle, IA-columns also have some shortcomings. Cross-reactivity means that an antibody has the ability to recognize not only antigens but also some related molecules (5, 6) In practise, an antibody is specific and selective for a class of compounds or for a target molecule and its metabolites, at least when the molecular size of the antigen is small. Another problem related to IA-columns is their capacity. Capacity is an indication of the analyte breakthrough when a certain amount of analytes has been loaded onto the column (7). It also refers to the total number of active antibodies immobilized on the immunosorbent and how strongly the analytes are bound to the antibodies (5, 6, 7). If the purity of the antibodies is high, there are more antibodies available. If immobilized antibodies have a random orientation or they are sterically hindred, there might be problems with capturing the analyte and this can evoke some problems in the analytical method.

The earliest IAC applications date from the middle of 1980s and since then development of immunoaffinity-based columns has had an immense impact on utilizing antibodies at least in trace analysis. In 1984, Groopman et al. developed a reusable column recognizing mycotoxins from human serum, milk and urine (8). Serum and milk were applied directly to the column but the urine samples needed pre-purifying in order to achieve a quantitative recovery. In the first decade of the 21st century, IAC has become widely and increasingly used as a specific extraction for quantitative analysis of endogenous and exogenous biomarkers, drugs, toxins, pesticides and environmental

contaminants. The combined use of IAC extraction and MS/MS separation has been demonstrated in several studies as being superior in accurate quantitative measurement of different substances in various biological matrices (9). In addition to food and feed, there are applications from urine, plasma, water, smoke and tobacco. Analysis of different toxins represent a large part of IAC applications and has had a major impact in the development of commercial single- and multi-compound IACs (7). Lattanzio et al. published a method for the determination of 11 mycotoxins in maize using a commercial multi-immunoaffinity column which contained antibodies for all of the toxins (10). For most of the toxins, recoveries were between 79 and 104 % but the recovery for HT-2 toxin was 180 % and T-2 toxin was not detected. It was suggested that carboxylesterase-enzyme would hydrolyse T-2 to HT-2 in aqueous conditions.

In conclusion, immunoaffinity columns offer an effective and rapid cleanup of several analytes from different sample extracts (7). There are many commercial IACs on the market but there is still a need for reusable columns containing several antibodies against different analytes. There are also challenges in the preparation of more specific antibodies against analytes and device techniques involving non-animal antibody production (11).

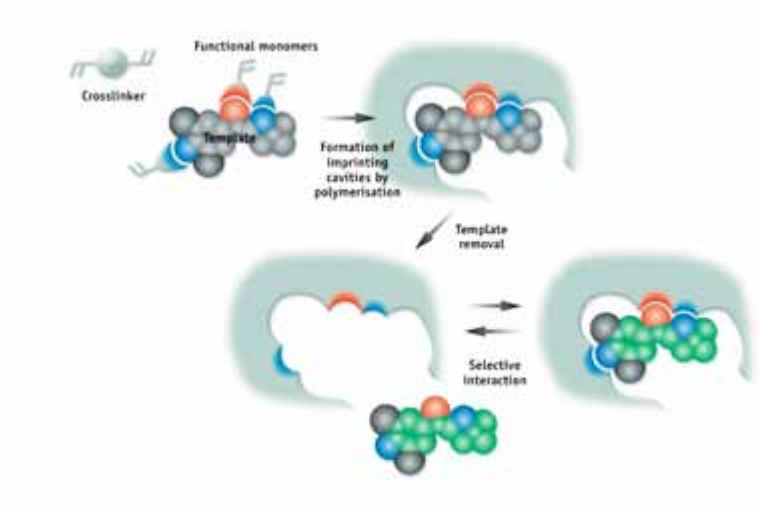
Molecularly imprinted polymers as SPE sorbents

Molecularly imprinted polymers (MIPs) are synthetic, "custom-made" cross-linked three-dimensional network-polymers with specific recognition sites (cavities) complementary in shape, size and functional groups to the target (template) molecule. Due to these specific cavities, MIPs enable, through re-binding, a highly selective extraction and/or pre-concentration of analytes even from very complex sample matrices, and as a result they can be considered as a kind of synthetic immunoaffinity-phase. Applications of MIPs include several analytical techniques (e.g. liquid chromatography, capillary electrochromatography, binding assays and biosensors). However, especially the development of molecularly imprinted solid-phase extraction (MISPE) has recently greatly increased, at the moment representing the most advanced application area of MIPs (12). In addition to a single target molecule, a selective MIP may also be developed and synthesized for a group of structurally related compounds (12), which will then enable multicompound analyses. Until recently, most of the MIPs have been developed and produced in research laboratories, but nowadays there are also some commercial suppliers of MISPE-sorbents for selected assays as well as tailor-made purposes.

A MIP is usually synthesised for a specific analytical use (12). The synthesis and characterisation of imprinted polymers has been reviewed in detail by Cormack and Elorza (13). In brief, the MIP-synthesis involves the complexation of print molecule (i.e. template, most usually the target-molecule itself) with functional monomers in a solution (porogen). The most commonly used functional monomers are methacrylic acid, 4-vinylpyridine, trifluoromethacrylic acid and N,N-dimethylaminoethyl methacrylate (14). After the complexation, the monomers are polymerised with the help of cross-linkers in the presence of an initiator. The cross-linker provides MIPs with high mechanical stability and chemical inertness, and a high degree of cross-linking is needed to make the polymer rigid and to retain the imprints (14). Commonly used cross-linkers

Figure 1.

The basic principle of synthesis and extraction mechanism of molecularly imprinted polymers (reprinted with the permission of MIP Technologies, www.miptechnologies.se).



include ethylene glycol dimethacrylate and trimethylolpropane trimethylacrylate (14). Finally, the template molecules are removed from the polymer-network by comprehensive washing to disrupt the interactions between the template and monomers, leaving the imprint available for specific binding of the analytes compatible with the template (12). A schematic of synthesis and retention mechanism of MIPs is presented in Figure 1. There are three different processes that can be used to synthesise MIPs depending on the interactions between the template and the monomer: i) non-covalent, ii) covalent or iii) semi-covalent imprinting (15). Currently, the MIPs used for SPE are in most cases prepared by non-covalent imprinting. This, however, gives quite low yields of specific binding sites and furthermore may lead to low capacity and non-specific binding (16).These non-specific interaction sites results from a proportionally high amount of functional monomers being used during the MIP synthesis and they may lead to co-extraction of matrix components as well as loss of MIP performance (17). Additionally, as the interactions involved in the non-covalent polymers are weak, the template should have several functional sites in order to strengthen the template-monomer assemblies (12) and achieve the best possible extraction efficiency.

The retention mechanism of target molecules on MISPE-cartridges may vary depending on the nature of the MIP. In MIPs produced by non-covalent imprinting process, the bonds involved are usually of a weak chemical nature: hydrogen- and π-π bonds as well as electrostatic and hydrophobic (van der Waals) interactions (14, 15) and highly functionalised templates with amino-, carboxyl- or keto-groups are preferred to achieve multiple additive weak interactions (14). Instead, covalent polymerisation generates a better defined and more homogenous recognition sites as the interactions involved are stronger and more stable (15). However, as the use of covalent bonding is limited, being specific only for particular functional groups, the non-covalent printing with weaker bonding is more applicable (14). Instead the semi-covalent imprinting refers to polymers in which the covalent bonding is used during the polymerisation, but in which non-covalent bonding accounts for the template-polymer interactions (15).

The use of a MIP as an SPE-sorbent is generally similar to conventional phases, i.e the method protocol includes the four common steps: conditioning of the column, loading of the sample, washings and elution. However, there are some characteristics of MIPs that need to be taken into consideration in the method development, as the clean-up is more critical in MISPE (15) as compared to conventional approaches. For instance, the choice of the solvent used in sample loading step is of crucial importance as it defines the nature of the interactions involved in the retention process of analytes. The highest selectivity is obtained when samples are dissolved and applied in the same solvent (porogen) that was used for the MIP's preparation as it is able to recreate exactly the same template-monomer interactions that took place during the polymerisation. As the porogen used in the polymer synthesis is usually non- to moderately polar and aprotic (18), the application of aqueous samples may pose problems in specific retention of the analytes in the column, as the conditions do not favour the formation of the same interactions. To overcome this drawback, a previous transfer (e.g. liquid-liquid extraction or SPE) of the analyte(s) to an appropriate solvent may be used (18). However, methods for applying aqueous samples to MIPs are constantly being developed (18) facilitating the procedure for this kind of samples. This is achieved by utilising a selective desorption (recognition of hydrophobic parts of the molecules) instead of selective adsorption with interactions of polar functionalities, which are preferred in non-aqueous media. In selective desorption, the analyte as well as hydrophobic interfering compounds are non-specifically adsorbed and a selective washing (i.e. desorption) of the MIP is needed before the elution to disrupt non-specific interaction between the analyte and the polymeric matrix. In this way, the specifically retained compounds are not washed off (14).

Additionally, the extraction procedure needs to be fully optimised in order to prevent non-specific, low-energy binding of analytes to the polymer external surface without impairing the specific interactions in cavities (12). This is usually achieved by carefully selecting the pH, as well as the nature and volume of the application solvent being used (15). A washing step of the clean-up procedure to remove the disturbing compounds is usually performed using the same solvent as was used in sample loading. In the elution step, a small amount of a modifier, such as trifluoroacetic acid, acetic acid or triethylamine, may be used to slightly change the elution strength and consequently limit the non-specific interaction at the polymer surface (12).

The use of MIPs as SPE-sorbents serves several advantages. As explained above, the phase itself is very selective and specific due to tailor-made recognition sites for analytes. This selectivity will result for lower matrix effects and consequently to lower LOD/LOQ-values (limit of detection/quantification), as well as better accuracy and improved precision of the method, as was shown when comparing MIPs with other polymeric phases in the analysis of amphetamine drugs from wastewater samples (19). MIPs are also extremely stable, robust and inexpensive e.g. as compared to immunoaffinity-sorbents and their handling and synthesis are also relatively easy. For instance, the sorbent may be dried during the extraction process, which cannot be done in the case of conventional SPE-sorbents, which lose their retentive properties when dried. The complete drying of the MISPE-cartridges enables a more efficient elution and more concentrated eluate without any aqueous phase present. The MISPE-cartridges may also be used several times for sample extraction or purification (15), although

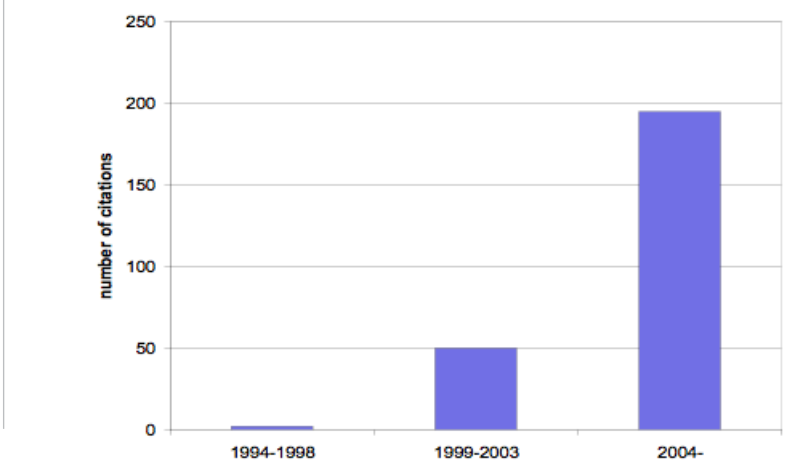
especially when applied in trace analyses, the reusability must be tested to avoid any erroneously positive results. Though most of the MISPE-methods published are off-line applications, also on-line methods are possible, as described for instance by Bjarnason et al. (20).

Despite the advantages described above, the disadvantages related to MIPs cannot be ignored. For instance, the removal of the template is often incomplete, despite extensive washing steps applied in polymer synthesis. This may lead to the leakage of template during the extraction process, which is a clear drawback especially in the case of trace analyses (15) where the possibility for erroneous positive result increases. However, there are certain approaches (e.g. the use of so-called dummy-analogue with structural similarity to the analyte of interest) that can be used to overcome this drawback as the template is not the same as the analyte. The sample matrix components may also present a problem when using MIPs, despite the high specificity of the polymer, as the accessibility of the analytes to the cavities may be hindered. For this reason, an additional sample preparation step (e.g. another SPE-phase) has been used to clean up the sample prior to its application onto MISPE. This same approach is also applicable when aqueous samples need to be analysed, as the transfer to the appropriate application solvent is also easily achieved (15). The problems relating to the water usability together with MIPs are also a major drawback, as discussed above, although, more and more applications using aqueous phases directly are being described. The disadvantages of MIPs also include the fact that although MIPs may suffer from restricted flow rates and plugging, vacuum cannot be applied to enable efficient interactions, which in turn increases the sample preparation time. The sample capacity of MISPE-cartridge is also usually lower when compared to other polymeric phases (15, 19). Furthermore, at least at the moment, MIPs are not suitable sorbents for very polar or high-molecular weight molecules, and therefore developments in polymer-synthesis and extraction of such molecules are needed (18).

The first MISPE-application was reported for pentanamide from urine samples (21). Since then, the applications of real sample reported in the literature have constantly increased as demonstrated in Figure 2. Reviews of the application areas reported are available for instance by Pichon & Chapuis-Hugon, Caro et al. and He et al. (12, 15, 22).

Figure 2.

The amount of citations at www.pubmed.com for "molecularly imprinted polymer" and "solid phase extraction" starting from the year 1994.



The most common applications include environmental, biological or pharmaceutical samples, whereas there are still less food-related applications. There is a wide range of sample matrices used for the extraction (e.g. sediments, river/tap water, urine, plasma, serum, tissues, milk, wine) reflecting the fact that MISPE is well suited for extraction/concentration of analytes of interest in sample matrices that have historically been considered to be extremely troublesome. A full list of analytes for which MISPE-approach has been used can be found in the reviews listed above.

Recently, combinatorial sample preparation techniques utilising MIPs together with solid-phase micro extraction (17), a technique which will be presented in more detail in another chapter of this book, have been reported. This approach will provide an interesting possibility for miniaturization of certain applications and analytical systems and in the future this kind of combinatorial sample preparation techniques using the MIP-approach can certainly be expected to increase.

It is to be anticipated that the applications of MISPE will be further increased in the near future. This is because there is a trend to try to achieve constantly lower LODs/LOQs-values as well as other critical method parameters. Additionally, as commercial, in some cases even tailor-made, MIPs with full protocol description will be available for application laboratories, the use of the cartridges will be made available even to the users with no knowledge or history with the imprinting process.

Dispersive SPE and QuEChERS

In one specific form of solid phase extraction (SPE), the sorbent will bind the sample impurities instead of the compounds of interest. The method is straightforward since the purified sample can be collected at the first step and no washing step or any particular elution step are needed. That kind of purification can be used for example to remove hydrocarbons and fats from oil samples by a normal phase sorbent. Modern techniques discussed in this section, dispersive solid phase extraction (dSPE) and QuEChERS (quick, easy, cheap, effective, rugged, and safe), are also based on the adsorption of impurities but the extraction is made inside a tube instead of a cartridge as is the case in conventional SPE.

In 2003, Anastassiades et al. introduced the QuEChERS method for pesticide residue analysis (23). Thereafter, Lehotay et al. demonstrated its effectiveness for extracting > 200 pesticides in fruits and vegetables, which were analyzed using gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) (24). The method was based on acetonitrile extraction followed by a dSPE cleanup step. In dSPE, the sample is extracted in a vial containing a small amount of SPE sorbent which is chosen so that it removes the matrix interferences but not analytes. Compared to conventional cartridge based SPE, dSPE is less solvent- and time-consuming and those advantages compensate the slightly better cleanup provided by cartridge SPE. After introduction, QuEChERS has become a routine technique in the pesticide laboratories worldwide and many companies have their own commercial QuEChERS-kits.

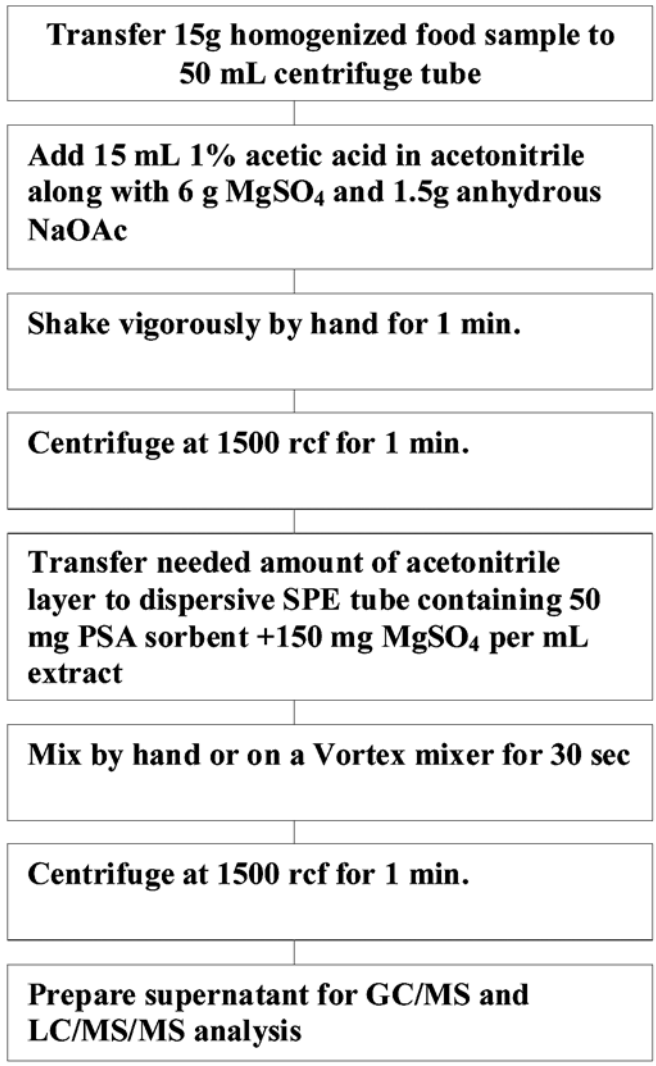
In 2007, a QuEChERS collaborative study was published, involving 13 laboratories from 7 countries (25). The QuEChERS procedure was tested for 26 representative pesticides at three duplicate levels in grapes, lettuces, and oranges. The study was successful,

and as a result, the method was adopted as an official first action method (AOAC Official Method). Figure 3 shows a flow diagram of the method, which at the same time can serve as a representative example of a typical QuEChERS extraction. A high-moisture sample is first extracted with a water-miscible solvent in the presence of high amounts of salts and buffering agents. After shaking or vortexing and centrifugation, an aliquot of the organic phase is transferred to the dSPE tube for further clean up. The dSPE tube is shaken for half a minute and centrifuged. Ideally, the supernatant can be analyzed directly after the extraction without concentration or solvent exchange.

Acetonitrile is the recommended solvent for the QuEChERS extraction. Acetonitrile is better separated from the water than acetone with the addition of salt and it co-extracts less lipids than ethyl acetate. Acetonitrile achieves also higher recoveries for

Figure 3.

Flow diagram of QuEChERS extraction for pesticide residues in grapes, lettuces and oranges (25).



acid-base pesticides than ethyl acetate. Mostly QuEChERS is applied to high-moisture samples and in case of dry foods water is added to the sample prior to extraction.

The purpose of the salts and buffering agents in the liquid-liquid extraction step is to induce phase separation and to stabilize labile compounds, respectively. Typical salts and modifiers are $MgSO_4$, Na_2SO_4, NaCl, citrate, sodium acetate (NaAc), acetic acid (HAc) etc. $MgSO_4$ is used as a drying salt, promoting the partition of compounds from water phase into the organic phase. NaCl and other salts assist in the salting-out by controlling the polarity of the extraction solvent. The type and amount of different salts have a great influence on the selectivity of partitioning. Anastassiades et al. investigated the effect of various salts and fructose in induced phase separation (23). In their study, optimal combination for the extraction of pesticides in fruits and vegetables was found to be 5 g $MgSO_4$ and 1 g NaCl which is a compromise to avoid co-extraction of polar matrix components but still able to achieve high recoveries of polar pesticides. The addition of citrus or acetate buffering salts to elevate the pH of sour fruits reduced dramatically the amount of co-extractives in the raw extracts.

Generally, the pH of the extraction must be controlled. Most of the pesticides are base-sensitive and stable and better extracted at pH <4. However, some pesticides are acid-sensitive and hydrolyze or protonate at lower pH, resulting in poor partitioning into the organic phase. Therefore, in the multiresidue methods, maintaining the pH at 4–5 is recommended as a compromise which gives adequate recoveries for the acid-sensitive pesticides and adequate stability for the base-sensitive pesticides. Lehotay et al. (25) preferred the use of combination of HAc/NaAc which gave high and consistent recoveries for a wide range of pesticides from matrices with pH 2–7. Liquid-liquid partitioning was carried out in a tube containing 1 ml of 1 % HAc in MeCN plus 0.4 g anhydrous $MgSO_4$ and 0.1 g anhydrous NaAc per g of sample. When investigating certain analyte types, it is useful to carry out optimization of the conditions to achieve greater specificity. For instance, formic acid (5 %, v/v) in acetonitrile as the extraction solvent and inclusion of citrate buffer helped in the partitioning of phenoxy acid herbicides into the acetonitrile phase (26).

Different SPE sorbents and their combinations can be utilized in the dSPE approach. Anastassiades et al. (23) compared pure and mixed sorbents of PSA (primary secondary amine), -NH2, alumina neutral, GCB (graphitized carbon black), polymer, -CN, SAX (strong anion exchange), and C18 in the determination of the pesticide residues in mixed fruits and vegetables, in mixed livers and in ground meat. PSA removed most strongly fatty acids and other organic acids but also pigments and sugars. However, the capacity of PSA may be limited if the samples contain more polar components such as sugars (e.g. strawberry extract). Therefore, adding salt (eg. NaCl) to the liquid-liquid partitioning step before dSPE is necessary to decrease or avoid the saturation of PSA. The combination of PSA and GCB also proved to be excellent for the removal of a variety of matrix materials, but there was loss of certain structurally planar pesticides as GCB retained them strongly. All amino-type sorbents will remove the same type of co-extractives as PSA and elevate the pH of the extract. Cleanup efficiency has been found to increase in the order mono- < di- < tri-amino sorbents. Acidic, neutral, and basic alumina sorbents have been found to remove similar co-extractives as amino sorbents but to a slightly lesser extent. The combination of C18

and alumina-N as the sorbent in dSPE improved the overall recoveries of the phenoxy acid residues in rice compared with C18 and C18-GCB (26). It was concluded that some fat, vitamins and minerals in the rice extract were cleaned and at the same time analytes from the active sites of the alumina-N sorbents were released.

Lehotay et al. (27) evaluated the QuEChERS method for intermediate fatty food samples (2–20 %); for milk, egg and avocado. In that study, PSA, GCB and C18 sorbents were investigated. Combination of PSA+C18 was shown to be very effective for the cleanup; and as was shown in the previous study with non-fatty foods, CGB can be employed for the cleanup if analytes of interest are not planar-ring pesticides. CGB has been successfully used in cleanup procedures to remove chlorophyll and carotenoid pigments, e.g. in spinach (28) and rice paddies (29). The good efficiency of C18 to remove fatty acids is clearly related to the fact that triglycerides are structurally very close to C18, both containing long carbon chains.

As mentioned above, dSPE and QuEChERS are widely used in the analysis of pesticides from non-fatty or intermediate fatty foodstuffs; such as from fruits and vegetables but also from milk (27), egg (27), cereals (30), wine (31), rice (29), honey (32) and meat (33). In addition, modified applications have been published for the pesticide analysis for example in oil and olives, sludge (34), soil (35) and tobacco (36).

In addition to pesticides, dSPE has been applied for the determination of veterinary drug residues in animal tissues (37), acrylamide in food (38), antibiotics in bovine kidney (39), milk (40) and wastewater (41), pharmaceuticals in whole blood (42), polycyclic aromatic hydrocarbons in fish (43) and estrogens in fish and shrimp (44).

A number of recent applications have provided strong evidence that in the future dSPE will preserve its position as a functional sample pre-treatment method and it can be used routinely also for the extraction of other commodities than fruits and vegetables and also for other analytes besides pesticides. There is no question of that dSPE cannot become a routine technique in an accredited laboratory and successfully transferred to other laboratories. ●

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10 Applications

Some Timelines in Separation Science
Sandy Fuchs, Engineer, Fuchs Konsultointi Oy
Päivi Laakso, Ph.D., Docent, Analytical Services Manager, Eurofins Scientific Finland Oy

Sample preparation in protein and peptide analysis, practical applications
Nisse Kalkkinen, Ph.D., Laboratory Director, University of Helsink

Sample preparation and analysis of dioxins and other persistent organic pollutants
Terttu Vartiainen, Research professor, National Institute for Health and Welfare
Panu Rantakokko, Ph.D., Principal research scientist, National Institute for Health and Welfare
Hannu Kiviranta, Ph.D., Unit head, Senior researcher, National Institute for Health and Welfare

Treatment of biofluid samples for liquid chromatographic analysis
Tapani Suortti, D.Tech., Senior research scientist, VTT Technical Research Centre of Finland

Generic sample preparation methods for drug screening
Ilkka Ojanperä, Ph.D., Docent, Laboratory director, University of Helsinki
Ilpo Rasanen, Ph.D., Forensic toxicologist, University of Helsinki

Organometals and sample preparation in environmental samples
Panu Rantakokko, Ph.D., Principal research scientist, National Institute for Health and Welfare
Riikka Airaksinen, M.Sc., Researcher, National Institute for Health and Welfare
Jari Kaikkonen, Ph.D., Docent, Chemist, National Institute for Health and Welfare
Hannu Kiviranta, Ph.D., Unit head, Senior researcher, National Institute for Health and Welfare

Some Timelines in Separation Science
Gas Chromatography

Sandy Fuchs | Päivi Laakso

1905

The separation of mixtures of gases and vapors

Sir William Ramsey

He discovered helium, which since 1868 had been known to exist, but only in the sun. This discovery led him to suggest the existence of a new group of elements in the periodic table. He and his coworkers quickly isolated neon, krypton, and xenon from the earth's atmosphere using selective adsorption or desorption from active charcoal.

Proc. Roy. Soc. A76 111 (1905)

1945–1947

The first modern gas solid adsorption chromatography study

Erika Cremer and her students *F. Prior* and *R. Müller*

A variation of the liquid adsorption chromatograph of Tswet. Using a tube packed with silica gel and carbon as column and hydrogen as carrier gas, separations of gas mixtures was performed.

1951

The first description of gas liquid partition chromatography

A.J.P. Martin and *A.T. James*

A publication about gas liquid partition chromatography for the separation of volatile fatty acids.

Biochem J. Proc. 48, vii (1951), Analyst 77,815 (1952)

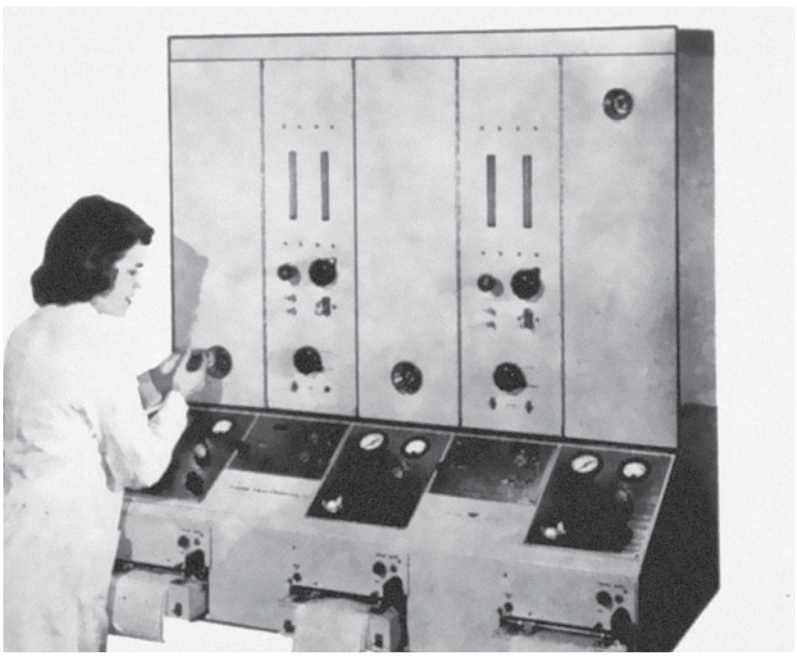
1955

The introduction of the first commercial gas chromatograph

M.J.E. Golay (Perkin-Elmer)

The Model 154 Vapor Fractometer isothermal GC was introduced May 1955.

The first GC developed by the Perkin-Elmer Corporation, was the Model 154 Vapor Fractometer introduced in May 1955.



The model 188 Triplestage Gas Chromatograph, introduced in 1957 by Perkin-Elmer Corp.

Source: 50 Years of GC at Perkin-Elmer - Pittcon 2005

1957

Open tubular (capillary) columns were introduced

Marcel Golay was an employee at Perkin-Elmer when he introduced capillary columns into gas chromatography and started the miniaturization of chromatography describing (theoretically) the basis for capillary columns. The miniaturization of gas chromatography started.

The relatively non-selective, high-sensitivity FID was introduced

J. Harley, W. Nel and *V. Pretorius*

Flame Ionization Detector for Gas Chromatography.

Nature 181,177–178 (18 January 1958); Nature 178, 4544, pp.1244 (1956)

1959

Introduction of the first glass capillary drawing machine

Denis Henry Desty applied for the patent 899909 (applied 9th April 1959 and issued 27th June 1962).

The first direct coupling of GC/MS

Time-of-Flight Mass Spectrometry and Gas-Liquid Partition Chromatography.

R.S. Gohlke, Anal. Chem. 31:535 (1959)

1969

The split-splitless injector by Kurt Grob was introduced

Kurt Grob and CE SID finally succeeded to introduce the first genuine Grob split-splitless injector showing results unachievable before, particularly in trace analysis which in those days was Grob's application area number one.

1979

The introduction of fused silica columns

In 1979, the landmark paper by *Raymond Dandeneau* of Hewlett-Packard announced the development of fused silica columns to the world.

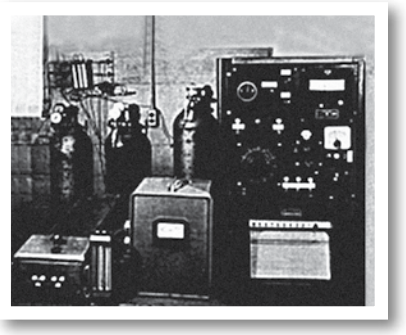
J. High Resol. Chromatogr. 2(6):351–356 (1979)

The Aerograph model 600 Hy-Fi GC, introduced at the 1961 Pittcon by Wilkens Instrument & Research Co. The upper part of the FID can be seen on the top.

Source:
Editor Leslie S. Ettre Milestones in Chromatography LCGC No,Am v20/n/1 Jan 2002

Development of Japan's first Gas Chromatograph 1956 by Shimadzu.

Source:
www.shimadzu.com/visionary/history/1917.html



The F&M Scientific Corp. Design and considerations of a gas chromatograph operable at temperature up to 1000° C is described (1960–1961) .

Source:
http://contrails.iit.edu/DigitalCollection/1961/WADDTR61-176.pdf

Some Timelines in Separation Science
Liquid Chromatography

Sandy Fuchs | Päivi Laakso

1901

Mikhail Tswett invented Chromatography

Mikhail Tsvett used liquid-adsorption column chromatography with precipitated chalk as adsorbent and petrol ether / ethanol mixtures as eluent to separate yellow and green chloroplast pigments. This method was described on 30th December 1901 at the XI Congress of Naturalists and Physicians in St. Petersburg. The term "chromatography" appears for the first time in print in his two papers about chlorophyll in the German botanical journal, Berichte der Deutschen Botanischen Gesellschaft.

Vol. 24, p384 (1906)

1941

The first description of partition chromatography

A.J.P. Martin and *R.L.M. Synge,* UK

By using paper as the solid carrier support and two liquid phases their system led to paper partition chromatography.

Biochem J. 35:1358–1368 (1941)

1958

The first LC was an amino acid analyzer

S. Moore, D.H. Spackman and *W.H. Stein*

First liquid chromatograph using an ion exchange column for the separation of AA's "Chromatography of Amino Acids on Sulfonated Polystyrene Resins", "Automatic Recording Apparatus for Use in the Chromatography of Amino Acids".

Anal. Chem. 30:1185–1190 (1958), Anal. Chem. 30:1190–1205 (1958)

Polymer fractionation by gel permeation chromatography GPC

J.H.S. Green and *M. F. Vaughan*

Separation of hydrophobic polymers on cross-linked polystyrene SCI p829 (1958)

Techniq. Polym. Sci. SCI, Monograph No. 17, Soc. Chem. & Ind., UK, 81 (1963)

First commercial amino acid analyzer

Beckman introduces their AA analyzer.

1959

The launch of a cross-linked dextran gel used for gel filtration GFC

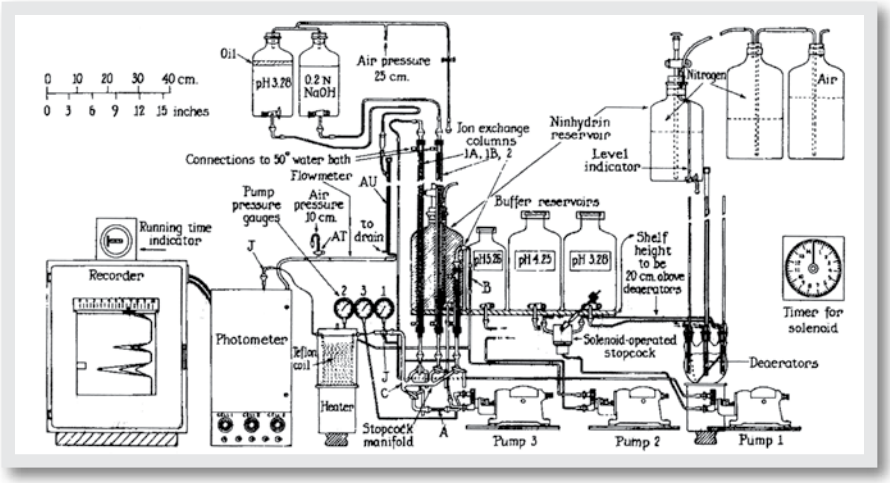
Jerker Porath and *Per Flodin*

Separation **Pha**rmacia **dex**tran was introduced. Gel filtration: a method for desalting and group separation.

Nature, 183:1657–1659 (1959)

D.H. Spackman, W.H. Stein and S. Moore Automatic recording apparatus for use in the chromatography of amino acids.

Anal. Chem. 30(7):1190–1206 (1958) Additional info: http://www.dipity.com/cenacs/Milestones-in-Chromatography



1963

First commercial liquid chromatography system was introduced

Waters comes on the market with their GPC 100 and obtains exclusive license to Dow's patent.

1965

The first HPLC instrument

C. Horvath, B. Preiss and *S.R. Lipsky*

The first modern HPLC instrument is generally credited to C. Horvarth working at Yale Medical School.

Anal. Chem. 39(12):1422–1428 (1967)

1969

The miniaturization of liquid chromatography started

Classification of finely particulate, porous sorbents down to 5 micrometers, the starting signal for HPLC (Merckosorb Si60/Si100).

1973

HPLC/MS

Themospray (C. Blakely 1973), moving belt interface (W.H. McFadden 1976) direct liquid introduction (M. Baldwin and E. McLafferty 1973).

Development of bonded silica phases for GC and LC

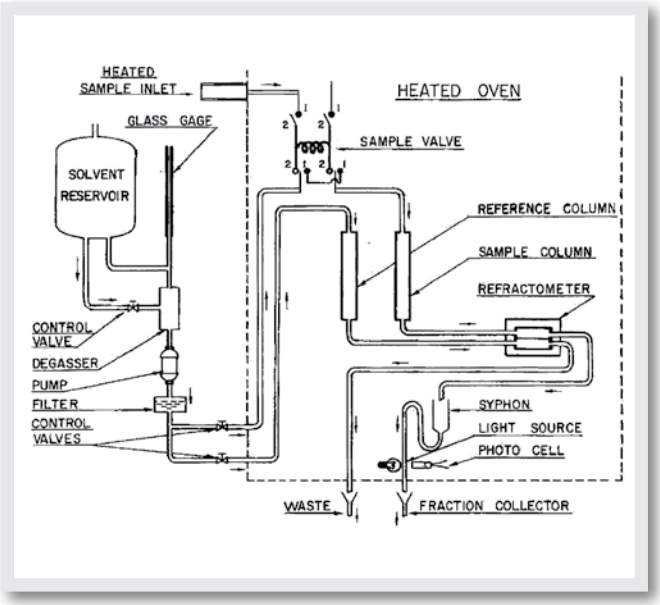
by *István Halász* e.g. Saarbrücken

1972–76 (packings: Hypersil, Lichrospher, µBondapak, Spherisorb, Zorbax, Vydac)

1994

First sub 2 micrometer HPLC packing material was introduced

Micra 1.5 micrometer nonporous HPLC columns became available 1994.



Waters liquid chromatograpfy assembly.

Flow schematic on page 4 of 1963 Instruction Manual for first GPC-100.

Second generation GPC-200 (1964).

Source: James Waters and His Liquid Chromatography People: A Personal Perspective by Patrick D. McDonald, Ph.D.

Some Timelines in Separation Science
Other Separation Techniques

Sandy Fuchs | Päivi Laakso

1809

Electrophoresis

F.F. Reuss, Mem. Soc. Imperiale Naturalistes de Moscow, 2: p327 (1809).

1958

Supercritical mobile phase

James Lovelock

Introduction of the electron capture detector for GC but also suggestion to use supercritical mobile phase in chromatography.

1964

Development of Headspace technique

G. Machata at University of Wienna

The first commercial instrument F-40 produced by Perkin Elmer 1967.

1965

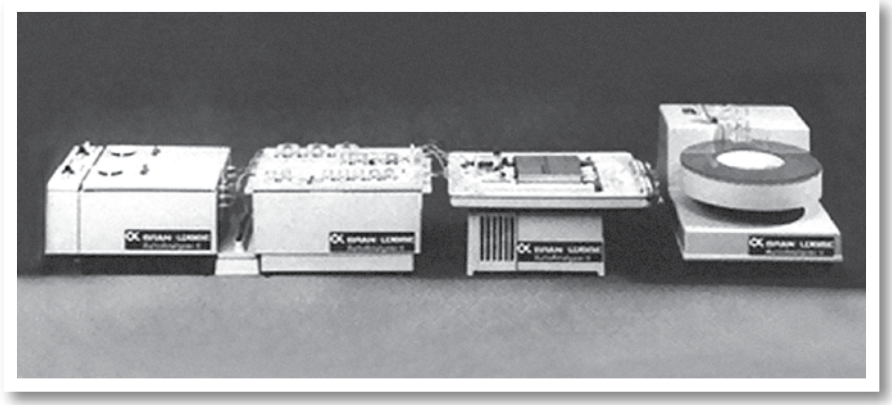
Development of purge-and-trap technique

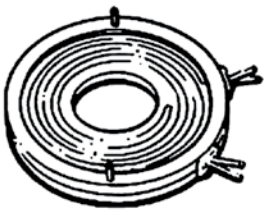
In the 1960's, P&T was used in the study of bodily fluids.
In the mid-to-late 1970's, P&T became a technique that was well-known and widely applied due the need to monitor VOCs in drinking water.

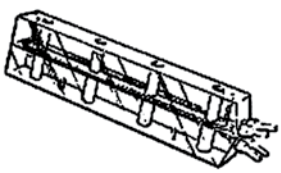
1966

The first TLC precoated plates using standardized sorbents

Introduced by *Merck Darmstadt.*







Dialysis

Thomas Graham conducted investigations on the diffusion of gases, osmotic force, and the fractionation of chemical fluids by dialysis.

Graham T: Liquid diffusion applied to analysis
Phil.Trans.R.Soc.London 1846,573–631 /1849,349–391 / 1854,177–228 / 1861,183–224 .4

Leonard Tucker Skeggs developed the continuous-flow analysis by introducing the AutoAnalyzer (1951). He utilized dialysis in the original Technicon AutoAnalyzer (1955). The artificial kidney of Skeggs was a compact system using dialysis sheets instead of huge coils of cellophane tubing.

Leonard Tucker Skeggs – A Multifaceted Diamond Chlin.Chem. 27/8, 1465–1468 (1981)

Source: www.seal-analytical.com, www.segmentedflow.com

1975

Use of microwave ovens for sample preparation

A. Abu-Samra, J.S. Morris and *S.R. Koirtyohann*

Wet Ashing of Some Biological Samples in a Microwave Oven.

Anal. Chem. 47:1475–1477 (1975)

1976

Solid Phase Extraction

M.S. Mills

U.S. patents available from 1976.

Field Flow Fractionation

J.C. Giddings, F.J. Yang and *M.N. Myers*

A versatile new separation method. Science 193 (no 4259):1244–1245 (1976)

1978

Development of cloud-point extraction

H. Watanabe and *H. Tanaka*

Talanta 25:p585 (1978)

1985

Development of supercritical fluid extraction

G.A. Mansoori and *J.E. Ely*

Density Expansion (DEX) Mixing Rules (Thermodynamic Modeling of Supercritical Extraction) J.

Chem. Phys. 82:p406 (1985)

Development of molecular imprinted solid-phase extraction

M. Glad, O. Norrloew, B. Sellergren and *K. Mosbach*

Use of silane monomers for molecular imprinting and enzyme entrapment in polysiloxane-coated porous silica.

J. Chromatog. 347:11–23 (1985)

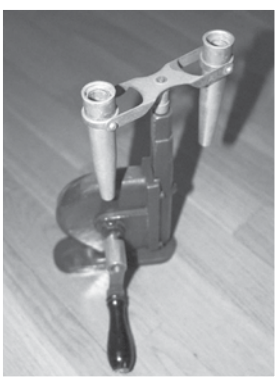
1989

Development of solid-phase micro-extraction

R.P. Belardi and *J. B. Pawliszyn*

The application of chemically modified fused silica fibers in the extraction of organics from water matrix samples and their rapid transfer to capillary columns.

Water Pollution Research Journal of Canada 24(1):179–91 (1989)



Source: Picture by *Stephan M. Höhne*: Innovations in centrifugation 60 years Beckman.

Centrifugation

Antonin Prandtl developed the dairy centrifuge in order to separate cream from milk in 1864.

The first centrifuges were hand driven. By 1920 new centrifuges were developed which used electrical power.

Theodor Svedberg received the Nobel Prize in 1926 for his work in colloid chemistry and the invention of the ultracentrifuge.

Mass Spectrometry Timelines

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A Mass Spec Timeline

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Sample preparation in protein and peptide analysis, practical applications

Nisse Kalkkinen

General

Almost without exception, food is a complex non-homogeneous mixture of a staggering range of chemical substances that makes it hard to isolate and determine analytes of interest. Even with the emergence of advanced techniques of separation and identification, it is rarely possible to analyze the substances of interest as such without manipulation of the original sample. The more sensitive and accurate the analysis techniques have become, the more attention has to be paid to the initial sample preparation and clean up before the final analysis. After original sampling, it is necessary, depending of the starting material, to prepare the sample e.g. by different extraction, precipitation, concentration, chromatographic or other methods before the final analysis. In general, before attempting to design a protein or peptide preparation or purification protocol, one should collect as much information as possible about the analyte itself as well as about the most important interfering impurities to be removed before the analysis. It is useful to gather information about molecular weight, pI, hydrophobicity, solubility, cellular localization, presence of glycans (glycoproteins) or other modifications. However, in practice, the most usual way is to examine the sample in preliminary trials using a

sequence of the most usual methods and tailor the methods and their order according to the obtained results. When optimizing the sample pretreatment and purification methods, it is important to have some way to follow the distribution and recovery of the analyte in the fractionation steps. Such properties include enzymatic activity, immunological properties (immunoblot) and mobility (apparent molecular weight) in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Some structural properties can also been monitored by mass spectrometry or amino acid sequence analysis by Edman degradation. In addition, a huge number of different protein structures are today available in different sequence databases, from which some of the properties of the proteins of interest can be predicted.

When fractionating a protein or peptide prior to the different analytical steps, one should also establish criteria with regard to its stability. Some important parameters affecting the stability and structure are pH, temperature (fractionating temperature, freezing/thawing), ion strength, buffer composition, organic solvents, oxygen and heavy metals. Special concern should be addressed to the presence of contaminating proteolytic enzymes which, if not inhibited, may cause proteolytic degradation of the proteins or peptides.

The number of fractionation steps in a successful preparation of a protein or peptide before its final analysis depends on the quality of the starting material and the amount and properties of the analyte itself. Different types of analyses have also requirements concerning the sample, its concentration and solvent. Very seldom can a protein or peptide be obtained in a pure form for analysis with only one fractionation step, even if the step is based on the biospecificity of the analyte. Usually a number of different steps, such as extraction, precipitation, concentration, different kinds of chromatographic and electrophoretic steps are needed. The yield of a protein of interest after multiple fractionation steps varies depending on the fractionation methods and the number of fractionation steps. Thus the absolute yield of the analyte always decreases with the increasing number of fractionation steps. For preparative purposes, it is important to optimize the yield in order to end with the maximal amount of the desired protein. For many analytical purposes, especially when using new emerging sensitive technologies, the quality of the analyte is more important than its total yield, which makes it possible to use a large number of fractionation steps in obtaining the final sample for analysis.

In most cases, at least in our analyses, interest is focused on one particular protein or its fragment. Recently, with the increasing importance of proteomics to the scientific community, the interest is has also focused on the whole protein content of an organism, the proteome, either in a qualitative or quantitative manner. It is important to remember that the problems in protein fractionation and purification are not solved by the acquisition of sophisticated laboratory instrumentation and column packings with high selectivities and efficiencies. It is also a demanding task to identify optimal conditions for protein extraction and concentration before the subsequent chromatographic or electrphoretic separations. When planning a sample pretreatment and fractionation method for the proteins of interest, the starting material is of importance. Some biological materials constitute themselves a clear or nearly clear protein solution suitable as such for further fractionation. There are many examples of this kind of material e.g. blood, milk, plant juices, and fermentation media after cultivation of micro-organisms.

In most cases, however, one has to extract the desired activity or structure from a tissue or a cell paste which involves homogenization of the starting material, extraction of the protein of interest and clarification of the recovered extract. During these steps, also considerable amounts of other contaminating molecular species, such as e.g. lipids, nucleic acids and phenolic substances are set free and the proteins of interest are also subjected to fragmentation by the simultaneously released proteases. Optimization of extraction conditions should thus favor the release of the desired protein and leave behind those contaminants that are difficult to remove during subsequent fractionations.

The extraction medium is usually a buffer solution with some additives. Normally the pH of the solution is that of the maximal stability of the protein and where it is most efficiently released from the cells or tissues. The buffer salts are selected according to the desired pH of the solution. An acceptable buffer capacity is obtained within one pH unit from the pK values of the buffer salts and most proteins are maximally soluble at 10–100mM ionic strength. The buffer capacity is important because proteins are buffering substances themselves and they may change the pH of the solution. For extraction of hydrophobic membrane proteins, either detergents or chaotropic agents should be used. These reduce the hydrophobic interactions between the proteins and membranes. The most usual detergents are either non-ionic (e.g. Triton X-100), anionic (e.g. sodium dodecyl sulfate) or zwitterionic (e.g. CHAPS). Sometimes also reducing agents (DTT, DTE) and chelating agents (EDTA) of heavy metal ions are added to the extraction solution. Reducing agents prevent oxidation of free thiol groups and EDTA binds heavy metal ions which can enhance oxidization of thiol groups and form complexes with the enzyme active site and thus interfere with the enzymatic activity of the protein. The most serious threats to protein stability are the endogenous proteases which may be extracted from the tissue or cell homogenate together with the protein of interest. The impact of these proteases is usually minimized by working quickly and in the cold and/or by adding specific protease inhibitors (e.g. diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, ethylenediamine tetra acetate or Pepstatin A) to the extraction solution. In addition, 1mM sodium azide may be added to prevent bacterial growth.

Clarification of any cell or tissue homogenate is usually performed by centrifugation in a laboratory scale refrigerated high-speed centrifuge operating in the range 40,000 g to about 500,000 g. The sample volumes may vary from some microliters up to several liters depending on the application.

Concentration of biological samples is frequently required during sample preparation and prefractionation. Concentration of protein samples can be achieved in several ways e.g. by freeze drying (lyophilization), precipitation or membrane ultrafiltration. Most protein and peptide fractionations are performed in water solutions, from which water can be removed to achieve a concentrated or dried sample. During lyophilization, the sample is kept frozen and water is evaporated from the sample under reduced pressure, resulting either in a dried or concentrated sample. Normally most proteins retain their biological activity in this procedure but the disadvantage is that non-volatile buffer salts are retained and concentrated in the sample and have to be removed or exchanged depending on the following fractionation step. Even after clarification by preparative centrifugation crude cellular or tissue extracts are, seldom suitable for direct application onto chromatographic columns. This is one reason to use precipitation steps to concen-

trate the proteins of interest and simultaneously remove other unwanted proteins and non-proteinous contaminating impurities. Precipitation of proteins in an extract can be achieved by adding salts, organic solvents or organic polymers under varying pH and temperature conditions. In our hands, the most common techniques are precipitation by ammonium sulfate or acetone. Ammonium sulfate, which is an antichaotropic salt, increases the hydrophobic effect of proteins in solution and promote protein aggregation by association of hydrophobic surfaces. The classical way to use ammonium sulfate is to undertake a fractional precipitation. Different proteins precipitate at different ammonium sulfate concentrations (% of saturation). In fractional precipitation, the protein of interest is recovered at a particular saturation of ammonium sulfate and thus fractionated from proteins and other substances precipitating at lower or higher ammonium sulfate concentrations. One advantage of ammonium sulfate is that it is easily soluble and stabilizes most proteins. Another method is to precipitate proteins with acetone. The method is suitable especially for low protein concentrations but requires usually large solvent volumes and low temperatures (usually -20 °C). A further disadvantage of acetone is that it is flammable and may cause protein denaturation.

Ultrafiltration is a widely used technique in sample treatment and preparation in biochemistry. Ultrafiltration membranes of different materials and different cut-off limits are available (e.g. Amicon, Filtron, Durapore), which separate and concentrate protein and peptide molecules of different sizes, ranging from 1,000 to 300,000 Daltons. In ultrafiltration the molecules exceeding the cut-off limit of the membrane are retained above the membrane while smaller molecules pass through it. The method is excellent both for the separation of salts and other small molecules (desalting, buffer exchange) from a protein fraction and for its concentration. By selection of the cut-off value of the membrane, the proteins of desired size and above can be concentrated, while smaller molecules are removed from the concentrate. The process is gentle, fast and the devices relatively inexpensive. Ultrafiltration is performed either by pressure of an inert gas (e.g. nitrogen) or by centrifugal force in units which vary in size for handling volumes from 100 microliters up to one liter. For concentration of larger volumes, continuous flow devices based of selective membrane penetration are also available.

Electrophoresis has become an important method for fractionation and separation of proteins and peptides for further analysis. Development of many analysis methods (especially mass spectrometry and nano LC) has made it possible to analyze minute amounts of proteins and peptides, prefractionated or separated by electrophoresis. The most widely used electrophoresis methods in protein chemistry are one dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE) and two dimensional polyacryamide gel electrophoresis (2-D PAGE). In SDS-PAGE, proteins or peptides are separated according to their molecular size in a polyacrylamide gel from which the proteins are visualized by staining and recovered by different methods for further analysis. In 2-D PAGE, proteins are first separated by isoelectric focusing (IEF) according to their isoelectric point (pI) followed by their separation according to molecular size by SDS-PAGE. Proteins from the bands or spots of interest can then be recovered and analyzed by different methods. Analysis of proteins and peptides separated by electrophoretic methods has become very popular in proteomic and other related analyses due to the development of methods in recovering the proteins or their

fragments from the gel. Proteins in the gel can be electroblotted onto a membrane (e.g. polyvinylidene difluoride, PVDF) from which it can be subjected to immunological studies or for N-terminal sequencing. Proteins can also be digested "in gel" by proteolytic enzymes (most often trypsin) and the tryptic fragments eluted from the gel and subjected to identification by using MALDI-TOF peptide mass fingerprinting, MALDI-TOF/TOF fragment ion analysis or nanoLC-ESI-MS/MS.

Separation of proteins and peptides by chromatography depends on their differential partition between the stationary phase and the mobile phase. Normally the stationary phase is packed in a column but an alternative method, especially in an early stage of the fractionation, is to stir the protein solution with the stationary phase batch-wise and pass the slurry through an appropriate filter and conduct the washing and elution steps on the filter. All chromatographic separation parameters can be used for protein and peptide fractionation with the order of the steps depending on the application. The different protein and peptide fractionation modes are based on protein and peptide shape and size (gel filtration), net charge and distribution of charged groups (ion exchange chromatography), isoelectric point (chromatofocusing), hydrophobicity (hydrophobic interaction chromatography, reversed phase chromatography), metal binding (immobilized metal ion affinity chromatography) and biospecific affinity for ligands (affinity chromatography). All these methods have very different requirements with regard to chromatographic conditions e.g. depending on ionic strength, buffer salt composition, pH and additives. Normally, one has to combine several chromatographic methods to achieve a desired or complete purification of a protein from a crude biological extract. The number and order of fractionation steps to reach the final goal varies depending on the application. In general, however, the absolute yield of the desired protein decreases with the increasing number of fractionation steps which has to be judged in each case. On the other hand, many protein analysis-, and characterization methods are extremely sensitive requiring only minute amounts of sample.

Selected applications

As a protein chemistry laboratory, we have during the years been involved in analysis of proteins and peptides from very many different sources including humans, plants, bacteria, molds, fungi, viruses etc. Some of these analyses have also been connected to food material, either food itself, its microbes or enzymes used in food processing. The following section describes some examples about different sample treatment- and fractionation methods connected to these analyses. The examples are selected to highlight the different kinds of approaches utilizing homogenization, clarification, precipitation and chromatographic sample preparations as well as methods taking advantage of the different solubilities of the analytes of interest. The examples illustrates also sample separation by 1-D SDS-PAGE and 2-D PAGE in order to recover proteins for further identification by mass spectrometry.

Fish proteins

The skin of Atlantic salmon (*Salmo salar* L.) contains novel cysteine proteinase inhibitors which may have a role in the defense of the fish against bacteria and viruses. In order to isolate these proteins, characterize them at the molecular level and to measure their

antibacterial and antiviral activity, we purified the proteins from salmon skin (1). The goal of the isolation was to obtain pure proteins in order to measure their molecular weight by mass spectrometry, to determine their partial amino acid sequences by Edman degradation for homology comparisons and for cloning of the genes, to measure their isoelectric point by capillary isoelectric focusing as well as to demonstrate the presence of N-and O-glycans on their polypeptide chains. Subsequently, the devised isolation procedure was utilized also in purification of similar proteins from other fish species (2), in a detailed structural analysis of the glycan structures of the glycoproteins (3) as well as in cloning and sequencing of the genes of these proteins (4). Each of the mentioned analysis methods has certain sample requirements and need some fractionations and pretreatments before the analysis itself. Since the inhibitors were part of the salmon skin, they had first to be homogenized mechanically (Waring Blendor type homogenizator) and extracted in 10 mM Tris/HCl (pH 7.4), 250 mM sucrose, 10mM ethylenediamine tetra acetate (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine, 15 mM sodium azide. This medium represents a typical protein extraction solution with a defined ion strength, pH, protein stabilizing agent (sucrose), chelator for heavy metal ions (EDTA), proteinase inhibitors (PMSF, benzamidine), and bacterioside (sodium azide). Tissue homogenizations and extractions are usually performed at +4 °C, if possible. The extract was then clarified by centrifugation at 6,000 g for 30 min at +4 °C. Further fractionation and purification of the proteins in the centrifugation supernatant was carried out in four chromatographic steps: papain affinity chromatography, gel filtration, anion exchange chromatography and reversed phase chromatography. Usually a combination of chromatographic separation methods with different specificities (molecular weight, net charge, hydrophobicity, biological activity) are used in order to reach the desired protein purity and environment for analysis. The order of the purification parameters varies in a case by case basis and depends on the properties of the protein as well as on the contaminating proteins to be removed. The purification cascade presented here for the cysteine proteinase inhibitors utilized the biological activity of the inhibitors towards one of their targets, papain (cysteine proteinase) as the first purification step which resulted in considerable enrichment of the proteins of interest. This kind of affinity purification step is recommended if a suitable biological interaction exists. The further separation parameters, gel filtration, ion exchange chromatography and reversed phase chromatography utilized the molecular size, net charge and hydrophobicity in order to gather pure proteins for further characterization. The last purification step, reversed phase chromatography resulted in protein samples which could be subjected as such to subsequent molecular characterization methods, mass spectrometry, Edman sequencing, SDS-PAGE, peptide mass fingerprint (PMF) analysis after trypsin digestion.

Allergens

Many food substances contain proteins or chemical substances which are able to induce allergic reactions. Almost 20 % of the population in the western world suffer from different types of allergies and are susceptible to the development of allergic reactions to food and/or environmental allergens. The prevalence of food allergy in the general population ranges from 1 % to 2 % in adults and from 6 % to 8 % in children. We have been involved in studies involving the isolation and characterization of allergens, mainly

from plant materials. We started our studies with the non-food allergens from natural rubber latex of rubber tree (*Hevea brasiliensis*) but extended the studies later to food allergens from potato (*Solanum tuberosum*). In addition, we have isolated and studied food allergens from rye, barley and wheat (5, 6), banana (7), and oilseed rape and turnip rape (8).

Potato allergens

Extraction and purification of potato allergens (9) is an example of a commonly used cascade of methods to isolate and separate proteins from plant material. The purification of one of the allergens, patatin (Sol t 1) started with peeling and mechanical homogenization of the pulp in 50 mM Tris/HCL (pH 8.0), 150 mM NaCl without further additives. This extraction solution is about neutral in pH and sodium chloride is added in order to achieve physiological ion strength. The homogenate was clarified by filtering through a Whatman 3M/M filter paper and the proteins in the clear filtrate were precipitated with 60 % saturated ammonium sulfate. Ammonium sulfate precipitation is a common way to recover and concentrate proteins in a native state from different tissue extracts or protein solutions. This precipitation removes most of the non-protein based impurities present in the early step protein extracts and after centrifugation, it is possible to dissolve the protein precipitate to almost any desired volume and buffer. The potato allergens were further fractionated by gel filtration on a Superdex 75 HR (GE Healthcare) column in 50 mM Tris/HCl (pH 8.0), 75 mM NaCl which is the most suitable method for molecular weight based separation in the range 10,000–100,000 Da. Gel filtration is a convenient way, in addition to size dependent fractionation, to obtain the proteins of interest into a suitable buffer for the next purification stage. The immunologically active (immunoblot analysis using sera of potato allergic patients) fractions from the gel filtration step were further subjected to strong anion exchange chromatography on a MonoQ HR5/5 (GE Healthcare) column in 50mM Tris/HCl (pH 8.0) using elution with a linear salt gradient (0–0.5M NaCl) in the equilibration buffer. The immunologically active fractions from anion exchange chromatography were further subjected to hydrophobic interaction chromatography (HIC) on an Octyl Sepharose 4 Fast Flow (GE Healthcare) column. Proteins were bound to the column in 10mM sodium phosphate (pH 7.0), 1M ammonium sulfate and elution performed with a decreasing linear gradient of ammonium sulfate (1–0M) in the 10mM sodium phosphate buffer. This sample fractionation and purification protocol resulted in a pure fraction of patatin (Sol t 1), one of the potato allergens, which was then finally characterized by mass spectrometry, N-terminal sequencing and immunological studies.

Potato tuber contains also several other allergens which could be purified principally with the same methods as patatin, though in a different order of the methods and with slightly different conditions (10, 11). For purification of four other allergens, which later were identified as Kuniz-type soybean trypsin inhibitors, we adopted the same initial peeling, homogenization and extraction procedure as used for isolation of patatin. Gel filtration on Superdex 75 was, however, now performed in 20 mM sodium phosphate (pH 6.0), 150 mM NaCl, followed by hydrophobic interaction chromatography (HIC) on a Phenyl Sepharose column equilibrated with 20 mM sodium phosphate (pH 6.0), 1.5M ammonium sulfate. Elution was

performed with a decreasing linear gradient of ammonium sulfate (1.5–0 M) in the 20 mM phosphate buffer. The immunologically active fractions from HIC, still containing various amounts of ammonium sulfate, were then subjected to cation exchange chromatography on a Mono S column in 50 mM Sodium acetate (pH 3.3). Before this step, immunologically active fractions from the HIC column were pooled and the buffer was changed to 50 mM sodium acetate (pH 3.8) with pressure membrane ultrafiltration (10k Omegacell, Filtron Technology, USA). Elution from the cation exchange column was performed with a linear gradient (0–0.5 M) sodium chloride in sodium acetate buffer. The immunologically active fractions from cation exchange chromatography contained the potato allergens which were further characterized by SDS-PAGE, immunoblotting, mass spectrometry and N-terminal and internal Edman sequencing.

Cereal allergens

Isolation of plant allergens from cereals (5, 6) represent sample prefractionation methods which take advantage of the different solubilities of the proteins. Wheat allergy reflects well the diversity of food-allergic diseases and is one of the top six food allergies that together account for about 90 % of food allergies in infants and small children. Cereal proteins can be fractionated according to their different solubility in different solvents. To extract cereal proteins with different solubility, ground grains were sequentially extracted with 50 % acetonitrile in 0.1 % trifluoroacetic acid, 50 mM sodium phosphate (pH 7.5), 150 mM NaCl (phosphate buffered saline, PBS) or 70 % ethanol. Cereal proteins are classified according to their solubility. Albumins are water soluble, globulins are salt soluble and prolamins (gluten) can be further divided into acid/alkali soluble gluteins and ethanol soluble gliadins. IgE-binding proteins (possible allergens) were first identified in the extracts by SDS-PAGE/immunoblotting. The immunoreactive proteins from the different fractions were then further purified by either a combination of gel filtration and reversed phase chromatography or by reversed phase chromatography alone. The immunoreactive fractions from reversed phase chromatography were then subjected to SDS-PAGE and the protein bands corresponding to positive reaction in immunoblotting were subjected to N-terminal sequence analysis for identification.

Electrophoresis in food sample preparation

Electrophoretic methods are widely used for monitoring protein patterns recovered from different chromatographic fractionation and purification steps but can also be used to recover proteins for quantification and/or identification. For relatively simple protein mixtures, 1-D SDS-PAGE is sufficient. If the protein mixture is more complex, e.g. representing e.g. a total cell or tissue lysate, 2-D PAGE is preferable due to its capability to separate up to several thousands of proteins which can then be quantified and identified by mass spectrometric methods. From a 1-D SDS-PAGE or 2-D PAGE, it is possible to determine relative quantities of individual protein bands or spots and to determine the identity of the proteins by enzymatic "in-gel" digestion followed by mass spectrometric identification. One example of the use of 1-D SDS-PAGE in analysis of food proteins is the investigation of enzyme-aided modification of chicken-breast myofibril proteins (12). The effect of laccase and transglutaminase enzymes on cross-linking, gelation, and thermal stability of these proteins were investigated. Both

enzymes cross-linked the chicken-breast myofibril proteins as detected by their 1-D SDS-PAGE pattern. The cross-linked proteins were then identified by MALDI-TOF/(TOF) peptide mass fingerprint analysis and peptide fragment ion analysis.

Proteomics on probiotic bacteria

The higher resolving power of 2-D PAGE was used to separate proteins of the probiotic bacterium *Lactobacillus rhamnosus* GG which is one of the most extensively studied and widely used probiotic bacteria in fermented foods (13). In that study, the proteomes of the bacterium grown on industrial whey-based medium and a laboratory MRS medium were compared in order to find medium related differences in the proteome. For 2-D PAGE, sample preparation and pretreatment differs from the treatments e.g. for chromatography. In this study the cultivated bacteria were harvested by centrifugation at +4 ºC and washed twice in ice-cold 50 mM Tris/HCl (pH 8.0). Bacterial cells were broken by bead beating with glass beads in 30mM Tris and resuspended in a buffer containing 7 M urea, 2 M thiourea, 4 % CHAPS in 30 mM Tris and incubated at room temperature for 60 min. Urea and thiourea are protein denaturing and solubilizing agents and CHAPS is a zwitterionic detergent used to dissolve the membrane proteins. Next, the samples were clarified by centrifugation at 16000 g for 30 min at room temperature and the collected supernatant processed using a 2-D Clean up kit (GE Healthcare). The purpose of the 2-D Clean up kit was to specifically recover the bacterial proteins so that they could be subjected to electrophoresis and to leave the other interfering cellular substances in the supernatant. The precipitated proteins were then labeled with specific fluorescent dyes (CyDye DIGE Fluor minimal dyes, GE Healthcare) to facilitate their relative quantification. The labeled proteins were dissolved in the urea buffer above and the proteins subjected to the first electrophoresis dimension, isoelectric focusing (IEF) followed by separation in the second dimension SDS-PAGE. These sample preparation and pretreatment methods resulted in a 2-D PAGE (2-D DIGE) suitable for relative quantification of about 1200 proteins, of which 156 were quantitatively altered (at least 1.5. fold change in relative abundance) depending on the growth medium. The proteins of interest were then subjected to mass spectrometric analysis and identified by correlation of the mass spectrometry results with the protein sequences deduced from the genome sequence of *Lactobacillus rhamnosus* GG.

Protein cross linking

Foods are multicomponent materials with complex structures. The structure of food is related to texture and physical properties, chemical and microbiological stability, diffusion properties, product engineering and nutrition. Proteins together with carbohydrates and fats are the main components affecting the textural properties of foods. Cross linking and aggregation of protein molecules into three-dimensional networks is an essential mechanism for developing food structures with desirable mechanical properties. Many different enzymes, such as transglutaminases, peroxidases, tyrosinases, lipoxygenases and laccases, are able to cross-link proteins. In addition, they have many other catalytic properties. The cross-linking has many effects and these are highly dependent on applications. As an example, we were involved in the purification and characterization of a fungal tyrosinase (14), in order to investigate in greater detail its catalytic properties and its possible use in the food industry. For

overexpression of the *Trichoderma reesei tyrosinase gene* tyr2, encoding a protein with a putative signal sequence, was overexpressed in the native host. The enzyme was secreted to the growth medium (about 0.3–1 g/l), from which it was purified with a multistep procedure for further characterization. Fungal growth media, especially after overexpression of a certain protein, are rather simple protein mixtures from which the protein of interest is relatively easy to purify. For further processing, the growth medium was concentrated by membrane ultrafiltration. The buffer of the concentrate was then changed to 10mM Tris/HCl (pH 7.3) by gel filtration on a Sephadex G-25 (Pharmacia Biotech) column. This step represents a procedure where the buffer is exchanged to a more suitable one for the next cation exchange fractionation on a HiPrep 16/10 CM Sepharore Fast Flow (Amersham Biosciences) column. The tyrosinase enzyme was bound to the cation exchange column in the 10 mM Tris/HCl (pH 7.3) buffer and could be eluted with a linear gradient of NaCl in the equilibration buffer. The fractions with tyrosinase activity were concentrated with membrane ultrafiltration (Vivaspin, 10kDa cut-off) and subjected to final purification with gel filtration on a Sephacryl S-100 HR (Pharmacia Biotech) column in 20 mM Tris/HCl (pH 7.5), 150 mM NaCl. The purity of the enzyme was verified by 1-D SDS-PAGE. The essentially pure enzyme in 20 mM Tris/HCl (pH 7.5), 150 mM NaCl was not, however, as such, suitable for some of the characterization analyses. Thus, it was further subjected to reversed phase chromatography on a C4 (Jupiter C4, 5µm, 300Å, Phenomenex) column in 0.1 % trifluoroacetic acid and elution with a linear gradient of acetonitrile. The method resulted in a fraction suitable for MALDI-TOF and ESI-Q-TOF mass spectrometric analysis as well as for N-terminal sequence analysis by Edman degradation. In order to achieve a detailed molecular characterization, the enzyme was reduced with dithiothreitol (DTT), alkylated with 4-vinylpyridine and desalted by reversed phase chromatography. The alkylated protein was then digested with trypsin and the tryptic peptides separated by reversed phase chromatography on a C18 (Jupiter C18, 5µm, 300Å, Phenomenex) column. Each purified peptide was further analyzed by Edman sequencing as well as with MALDI-TOF mass spectrometry. These analyses confirmed the identity of the expressed polypeptide chain including the N- and C-terminal processing sites of the precursor protein. The enzyme was also posttranslationally modified (glycosylated). To analyse the glycan structure, the tryptic glycopeptide was first isolated by affinity chromatography on a ConA (Amersham Pharmacia Biotech) column equilibrated with 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The bound glycopeptide was eluted with 0.5 M methyl-α-D-mannopyranoside in the equilibration buffer and desalted for MALDI-TOF/TOF fragment ion analysis using a reversed phase C18 ZipTip (ZTC 18M Millipore Corporation). MALDI-TOF/TOF analysis of the affinity chromatography purified glycopeptide confirmed the glycosylation site of the protein as well as the type of glycan. ●

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Sample preparation and analysis of dioxins and other persistent organic pollutants

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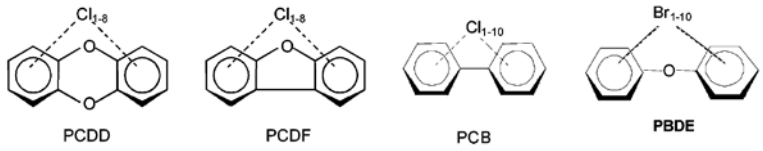
Introduction

Persistent organic pollutants such as polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/F, dioxins), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and several others have given tise to concern since 1980's when dioxins were found to be present in breast milk. Nowadays, it is known that in addition to breast milk also human blood and especially human fat contains hundreds of organic persistent pollutants.

The PCDD/Fs are two groups of tricyclic, planar, aromatic compounds with varying levels of chloride substituents in the benzene rings. Theoretically, 75 different PCDDs and 135 different PCDFs can be formed. They all are nonpolar, lipophilic, stable chemicals, poorly water-soluble and the solubility decreases with an increasing chlorination level. The substances are largely sediment bound a tendency which increases with increasing chlorination level. PCBs can contain from one to ten chlorine atoms in the two benzene rings. They form a group of oily and stable chemicals and they have been used because of their stability and low flammability as insulating materials in electrical equipments, as plasticizers in plastic products, and for a variety of other industrial purposes.

Figure 1.

The structures of polychlorinated dibenzo-p-dioxins (PCDD), dibenzofurans (PCDF), biphenyls (PCB), and polybrominated diphenyl ethers (PBDE).



They can contain small amounts of dioxins as impurities (synopsis). PBDEs contain one to ten bromine atoms in the benzene rings. The structures of dioxins, PCBs and PBDEs are presented in Figure 1.

Origin of dioxins

Dioxins can be formed as by-products in numerous chemical manufacturing processes or as by-products in thermal and pulp bleaching processes in which sources of chlorine, organic matter, and sufficient energy are involved. From the point of view of sediment contamination, a significant source of dioxins is the use and manufacture of chlorophenols, which have been used as fungicides, herbicides, insecticides, and as precursors in the synthesis of other pesticides (1–5). In Central Europe, pentachlophenol products and in Finland tens of thousands of tons a tetrachlophenol product marketed under the name Ky-5 have been used.

A term "toxic equivalency quantity (TEQ)" has been introduced mainly for regulatory purposes. The TEQ describes the relative toxicity of a single PCDD/F or PCB congener related to the 2,3,7,8-chlorine substituted PCDD congener (TCDD), which is the most toxic of all congeners. TEQ value is calculated as follows: $TEQ = \sum TEF_i \cdot C_i$, where C_i is the concentration of congener i, and TEF_i is the corresponding toxic equivalency factor. In the late 1980s, the final analytical results of dioxins and dioxin like PCBs were presented using I-TEFs (international toxicity equivalent) (6). After the year 1998, WHO-TEFs have been used (WHO toxicity equivalent) (7). In 2005, WHO re-evaluated the WHO-TEF and gradually they will be introduced into legislation (8). As new scientific data emerge, these TEF values will again be subject to change.

The main degradation processes in the environment are photolysis where PCDD/Fs react with OH radicals and biodegradation. Photolysis is of importance only in the atmosphere and the surface layer of soil (9). The study of a sediment core from the Baltic Proper (10) concluded that half-life at the furan isomer group decreased with an increasing level of chlorination. The tetrachlorodibenzofuran (TCDF) isomer group a the half-life of 80 years and octachlorodibenzofuran (OCDF) had the lowest value but was still very persistent, with a half-life of 30 years. The half lives of PCDDs are somewhat longer, varying from 100 years of tetrachlorodibenzo-p-dioxins (TCDD) to 170 years of hexachlorodibenzo-*p*-dioxins (HxCDD) (10). Consequently, the persistence of these substances in river, lake, and marine sediments is long enough to pose that it will pose a risk to wildlife and humans for centuries (11).

Health effects

Dioxins and PCBs, being fat-soluble, accumulate in the human body. Our body is not able to metabolize dioxins and excretion happens slowly via faeces, depending on the half life of the congener. PCBs instead are partly metabolized for example as hydroxy-PCBs and excreted via urine. The most efficient means for removing dioxins and PCBs is only possible in women during breast feeding. In humans, a wide variety of health effects have been linked to high exposure to dioxins, including several cancers, mood alterations, reduced cognitive performance, diabetes, changes in white blood cells, dental defects, endometriosis, decreased testosterone and (in neonates) elevated thyroxin levels, and decreased male/female ratio of births (12). Dioxin and PCB have also been linked to cryptorchidism, i.e. undescended testis, which is a common urogenital abnormality in newborn boys. In animal studies, prenatal and lactational exposure to dioxins has been associated with delayed testicular descent. At present, the effects have definitively been proven in the case of chloracne and dental defects. Polychlorinated biphenyls (PCBs) have well known endocrine disrupting effects on the thyroid function. Animal studies as well as human observations of highly exposed subjects have shown depression of the peripheral thyroid hormones following exposure to PCBs.

Dioxins in Finland

If one consideres at the situation in Finland, production of chlorophenols was previously the main source of PCDD/Fs into the Finnish environment. Burning of wastes has so far not been intensive in Finland but other burning processes have been significant sources. Bleaching of pulp with elemental chlorine was also a significant source of dioxins into the environment, but since new technologies were introduced by the pulp industry which enabled chlorine free bleaching, this source has also virtually disappeared.

The composition of the Finnish chlorophenol product Ky-5 varied, the main components being 2,3,4,6-tetrachlorophenol (78–83 %), 2,4,6-trichlorophenol (7–14 %), and pentachlorophenol (6–10 %) (13). The main fraction of PCDD/Fs in Ky-5 consisted of chlorinated furans (some 87 % of the total concentration of PCDD/Fs and 95 % of I-TEQ, (14)). The product consisted mainly of octa-, 1,2,3,4,6,8,9-hepta-, 1,2,3,4,6,7,8-hepta, 1,2,4,6,8,9,-hexa-, and 1,2,3,4,6,8-hexachlorodibenzofurans totalling from 65 to 200 µg/g (14, 15). During the manufacturing process the impurities crystallized on the surfaces of the manufacturing apparatus from where they were flushed directly in the river Kymijoki during the monthly cleaning operations, probably until late 1960s. Later, at least part of the precipitate was collected with filters and transported to a local landfill sit

Exposure and food

The population is exposed to persistent organic pollutants (POPs) mainly via fatty food. In Central Europe meat, dairy products, and fish are the most important food items but in Finland, where the environment is quite clean from POPs, exposure occurs mainly via fish. A Finnish survey on ten market baskets consisting of almost 4,000 individual food items showed that the average concentrations of dioxins in different market baskets

Figure 2.

The contribution of different food items as TEQs to the Finnish intake of PCDD/Fs and PCBs.

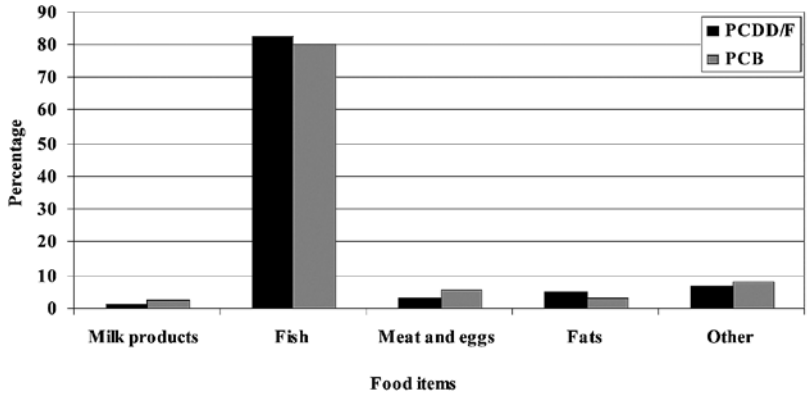


Figure 3.

Timetrend of intakes of dioxins (diamonds) and PCBs (squares) as pg WHO-TEQ/kg bw/day in UK (open diamonds and squares) and in the Netherlands (closed diamonds and squares) (17).

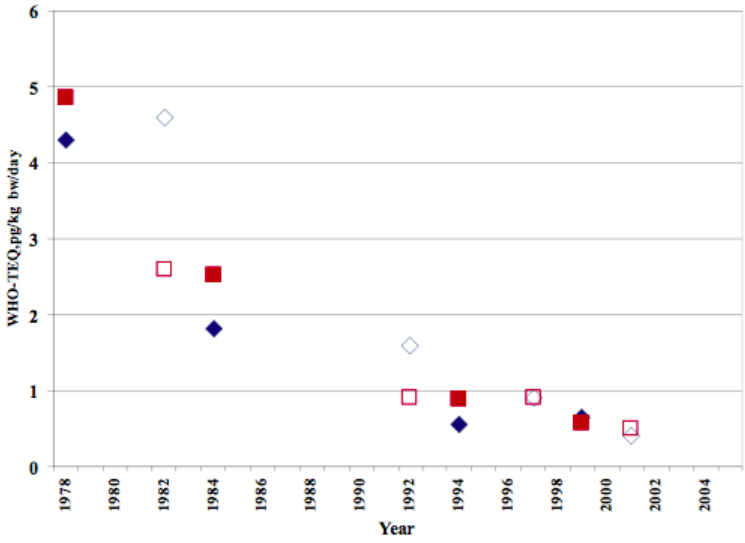
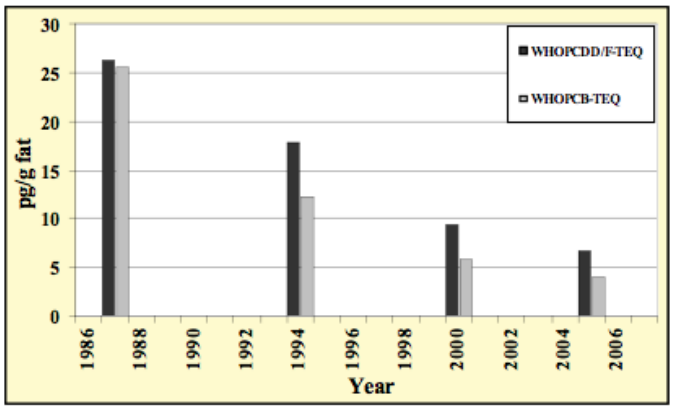


Figure 4.

Timetrend of dioxins and PCBs as TEQs in Finnish breast milk between 1987 and 2005 (17).



ranged between 0.0057 and 5.6 pg/g fresh weight (fw) and of PCBs from 39 pg/g to 25,000 pg/g fw. The fish basket dominated in the concentrations of dioxins and PCBs, and also in concentrations of PBDEs i.e. that basket ranged from 0.82 to 850 pg/g fw. Daily intake of sum of PCDD/Fs and PCBs as calculated to WHO toxic equivalents was assessed to be 115 pg which was 1.5 pg WHO-TEQ/kg bodyweight using an average mean weight of 76 kg for the study population in Finland in 1997. The contribution of fish to the intake of PCDD/Fs was between 72 and 94 %, depending whether lower or upper bound concentrations were used. With PCBs, the contribution of fish was about 80 % (Figure 2). The intake of PBDEs 44 ng/day was comparable to intake assessments in other countries (16). Since the 1980s, the intake of dioxins and PCBs has decreased in the whole of Europe, and also in Finland, being currently only one third or less of that in the 1980s (Figure 3).

In order to generate reliable background occurrence data on dioxins and PCBs, the Commission of the European Community has a recommendation for member countries to monitor these substances in foodstuffs. A minimum number of analysed samples per country per year in different food categories is recommended, in Finland the number of annual samples amounts to 45. In Finland, along with PCDD/Fs and PCBs, the occurrence of PBDEs has been measured from fish, meat, milk, cheese, egg, and oil and fat samples since 2003.

Concentrations of POPs in Finnish population

Breast milk samples have been monitored in Finland since 1985. Dioxin and PCB concentrations have decreased during twenty years period by 80 % (Figure 4) (17). Adipose tissue concentrations of dioxins and PCBs in a sample of 420 individuals representing general Finnish population in southern Finland showed that the mean (median) concentrations of WHO-PCDD/F-TEQ and WHO-PCB-TEQ were 29.0 (24.1) and 20.7 (16.7) pg/g fat, respectively. The concentrations clearly correlated with age. Expressing the concentrations as a function of the subject's ages revealed that the exposure of Finns had declined over the last 30 years. A downward gradient was found in the concentrations from Baltic Sea coast to inland areas in Finland, and this was assessed to be due to higher consumption of the Baltic Sea fish, especially Baltic herring in the coast areas when compared to inland areas. Linear regression models for natural logarithm WHO-PCDD/F-TEQ, natural logarithm WHO-PCB-TEQ, and natural logarithm WHO-total-TEQ, explained 70 %, 69 %, and 72 % of the variability, respectively. Age, lactation, place of residence, and fish consumption frequencies were significant predictors in the models (18).

Fishermen

The professional fishermen consume 85 % more fish than males of the general population, resulting in higher exposure to persistent pollutants but they also have 59 % higher intake of fish-derived omega-3 PUFAs. The fishermen's wives consume 45 % more fish and have 29 % higher intake of fish-derived omega-3 PUFAs than females of the general population. The mortality study from Finland showed that the fishermen and their wives had lower mortality from all causes (SMR 0.78, 95 % confidence interval 0.73–0.82, and 0.84, 0.76–0.93 respectively), and from ischaemic heart diseases (0.73, 0.65–0.81, and 0.65, 0.50–0.83) than the general population. It seems that consumption of contaminated Baltic fish results in a positive net health effect at least in the adult population (19).

Preparation of samples for analysis of PCDD/Fs, PCBs, and PBDEs

Sediment samples must be collected from stratification areas. The sediment cores are recommended to be collected with a pistonless corer and to be divided into slices. Dating of the slices is recommended. The samples are lyophilized and homogenised before laboratory analyses. Analyses from the Gulf of Finland have shown that the total amount of PCDD/Fs was 16.4 kg as WHO-TEQs and the highest load was between years 1960 and 1986 (20, 21).

Milk

Human milk is a good surrogate for human POP-exposure through food and (local) environmental levels since sampling has been performed according to WHO's guidelines in all countries and different years (22). The results provide information on time trends of exposure and contamination in the study areas. The collection of breast milk should be started on week two to four after the delivery and only ten to twenty ml per day needs to be collected. The residence time of mothers must have been at least five years in the study area. For environmental monitoring, only primiparae mother's milk is used. If the samples will be pooled, the parity, living history etc must be known (22). Pooling must also be organized according to WHO guidelines. Samples can be stored frozen for tens of years without loss of POPs. Cow milk samples are easier to collect but the collection technique depends on the purpose of the survey.

Meat

Meat samples must be representative to the study area and enough samples must be pooled in order to obtain reliable results. For official results, sampling and pooling must comply with the Commission directive 2002/69/EC. The market basket is a useful way to minimize the sample number for population exposure assessment. The samples are possible to be stored as such, after freeze drying or after extracting fat from the pooled samples (in all cases frozen at -20°).

Fish

Fish is a special case from food. There is a need to analyze fish samples as such or for human exposure calculations. In both cases, the heads and guts are removed. Homogenates are prepared comprising either the whole, cleaned fish (usefully Baltic herring, sprat, vendace, smelt) or a slice of 50–150 g that is dissected from just behind the dorsal fin (larger fish species). To estimate the influence of skin removal on POP concentration, fish individuals can be analyzed with and without skin. Many times also pooled fish samples are analyzed according to size, age etc. The weight, length and sex of fish have to be determined.

Blood and human tissue

For exposure analyses, in addition to breast milk, it is also possible to analyse blood and tissue samples for POPs. Tissue samples have been often taken during a surgical operation for purposes other than POPs sampling e.g. appendicitis operation. The amount of fat samples is usually about 0.5 g. The problem in blood samples is that blood contains only about 0.5 % of fat. With standard methods of analysis, to gather one gram of fat, 200 ml of blood, is needed to obtain reliable results for all dioxin and furan congeners. However, in most cases it is impossible to take so much blood from one individual. In addition, sometimes analyses must be made from (old), very tiny (e.g. 0.1 ml blood) and numerous samples, such as thousands of small samples for epidemiological analyses.

The only way to analyze a large number of tiny blood samples is to use fast methods that determine only one or a few representative congeners from the different POP groups (see below "fast analyses"). It must be noted that fat content in blood also varies after eating of (fatty) food. In order to obtain results from the person's stock fat, fasting sample must be collected. Sampling of breast milk, blood or human tissue always requires ethical permission.

The procedure for (large) blood sampling is normally as follows: After a 12-hour fast 50 to 200 ml of venous blood from each subject is drawn into centrifuge tubes (no anticoagulants or serum separator). The samples are allowed to clot for at least 40 min, and centrifuged for 20 min. The serums are transferred into glass vials and coded, the codes are broken only after the results have been reported.

Laboratory analyses

Different types of samples, typically containing 1–3 g of fat, are spiked with 16 toxic, ^{13}C-labelled 2,3,7,8-substituted PCDD/F congeners, variable number of ^{13}C-labeled PCBs (preferably non-*ortho* PCBs 77, 81, 126, 169, mono-*ortho* PCBs 105, 114, 118, 123, 156, 157, 167, 169 and so called indicator PCBs 28, 52, 101, 138, 153, 180), and with variable number ^{13}C-labelled PBDEs (preferably the most abundant BDEs 28, 47, 99, 100, 153, 154, 183 and 209) before extracting the samples. Analytes measured comprise of 17 2,3,7,8-chlorine substituted "toxic" PCDD/F congeners, dioxin like PCBs (non-*ortho* and mono-*ortho* PCBs), indicator-PCBs, a variable number of other PCBs, and a selection PBDE congeners in addition to the most abundant ones. Sometimes also OH-PCBs, the metabolites of PCBs in humans, are analyzed, usually ten congeners (OH-PCB54, OH-PCB104, 4`-OH-PCB108, 4-OH-PCB107/118, 4`-OH-PCB130, 3-OH-PCB138, 4-OH-PCB146, 4`-OH-PCB172, 3`-OH-PCB180 and 4-OH-PCB187).

Laboratory analyses for POPs are rather similar after extraction, but different matrices need different extraction methods (see below). Due to the large differences in the concentrations, PCBs (and PBDEs) have to be separated from PCDD/Fs even if only dioxins are going to be analyzed. Nonetheless, high resolution gas chromatography and high resolution mass spectrometry analyses are obligatory, because even very small residues of PCBs in the PCDD/F fraction are able to leading to false or too high PCDD/F concentrations.

Extraction of POPs from sample matrices

Sediment samples are Soxhlet-extracted with a mixture of ethanol and toluene (e.g. 30:70 v/v) for 20 h. Also faster extraction techniques with the same solvent system, such as Twisselman hot extraction or Accelerated Solvent Extraction (ASE) can be used. Co- extracted sulphur from the sediment samples is precipitated with activated copper powder.

After homogenisation, solid samples other than sediments or soil (meat, fish) are freeze dried and fat is extracted using similar extraction systems as for sediments. Liquid samples (water, juice) are extracted with liquid-liquid extraction (hexane or diethyl ether-hexane). Fat from milk samples is extracted with a mixture of diethyl ether and hexane after addition of sodium oxalate and ethanol to remove calcium and to precipitate the proteins. The fat content is determined gravimetrically after the exchange of solvent to hexane. Oil samples are dissolved in hexane.

Proteins from serum are precipitated with ethyl alcohol and ammonium sulfate. Fat is extracted with hexane, and the fat content is determined gravimetrically. The analysis method involves multiple cleanup steps (see below), and finally high resolution mass spectrometry is used for quantification. All the results are reported on a fat basis, and limits of quantification (LOQ) for PCDD/Fs, non-*ortho*-PCBs, and other PCBs are 0.5–5, 1.5, and 50 pg/g, respectively, depending on the congener studied.

Human fat is extracted from tissue samples similarly to other solid samples. If also PCB metabolites, OH-PCBs, need to be analyzed in human samples, they are spiked with a set of five ^{13}C-labeled OH-PCB internal standards (OH-PCB 61, 120, 159, 172, and 187). The solvent is evaporated to dryness and the solvent is changed to hexane. The fat percentage of the sample is determined gravimetrically.

Detailed procedure to fractionate POPs

In general, sample extracts are purified and fractionated with three columns: a large column of multiple layers of silica gel (neutral, H_2SO_4-impregnated and KOH- impregnated silica), and smaller columns of activated carbon and aluminium oxide (16). Also automated sample purification and fractionation systems rely on the same principles (23). Major impurities can also be removed with gel permeation chromatography (GPC) instead of multilayer silica (24). Multilayer silica column allows all neutral POPs to pass through while retaining the vast majority of co-extracted matrix components, such as fat. In the GPC column, co-extracted matrix components pass first through and analytes are collected in the later fraction. An example is given for the cleaning procedure where also the PCB metabolites, OH-PCBs, in human milk or tissue samples will be analyzed.

In order to separate OH-PCBs from potential methoxy metabolites of PCBs and from non-metabolised PCB congeners, the sample in hexane is extracted with 1 molar (M) potassium hydroxide (KOH) in ethanol: water solution (1:1). The hexane fraction is defatted in a silica gel column containing acidic and neutral layers of silica, and all analytes are eluted with dichloromethane (DCM): cyclohexane (c-hexane) (1:1). The KOH solution including OH-PCBs is acidified with 5 M hydrochloric acid (HCl) until the pH is < 2, after which the solution is extracted with hexane and dried by adding sodium sulphate (Na_2SO_4). The volume of hexane solution is evaporated to 0.5–1 ml and diazomethane is added to form methoxy derivates of the OH-PCB compounds. The sample in hexane is purified with a column including silica gel impregnated with sulphuric acid (H_2SO_4), from which it is eluted with hexane. Recovery standard (PCB 159) is added before analysis and hexane is replaced with 20 µl of nonane.

From the (remaining) hexane fraction or from fat purified hexane fraction, PCBs are separated from other compounds such as PCDD/Fs using activated carbon column (Carbopack C, 60/80 mesh) containing Celite (Merck 2693). The first fraction including PCBs is eluted with DCM:c-hexane (1:1) followed by a back elution of the second fraction (including PCDD/Fs) with toluene. The eluent including PCBs are evaporated using nonane as keeper and PCB-fraction in *n*-hexane is further cleaned by passing it through an activated alumina column (Merck 1097). The PCB fraction is eluted from the alumina column with 2 % DCM in *n*-hexane, and after changing the eluent again to *n*-hexane, the eluent is transferred to another activated carbon column (without Celite) in order to separate non-*ortho* PCBs from other

PCBs. Dichlorimethane (50 %) in *n*-hexane is used to elute other PCBs while non-*ortho* PCBs are back eluted with toluene. Recovery standards (^{13}C PCB 60 for non-*ortho* PCBs and PCB 159 for other PCBs) are added prior to analysis; the solvent is replaced by 10–15 µl of nonane for non-*ortho* PCBs and by 300 µl of *n*-hexane for other PCBs.

Quantification of samples

The quantification of analytes is performed by selective ion recording using a high resolution mass spectrometer (e.g. Waters Autospec Ultima) at a resolution of 10,000, equipped with a high resolution gas chromatography with fused silica capillary column (one of the best being DB-DIOXIN, 60 m, 0.25 mm, 0.15 µm). The laboratory reagent and equipment blank samples are treated and analyzed by the same method as the actual samples. They must be lower than the predetermined set values if one wishes to obtain reliable results.

Limits of quantification (LOQ) depend on the compound being studied, the amount of sample taken for analysis and reporting format (per fat, per fresh weight, per dry weight). For PCDD/Fs and non-*ortho* PCB, LOQs are typically in the sub pg/g range, and for other POPs in the sub ng/g range. Recoveries for internal standards must be from 60 % to 120 % for all congeners for all sample matrices.

It is recommended that the laboratory is accredited (e.g. EN ISO/IEC 17025) and the scope of accreditation shall include dioxins, non-*ortho*-PCBs, PCBs, and PBDEs. Several international intercalibration studies are arranged annually. Several reference materials are available including sediment, milk, fish, and meat.

Fast methods for tiny blood samples

Fast methods for the analysis of POPs are required in cases where a large number of tiny samples need to be analysed or are available e.g. in epidemiological studies. A large number of methods have been developed e.g. based on. solid phase extraction (SPE) or solid phase micro extraction (SPME), but also innovative liquid-liquid extraction (LLE) methods can be very simple and fast. All methods use GC-MS for separation and detection of the desired POPs. An example of a recent LLE method is described below.

Serum samples (200 µl) are pipetted into 8 ml glass test tubes (10mm outer diameter). Internal standards in 0.2 ml of toluene are added (2.0 ng of ^{13}C-p,p'-DDE and 0.4 ng of ^{13}C-PCB-153) followed by 0.5 ml of ethanol. The samples are sonicated for 5min to precipitate the proteins and equilibrate the internal standards.

To extract the analytes, 2ml of hexane are added to the serum–ethanol mixture and the samples are shaken at 2000 rpm for 10 min in a shaker. To cleanup the samples, 1.0 ml of 15 % sulphuric acid silica is added to the serum–ethanol–hexane mixture, and samples are shaken for another 5 min. After this, the test tubes are centrifuged for 2 min at 3500 rpm. A solid precipitate containing water, ethanol, proteins, fat and other biogenic compounds adsorbed to 15 % sulphuric acid silica are packed to the bottom of the test tube. Clear hexane from the top is poured to another test tube. Samples are evaporated to about 0.5 ml under a gentle stream of nitrogen, and transferred to autosampler vials. The recovery standard, 200 µl of ^{13}C-PCB-128 solution is pipetted to GC autosampler vials, and samples are evaporated to 200 µL of toluene as the final volume (25). Instrumental determination can include one or two different congeners from several POP groups, such as PCBs, PBDEs, organochlorine pesticides etc. ●

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Treatment of biofluid samples for liquid chromatographic analysis

Tapani Suortti

The principle for selection of sample preparation procedure

The optimal sample preparation method produces the sample components in a solution which is suitable for direct injection into the chromatographic instrument with a high yield and in a sufficiently stable environment that instability of the component before the actual analysis has no significant impact on the results.

The most problematic sample in the group of biofluids is serum because of its high protein content and enzymatic activities. In most cases urine and cerebrospinal fluid samples may be directly injected into an HPLC column, often with very minimal sample treatment, perhaps only dilution of sample with mobile phase and in case of urine, also enzymatic release of conjugated metabolites if non-conjugated forms are to be analyzed. The treatment of serum samples is more complicated as direct injection of serum, even diluted into a normal RP-HPLC column results into plugging of the column and the loss of its efficiency.

The selection of the method for serum sample preparation

The pH of serum is 7.35–7.45. The most important component in serum with regard to sample preparation is its high protein content (~7 %) and especially albumin (~4 %). Not only will the precipitation of albumin in the column under ordinary chromatographic conditions lead to rapid column deterioration, many of the analytes are bound into albumin other via non-conjugated bounds or simply adsorbed on the protein. Another important factor aspect is the enzymatic activities present in serum, which may lead to erroneous results. The selection of proteins removal from samples can be done with six different ways:

- precipitation at low pH
- precipitation at high pH
- precipitation with organic solvents
- use of solid phase extraction columns either off-line or on-line
- extraction of sample components with water inmiscible organic solvents.
- mechanical removal of high molecular weight proteins (ultrafiltration)

There are very few studies on different serum protein precipitation methods. The publication of Quareshi&Quareshi shows (1) how great the effects can be of different methods in influencing the concentrations of a chemically very similar group such as physiological amino acids present in serum.

Precipitation at low pH

The precipitation of serum proteins with low pH buffers has a strong link with amino acid analysis. In these techniques traditionally 5-sulphosalicylic acid (10 %) or 1M perchloric acid is added to the sample and sample is simply centrifuged and directly injected into instrument. Since albumin is denaturated, the compound adsorbed on it, are released into solution. The low pH of solution inactivates the enzymes though if the pH of the solution then increased they may recover part of their activity. The other low pH precipitating solutions used include trifluoroacetic acid, trchloroacetic acid and metaphosphoric acid. The choice of the precipitating agent seems to be based on the traditional methods which were in use long the introduction of before modern high performance liquid chromatographic techniques. As the use of these low pH precipitating solutions may destroy some of the compound of interest also dilution with pH 5 buffer (the PI value of albumin is 4.7) followed by centrifugation may make samples suitable for injection into a chromatographic column. However in this case, albumin is not denatured and thus the adsorbed compounds with the albumin will co-precipitate. As many of modern reverse-phase columns can tolerate a pH as low as 1, low pH precipitation will deliver samples that may be directly injected into a column with virtually 100 % removal of proteins and the precipitating agent will elute in the void volume. If chromatographic condition hinders the injection of low-pH samples, the adjustment of pH by dilution with suitable buffer is a simple task. This kind of precipitation is very suitable especially, for most polar serum components.

Precipitation at high pH

The increase of pH of serum with addition of strong alkali also denatures proteins but such methods are limited to compounds which are stable at high pH, like nitrite/nitrate and bile acids.

Precipitation with organic solvents

The most popular protein precipitant of serum proteins has been acetonitrile. The addition of an equal amount of acetonitrile to undiluted or 1–5 fold diluted serum was almost a standard procedure for many drugs. Since acetonitrile precipitates most of the proteins along with denaturation, precipitation with acetonitrile results in samples where medium polarity compounds are released from albumin and extracted into solution with high solvating power. According to the author's experience 50 % acetonitrile is not a good enough precipitant and the samples will have adverse effect on HPLC column's efficiency, especially if the samples need to released from acetonitrile and concentrated in order to obtain higher sensitivity. With 67 % or 75 % acetonitrile, such problems are not encountered. However 50 % acetonitrile may be used with plasma to obtain a fraction enriched with hydrophobic proteins <30000 dalton free from high molecular weight proteins. The removal of acetonitrile in order to analyze polar compounds will impair some of the enzymatic activities present in serum.

The other precipating solvents occationally used are methanol and isopropanol. The use of methanol for protein precipitation requires higher concentrations than acetonitrile, which results in samples contaminated with non-polar compounds, thus requiring more frequent column cleaning. The use of isopropanol requires a lower concentration than acetonitrile (~30 %) but it more suitable for analysis of non-polar compounds like retinols to keep them in solution, making direct injection possible.

Use of solid-phase extraction column on-line or off-line

The use of solid phase columns in protein removal is based on the much larger molecular size of proteins in comparison with ordinary analytes. As a consequence the access of proteins to solid phase pores is hindered and their mass transfer is slow since they do not bind to the solid phase.

When analyzing either acidic or basic components, very efficient sample cleaning may be obtained by retaining the compounds of interest in an ion-exchange cartridge the pH of the plasma needs to be adjusted (addition of formic acid or ammonia does not precipitate proteins), and then there is washing of polar non-ionized components with buffer or water, non-polar compounds with organic solvents and finally the release of the analytes by washing with a low or high pH-buffer. This kind of complex systems care is normally implemented off-line. Simpler but efficient systems are widely used i.e. firs step - bind+protein removal. second step-elution to analytical column.

Extraction of samples with organic solvents

The extraction of the sample in a non-ionized state into an organic solvent and then back-extraction into aqueous solution in ionized states is an old traditional technique. Since it is prone to recovery problems and it normally involves tedious manual operation, still it is not popular in modern methods. The simple extraction into organic solvent is anyhow widely used in plasma lipid analysis either by the method of Folch, Lees & Sloane Stanley (2) or the method of Blight & Dyer (3). Both of these extraction techniques use methanol/chloroform as the solvent. A recent method using methyl-tert-butyl ether to replace chloroform/methanol mixture has been published.

Miscellar chromatography

The use of miscellar mobile phase direct injection of serum into ordinary reverse phase column is possible and it does not cause without problems of proteins precipitating and damaging columns. Since only the free form of compounds can be determined, any interaction with albumin or other serum proteins will greatly affect the results. The same problem rises when plasma proteins are removed by ultrafiltration or ultracentrifugation.

Other methods

Some novel methods having very high selectivity including molecular imprinting stationary phases and stir bar extraction has been published but their widespread use is hampered by the poor availability of commerciale materials. ●

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Generic sample preparation methods for drug screening

Ilkka Ojanperä | Ilpo Rasanen

Introduction

Drug screening is used here as a general term for the comprehensive qualitative analysis of low molecular weight (MW = 110–700), xenobiotic organic compounds, such as medicines, illicit drugs, designer drugs, pesticides and metabolites, in complex materials by chromatography -based techniques.The societal areas utilizing drug screening include forensic, clinical and veterinary toxicology, sports doping testing, environmental hygiene and food safety. The sample preparation methods described here are especially related to human materials like blood, urine, hair and solid tissues. Importantly, drug screening is not used here to denote the discovery of new drug candidates within pharmaceutical research.

The scope of drug screening is very much dependent on the application field. For instance, in forensic toxicology related to a cause-of-death investigation, every xenobiotic compound is a potentially harmful poison if administered in excess. The screening methods should be able to encompass some 500–1000 substances with sufficient sensitivity and specificity. For example, the types of compounds tested for driving under the influence of drugs is limited to substances that impair driving performance, while

doping control in sports concerns only the substances on the prohibited list of the World Anti-Doping Agency. Drug testing in the workplace, in prisons, and at schools is usually restricted to illicit drugs. In the industrial countries, the majority of toxicologically relevant substances are medicines and controlled drugs, while pesticides are important in developing countries.

The standard handbook on analytical toxicology written by Baselt (1) covers approximately 800 relevant organic toxicants, of which about 80 % contain nitrogen. Examples of major non-nitrogen containing drugs include gamma-hydroxybutyrate, cannabinoids, ibuprofen and warfarin. Drugs acting on the central nervous system (CNS) are particularly important as many of them may cause addiction and exhibit pronounced toxicity. Features of a successful CNS-active agent include sufficient lipophilicity, molecular weight <450 and a neutral or basic character.

The analytical techniques most often involved in drug screening are gas chromatography (GC) with nitrogen specific (NPD), electron capture (ECD) or mass spectrometric (MS) detection. Liquid chromatography (LC) can be combined with photodiode-array detection (DAD) or with different forms of MS. The MS techniques are superior in terms of identification power, because the characteristic masses and fragmentation experiments can be utilized in addition to chromatographic retention data. In GC-MS, large libraries of electron ionization spectra are commercially available. Accurate mass measurement by LC coupled with a time-of-flight MS analyzer (LC-TOFMS) is a means for tentative substance identification without reference standards. However, if quantitative analysis is requested in connection with screening, GC-NPD and LC-DAD are the methods of choice due to their better stability of quantitative calibration. Serum or urine immunoassays can be used without sample preparation for a limited number of drugs and drug groups supporting the chromatographic screenings.

Analytical properties of drugs

In the development of generic extraction methods, it is important to examine at the main analytical properties of drug substances. After searching the literature for the structural formula and existing chemical and pharmacological information, the following points should be examined: 1) Functional groups related to acid-base behaviour, other functional groups allowing proton exchange, double bond conjugation and fluorescence; 2) proportion of saturated carbon to heteroatoms reflecting water solubility and polarity; 3) volatility; 4) therapeutic concentration range in blood; 4) metabolism; 5) existing analytical methods.

It is practical to divide drugs into acidic/neutral and basic substances and develop the sample preparation methods accordingly. In addition, there are amphoteric compounds that can act as acids and bases and quaternary ammonium compounds that are positively charged through the whole pH range. The number of basic drugs on the market is much higher than that of acidic and neutral drugs. The therapeutic concentration range of basic drugs in blood is generally lower than that of acidic and neutral drugs. Interestingly, new drugs are usually more complex, more hydrophilic and more complicated to analyse as a part of a comprehensive screening method than their older counterparts.

A logarithmic measure of the acid dissociation constant (pKa) is one of the most important pieces of information required for an analyte. For bases, it is conventional to use the pKa of the conjugate acid. An acid is 50 % ionized when pH = pKa, it is 91 % ionized when pH = pKa + 1, and 99 % ionized when pH = pKa +2. Acids include carboxylic acids (pKa ≈ 4–5), sulfonamides (pKa ≈ 10), phenols (pKa ≈ 8–11) and imides (pKa ≈ 8–11). Bases include pyridines (pKa ≈ 3–5), prim-, sec- and tert- arylamines (pKa ≈ 3–5), prim-, sec- and tert- aliphatic amines (pKa ≈ 8–11) and guanidines (pKa ≈ 11–12). Polar structures without significant acid-base properties include alcohols and amides.

Water solubility is another very important property. Generally, the more lipophilic compound, the easier it is to extract from aqueous material. The partition coefficient P is a ratio of concentrations of un-ionized compounds between water and octanol at equilibrium, while the distribution coefficient D is the ratio of the sum of the concentrations of all forms of the compound in each of the phases at a specific pH. It is common to use, the logarithmic forms logP and logD. The higher the value of logP or logD, the higher the lipophilic character. Experimental determination of pKa, logP and logD is laborious and prone to errors, but sufficiently accurate algorithms have been developed for predicting these properties in silico (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada).

Figure 1 shows the predicted logD curves for a few structures commonly found in drug substances. At low pH range, acids exist as neutral species resulting in higher logD values, whereas with bases, the situation is the opposite. Amphoteric compounds can be neutral in a narrow pH range provided that the pKa of the basic group (conjugate acid) is lower than the pKa of the acidic group, as is often the case with aminophenols. The logD -lowering effect of heteroatoms can be seen in these examples.

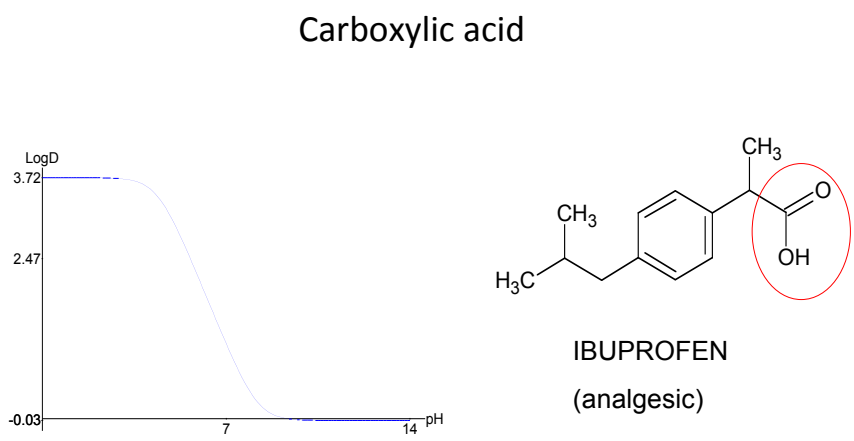
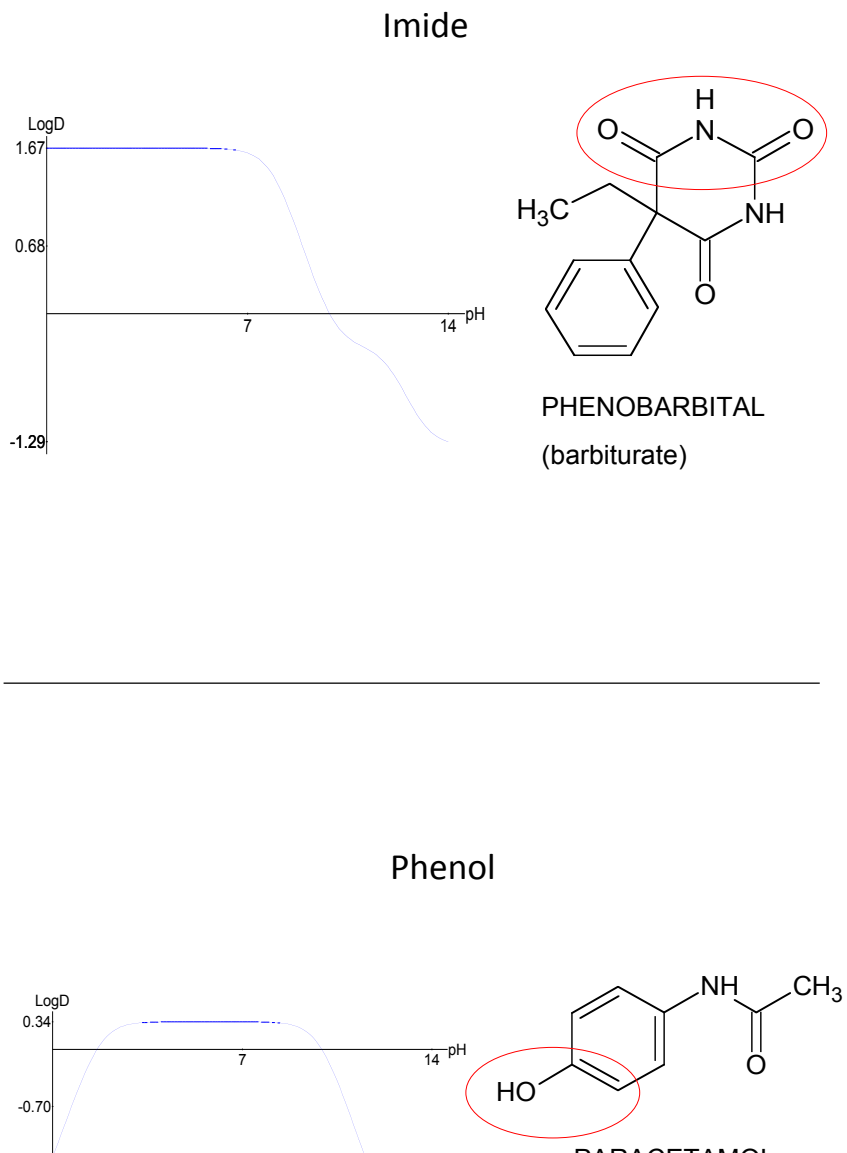


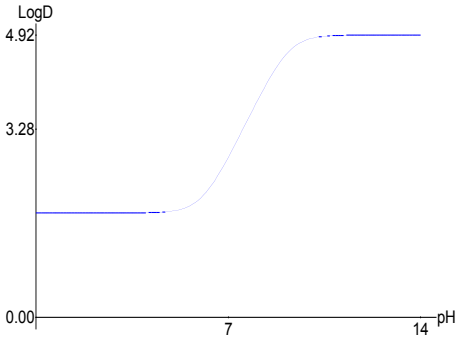
Figure 1.

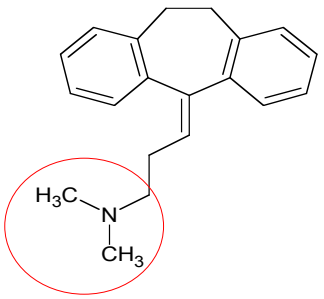
LogD as a function of pH for a carboxylic acid, imide, phenol, aliphatic tert-amine, guanidine and aminophenol predicted with ACD/ logD software (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada).

Figure 1.


Imide
LogD
1.67
0.68
-1.29
7
14
pH
H
O
N
O
H_3C
NH
O
PHENOBARBITAL
(barbiturate)
Phenol
LogD
0.34
-0.70
-2.79
7
14
pH
NH
CH_3
O
HO
PARACETAMOL
(analgesic)

Aliphatic *tert*-amine

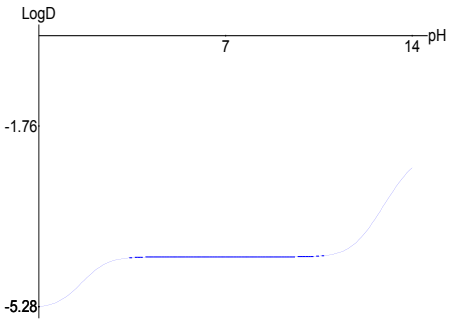


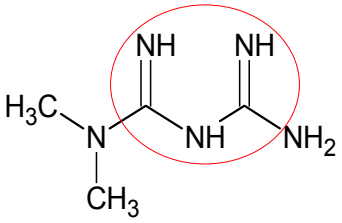


AMITRIPTYLINE

(antidepressant)

Guanidine

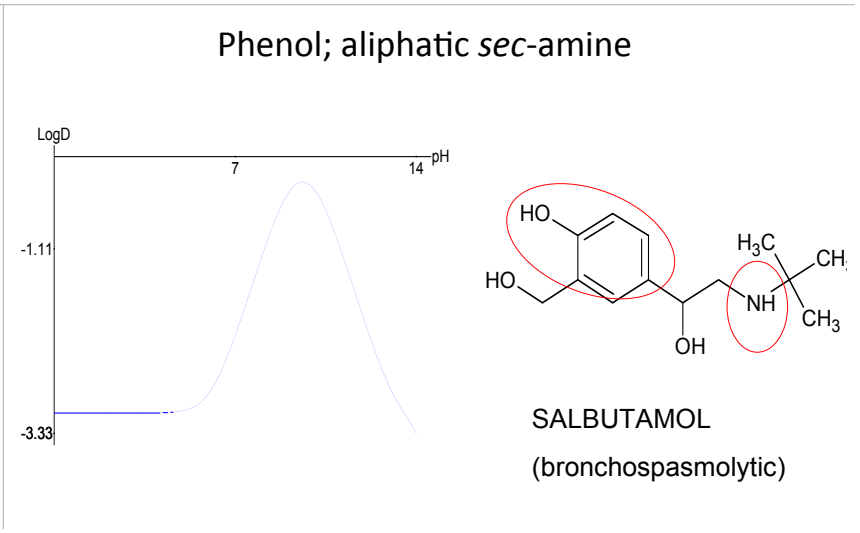




METFORMIN

(antidiabetic)

Figure 1.



Sample preparation

General

Due to the large number of analytes with differing analytical properties, sample preparation for comprehensive drug screening usually relies on a few non-selective generic methods. The situation contrasts with target analysis, in which the methods can be optimized and validated for a limited number of analytes. In the sample pre-treatment step, the sample is homogenized, measured by volume or weight and the analytes may be liberated from conjugates by hydrolysis if necessary. In the extraction step, the analytes are separated from matrix components using one of the many techniques available, notably liquid-liquid extraction (solvent extraction, LLE) and solid-phase extraction (SPE). Recently, several solvent-free and miniaturised extraction techniques have been developed (2), most importantly the solid phase microextraction (SPME), but these techniques are at their best with a limited number of analytes.

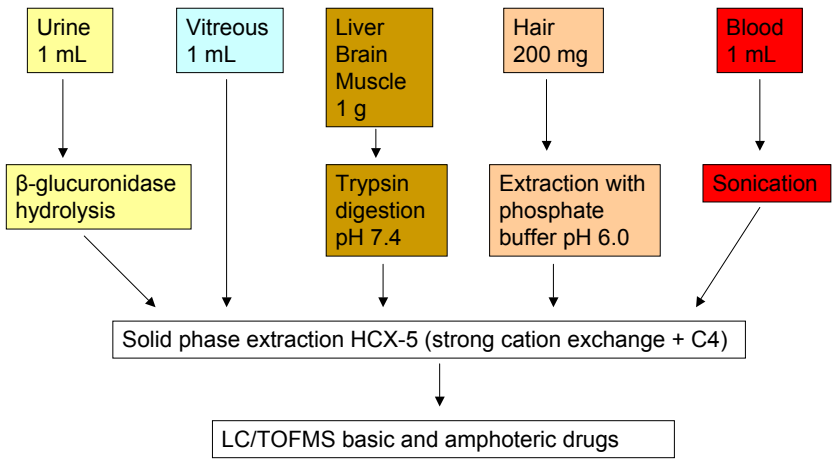
When developing generic extraction procedures, one should be aware of the general strategies for solvent selection. Perhaps the best illustrative is the Rohrschneider-Snyder solvent classification scheme that arranges solvents according to their polarity and divides them into eight selectivity groups (3). In general proton donors are suitable solvents for basic drugs and proton acceptors for acidic drugs. The addition of a few percent of alcohol, such as isopropyl alcohol, improves the recovery of polar drugs but inevitably leads to less clean extracts. Although empirical methods are predominantly used in the development of extraction procedures, also theoretical models are available for the selection of proper conditions for many types of procedures (4).

Sample pre-treatment

Figure 2 shows a schematic diagram of different sample pre-treatment procedures for common biological materials in analytical toxicology. The same SPE and LC-TOFMS method can be used following all sample pre-treatment procedures. Urine samples typically contain a considerable amount of polar phase I metabolites in addition to the

Figure 2.

Schematic diagram of different sample pre-treatment procedures for common biological materials in analytical toxicology.



parent drugs; sometimes the parent drug is not detectable at all. Especially drugs with hydroxyl and amino groups tend to form phase II metabolites by glucuronide and sulfate conjugation. The cleavage of conjugates is best carried out by enzyme hydrolysis. Acid hydrolysis is also applicable, but for instance many benzodiazepines are converted to the respective benzophenones with this kind of treatment.

Solid tissues, including liver, brain and muscle, should be homogenized before extraction. Liver accumulates lipophilic drugs that may be found there in high concentrations. However, liver is not homogenous and the concentrations vary between the regions. The fat content of liver can be high depending on nutrition and diseases. Liver can also be cirrhotic with high amounts of connective tissue. Consequently, the analysis of liver samples may be difficult due to variations in the material. Brain and muscle are more uniform in quality. Traditionally, mechanical homogenization methods have been used for solid tissues, but aerosol formation and carry-over are the drawbacks of the technique. Instead, digestion with trypsin or some other proteolytic enzyme has proved to be an efficient way of liberating drugs into solution. The resulting slurry is then filtered and extracted using a suitable technique.

Several pre-treatment methods have been developed for hair samples. First, the material should be washed with an organic solvent or shampoo to remove external contamination. Digestion with sodium hydroxide is ideal for liberating basic drugs, but the procedure also hydrolyzes some analyte structures, particularly esters. A more general and gentle approach is incubation with methanol or phosphate buffer following mechanical homogenization.

Liquid-liquid extraction (LLE)

LLE is the most traditional but still the most universal technique for non-selective extraction. Instead of separation funnels, LLE can be performed in a multi-tube vortex mixer using a rack of test-tubes. Typically, the liquid volumes for each tube are 0.5 ml of blood or urine, 0.5 ml of buffer and 0.5 ml of solvent. After centrifugation, the organic phase is ready to be injected into GC. The key factors to be optimized are the

type of solvent, ion strength, pH, phase volumes and extraction time. Typical extraction solvents are butyl acetate, ethyl acetate, methyl-tert-butyl ether, dichloromethane and n-butyl chloride. Of these, dichloromethane is the only solvent forming a lower organic phase. Possible emulsion problems can usually be avoided by adjusting the phase ratio and ionic strength. The cost of LLE is low and reproducibility is usually good, however, the technique is difficult to automate. Mechanical LLE using diatomaceous earth as a solid support is a means of making the technique amenable to automation.

Figure 3.

Analysis scheme for quantitative drug screening in blood by LLE and GC analysis.

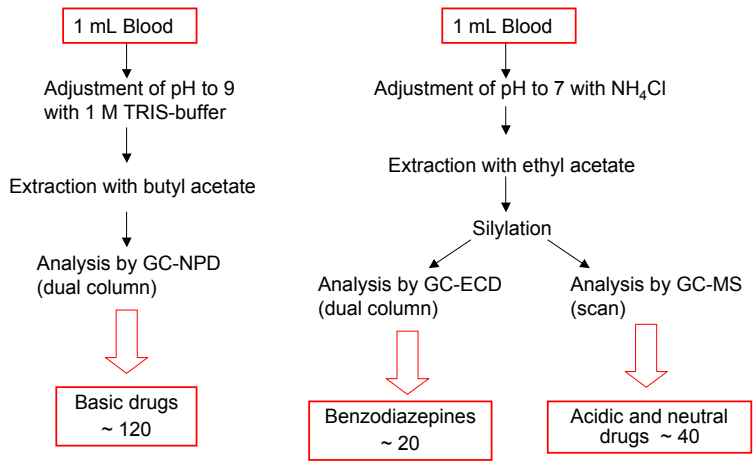
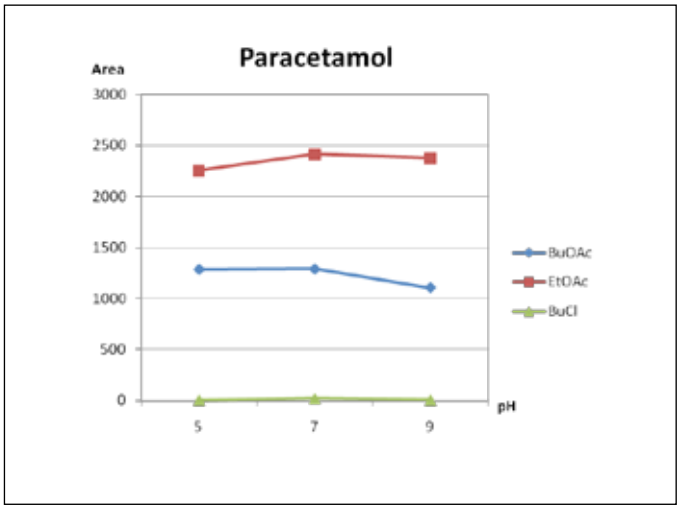
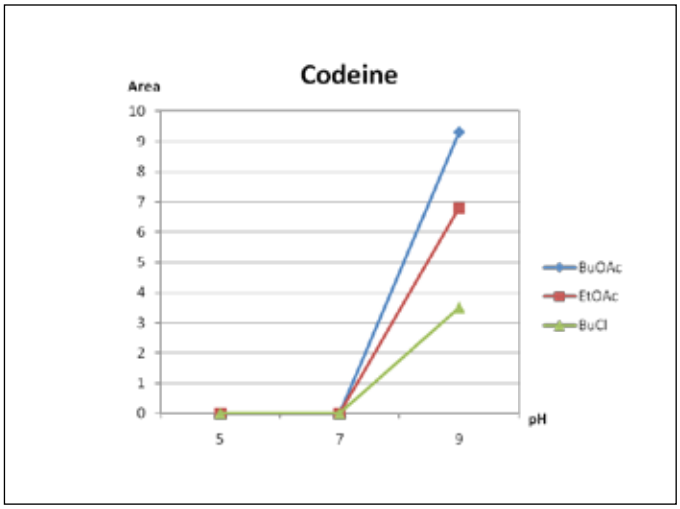


Figure 3 shows a simple and efficient analysis scheme for quantitative drug screening from 2 x 1 ml of blood, applying LLE followed by GC analysis (5). The following examples demonstrate the effect of pH and extraction solvent choice on recovery and background noise. A blood sample containing paracetamol, amitriptyline and codeine, with other drugs, is extracted at three different pH values with three different solvents. Figure 4 compares the extraction methods by peak area in GC-NPD. Paracetamol is a fairly hydrophilic compound with a pKa of 9.9 (phenol) and it exhibits a steady recovery through the pH range tested. Ethyl acetate is the most polar of the solvents, giving also the highest recoveries for paracetamol. Codeine is a tertiary aliphatic amine with a pKa of 8.2 and requires a basic pH to be extracted in its neutral form. Amitriptyline is also a tertiary amine with a pKa of 9.2, but due to its very lipophilic chacter it can be extracted even at pH 7 with a reasonable recovery (6). Dual-column GC-NPD chromatograms of the pH 9 extracts are shown for ethyl acetate (Figure 5) and butyl acetate (Figure 6). The background noise is somewhat higher with ethyl acetate than with butyl acetate, as expected.

Figure 4.

Comparison of LLE methods using three different solvents at three different pH values by peak area in GC-NPD.





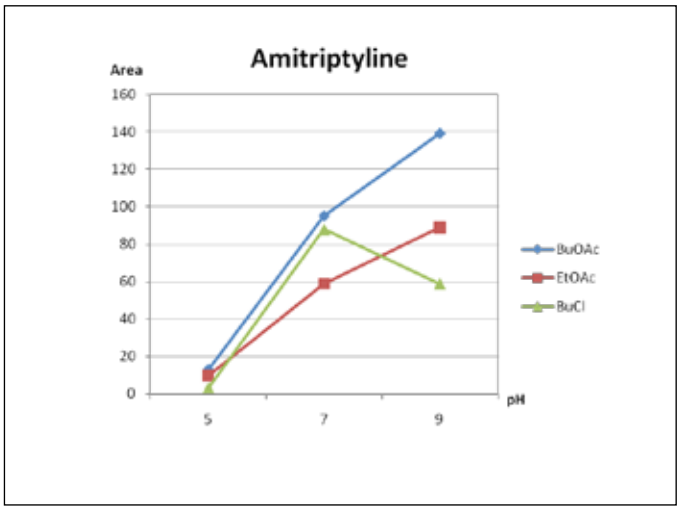


Figure 5.

Dual-column chromatogram of a blood sample extracted with ethyl acetate at pH 9. Findings in blood: paracetamol 57 mg/l (1), caffeine 2 mg/l (2), amitriptyline 2.4 mg/l (3), nortriptyline 0.3 mg/l (4), codeine 0.27 mg/l (5), dibenzepine internal standard 1 mg/l (6), zopiclone (7).

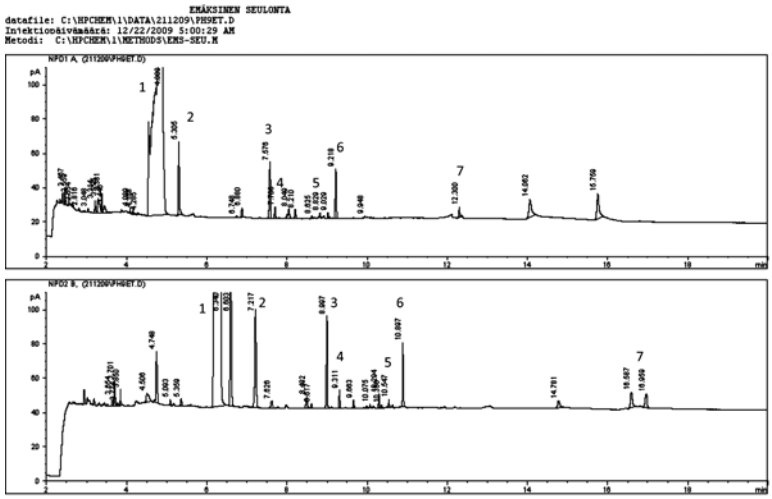
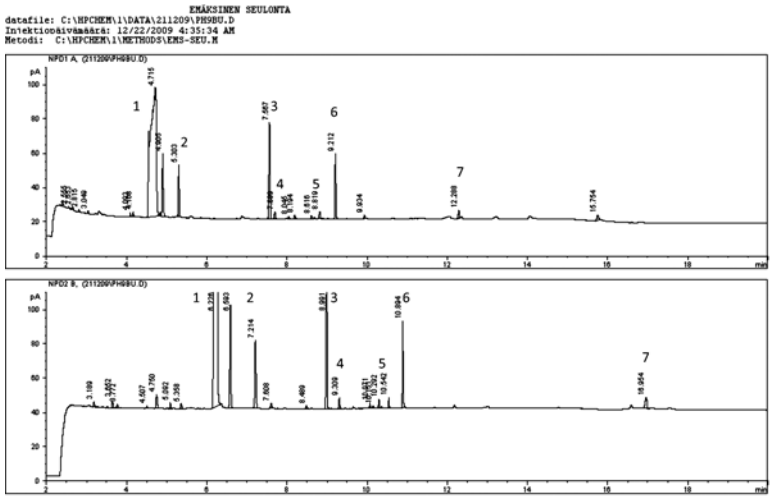


Figure 6.

Dual-column chromatogram of a blood sample extracted with butyl acetate at pH 9. Findings as in Figure 5.



Protein precipitation

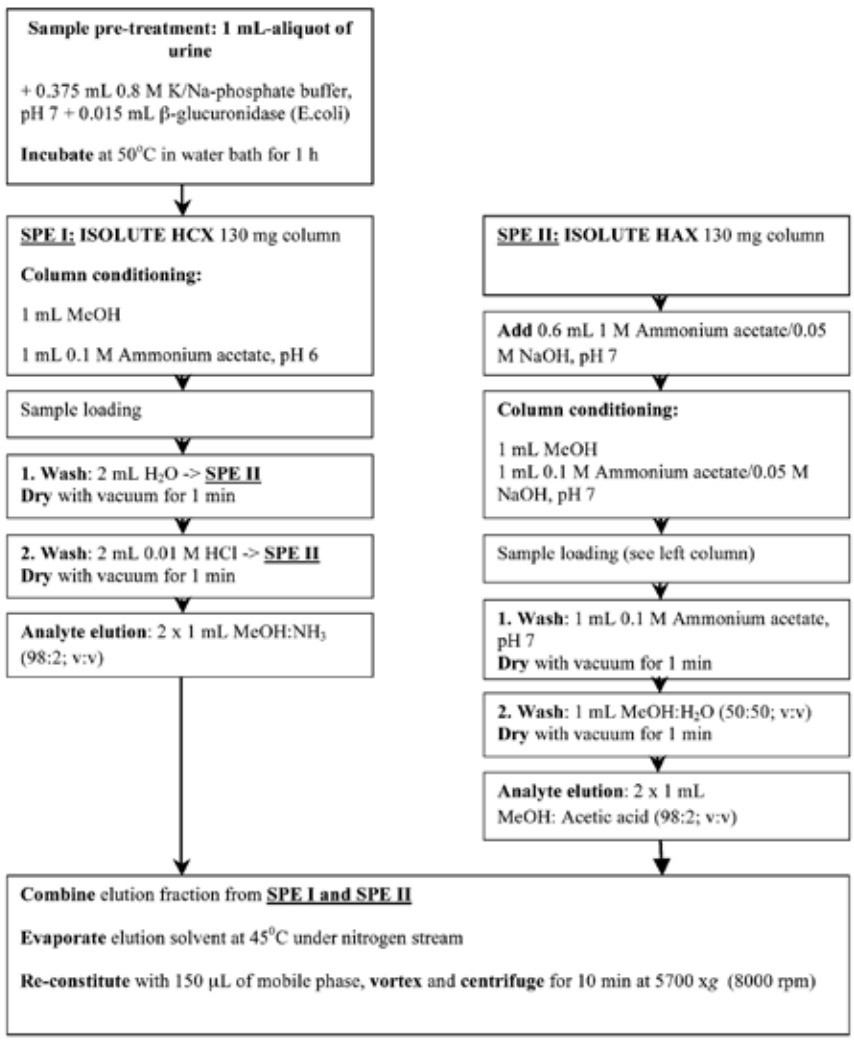
For polar compounds, LLE does not necessarily give satisfactory extraction recoveries from blood or tissues (7). Sample preparation by protein precipitation will then be the method of choice, provided that the drug concentration is sufficiently high for the analytical technique used. Acetone, acetonitrile, methanol and trichloroacetic acid are widely used reagents for protein precipitation. The choice of reagent depends on the analytical technique and if the supernatant is injected directly or after evaporation.

Solid-phase extraction (SPE)

SPE is also an established sample preparation technique for drugs. However, it was not perfectly suitable for comprehensive drug screening until the mixed-mode sorbents became available, such as the cation exchange/C8 phase. Normal phase or reverse phase sorbents alone could not encompass a sufficiently wide range of analytes. The key factors to be optimized are the type of sorbent, type of clean-up, type of elution solvent, pH, phase volumes and flow rate. Important factors include also sorbent equilibriation and possible sorbent drying. Consequently, there are numerous combinations to be tested during the method development, and this can make implementation slow. A continuous follow-up of the emerging proprietary sorbent materials is necessary if one wishes to stay up-to-date with the products.

Figure 7.

Generic SPE sample preparation method for comprehensive LC-TOFMS urine screening in doping analysis (8).



Due to higher complexity of SPE, the risk for irreproducible results is higher than with using LLE. SPE has a high concentrating capacity if large sample volumes are used, such as in environmental water analysis. SPE is amenable to automation, and it works best with large numbers of samples. The technique allows direct application of serum, plasma and urine, but whole blood must be diluted, sonicated and centrifuged prior to application. Samples containing solid particles easily clog the SPE column.

Figure 7 shows a generic SPE sample preparation method for comprehensive LC-TOFMS urine screening in doping analysis. Using two mixed-mode SPE methods, a very broad range of compounds with different polarities and acid-base behaviours can be extracted from a single 1 ml urine sample (8). ●

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Organometals and sample preparation in environmental samples

Panu Rantakokko | Riikka Airaksinen | Jari Kaikkonen | Hannu Kiviranta

Organometal compounds and environmental health

Organometal compounds are a heterogenous group of compounds containing at least one chemical bond between carbon and a metal. The metals, that typically form alkylated species and that are relevant from an environmental health perspective, are mercury, lead, tin and to a lesser extent, arsenic and antimony (1). Since organometal compounds are often more lipophilic and bind more readily to biomolecules than their respective elements, they are also more toxic and bioaccumulative (2). The main sources of organometal compounds into the environment are anthropogenic, e.g. coal combustion and various industrial processes, but natural formation also occurs through volcanic and bacterial processes (3). This chapter will focus on two organometals, methylmercury and organotin compounds, that are the most significant from an environmental health perspective and that we are familiar with in our laboratory.

Several unfortunate industrial accidents and disasters have made it possible to investigate acute high-dose effects of organometals on human health. Methylmercury and organotin compounds are known to be particularly toxic to the central nervous system (4). For this reason, human exposure has been restricted by setting maximum intake values for both of these compounds (5, 6). It is still uncertain whether average

lifetime exposure to these compounds has adverse health effects at the population level. However, there is an increasing body of evidence showing adverse health effects among fetuses and children exposed to moderate doses (7–9). Furthermore, well-characterized prospective studies are needed to investigate the human health effects of organometals at a population level.

It is important to develop and maintain robust, accurate and sensitive methods for the analysis of organometal compounds from both environmental and biological samples. The high variation in structural and chemical properties of organometals poses a challenge for their analysis, especially for their simultaneous determination.

Methylmercury

The toxic properties of mercury have been well known for centuries. Along with the industrial revolution of the last century, several tragic poisoning episodes related to accidental emissions revealed mercury to be one of the most hazardous environmental contaminants. After decades of emissions from fossil fuel combustion and various industrial processes, mercury has become ubiquitous in the biosphere.

The global annual emissions of mercury reached 5000 tons in 2000 (10), approximately half of which was of anthropogenic origin. The main anthropogenic sources of mercury are coal-fired power plants, waste incineration, chlor-alkali plants and gold production, while natural emissions are mostly related to volcanic activity. Over the past few centuries, mercury emission from anthropogenic sources has increased between 2 to 5 fold, and approximately two-thirds of the mercury currently present in the environment is of anthropogenic origin (11).

Mercury is emitted into the atmosphere in its volatile and stable elemental form (Hg^0) (12). Due to its non-reactive nature, it has a relatively long atmospheric residence time, enabling long-range transport into areas far from point sources. Elemental mercury is oxidized photochemically in the presence of reactive halogen species (13) into a water-soluble, reactive and somewhat toxic Hg^{2+} ion, which is quickly absorbed in rain droplets, snow, or small particles. Through wet and dry deposition, mercury is accumulated into soils and sediments, where microbes such as sulphate- (14) or iron-reducing (15) bacteria mediate the biological methylation of Hg^{2+} into highly toxic methylmercury (CH_3Hg). Methylmercury has high lipid solubility and high affinity towards the sulfhydryl (-SH) groups of amino acids. Therefore, it is readily absorbed by organisms, difficult to eliminate and effectively biomagnified, especially in aquatic ecosystems (16).

Food, and especially large predatory fish at the top of the aquatic food chain, are the main source of methylmercury for humans. Methylmercury is absorbed through the gastrointestinal tract into the bloodstream almost completely (17), and distributed to all tissues. Methylmercury is readily complexed with sulphur-containing amino acids, especially cysteine, and actively transported across cell membranes, the blood-brain barrier and the placenta via amino acid carriers (18). Methylmercury is accumulated into hair, brain and foetus. The blood to hair ratio of methylmercury is approximately 1:250 (5) and the cord blood:maternal blood ratio is 1:1.7 (19). In blood, most of the methylmercury is located in the red blood cells. The elimination of methylmercury oc-

curs mainly via the fecal route, and it undergoes enterohepatic cycling. The biological half-life of methylmercury in the human body is 39–70 days (20).

When considering low, chronic exposure to methylmercury, foetuses, infants, and young children are the most vulnerable groups. In epidemiological studies, fetal exposure to moderate levels of methylmercury has been associated with impaired neurological development (7–9). In an attempt to define the level of exposure without recognized adverse effects during a lifetime, the U.S. Environmental Protection Agency has set a reference dose (RfD) of 0.1 μg/kg body weight (bw) day^{-1} (5), corresponding to a level of 3.5 μg/l methylmercury in mother's blood (19). In addition to the neurotoxic effects, high exposure to mercury has been associated with adverse cardiovascular effects in adults (21).

Organotin compounds

Organotin compounds (OTCs) are a large class of compounds with widely varying properties, and they have been used for many different purposes. Mono- and di-substituted compounds, e.g. monobutyltin (MBT), dibutyltin (DBT), mono-n-octyltin (MOT), and di-n-octyltin (DOT), are used extensively as heat and light stabilizers in the production of polyvinyl chloride (PVC) polymers and as catalysts in the manufacture of polyurethane and silicone elastomers. From these products, OTCs can gradually leak into the environment as a result of degradation and flushing. Trisubstituted OTCs, tributyltin (TBT), and triphenyltin (TPhT) have a wide range of uses, mostly associated with their strong biocidal activity towards aquatic organisms, such as bacteria, fungi, algae, molluscs, and crustaceans. From an environmental point of view, most attention has been given to the widespread TBT and TPhT pollution of waters, sediments, and aquatic biota, resulting from their use in antifouling paints in boats and ships. TBT and TPhT are highly toxic to many aquatic species, and the most sensitive endocrine effect, imposex, occurs in some molluscs at TBT concentrations as low as 1 ng/L (22, 23). Due to their toxic effects, the use of organotin compounds in antifouling paints has been banned in the European Union since the beginning of 2008. Additionally, TBT is on the list of priority substances in the field of water policy (24) and Environmental Quality Standards (EQS) in surface waters of 0.2 ng/L for the annual average and 1.5 ng/L for maximum allowable concentration, respectively, have been proposed (25). A scientific panel of the European Food Safety Authority (EFSA) has assessed the consumer health risks associated with exposure to OTCs in foodstuffs. The most critical toxicological endpoint in their risk assessment was immunotoxicity. Due to immunotoxic similarities, a Tolerable Daily Intake (TDI) of 250 ng/kg body weight was established for the sum of TBT, dibutyltin (DBT), TPhT, and dioctyltin (DOT) (6). For humans, fish is the main source of OTCs (26, 27).

General aspects of sample preparation

The chemical characteristics of organometallic compounds vary widely, depending on the metal and the number and type of the organic ligand. For example, the water solubility of the compounds ranges from being readily soluble (e.g. monomethyltin,

charge +3) to highly insoluble (e.g. tetrabutyltin, neutral). Additionally, their affinity to environmental matrices may vary substantially even within compounds of the same metal. Furthermore, due to the distinct characteristics of organometals, obtaining good recoveries and high accuracy when a large number of organometal compounds need to be analyzed simultaneously is a very demanding task. One example of the problematic characteristics is the high affinity of organomercurials to sulphur-containing groups in proteins in biological samples. For this reason, compounds of a particular metal are usually analyzed separately from the other metals, although a few methods for simultaneous analysis of organotin, organomercury and organolead compounds have also been developed (28, 29).

Quantification

The stability of organometallic compounds during the storage and preparation of samples is an important issue. Irreversible absorption of compounds to container walls (30) and redistribution of organic ligands (31) may take place during the storage of both real samples and model compounds. This underlines the importance of correct storage conditions. In addition, in order to achieve good extraction efficiency, sediment and biological matrices may require harsh extraction conditions, which may result in partial degradation, loss, and even artificial formation of organometallic compounds. Artificial formation has been shown to be of particular importance in the determination of methylmercury in sediments and water samples rich in organic matter, where methylmercury may be formed from inorganic mercury if high temperatures are applied during sample preparation (32, 33). Ideally, an internal standard, added before the sample preparation, will correct the results for these events.

Structurally similar organometals as internal standards

For a given organometal compound, a differently substituted compound of the same metal is often used as the internal standard. For example, tripropyl tin has been used as an internal standard in the analysis of nine alkylated and phenylated organotin compounds from water, sediment and tissue samples (34), and ethylmercury has been used in the analysis of methylmercury from mussel samples (35). The advantage of this approach is that simple and cheap, non-mass spectrometric detectors can be used after separation, typically gas chromatography. However, even small differences in the structures of organometals can often evoke marked variations in their behaviour during sample treatment, increasing the number of structurally similar compounds required as internal standards.

Organometals with isotopically labelled ligands as internal standards

Another, less common approach is to use analogues with isotopically labelled (most often deuterated) ligands for each organometal compound to be analysed. This approach accurately corrects for the uncertainty arising from the differences in chemical structure and the properties of sample matrix. It also corrects for the redistribution of ligands, which occurs both for native and labelled compounds. One of the major benefits of this approach is that the calculation of results is very simple, based only on the use of relative response factors of each native and labelled compound from calibration standards and samples. Increasing the number of compounds in the same analysis does not make the

calculation of results any more complicated. The major drawback is that one cannot account for the possible artificial formation of organometals during sample treatment. Some applications using isotopically labelled compounds have been published e.g. for organic tin compounds from different matrices with good results (26, 27, 36, 37).

Organometals with isotopically enriched elements as internal standards

In the speciated isotope dilution (SID) approach, an isotopically enriched trace metal is used instead of deuterated or ^{13}C-labelled ligands. This opens up new possibilities for quantification, as different isotopes of the metal can be used for labelling different organometal compounds of the same element. The addition of the enriched solution of organometals (spiking solution) to the sample is the critical stage, and accurate quantification requires that full equilibrium between the native analyte and the enriched spiked compound is reached at the beginning of the analytical procedure. However, once the isotopic equilibration has taken place, any loss during the subsequent steps will not affect the final results, because the final analytical information is calculated from the elemental isotope ratios exhibited by the native and enriched species rather than from the absolute amount of these species. This enables non-quantitative, separative reactions to be used during sample preparation and renders unnecessary the complete isolation of the species under investigation. With regard to species detection, matrix effects and detector instabilities are totally compensated for, since all isotopic forms of the same organometal would suffer from these effects in an identical way. Consequently, the SID approach provides extremely accurate and precise trace element speciation data. In order to evaluate ligand redistribution, a spiking solution containing all of the species under evaluation may be used, isotopically enriched with different isotopes. In this way, any interconversion reactions taking place will lead to changes in the elemental isotopic composition of the individual organometal (38). Due to all of the aforementioned advantages, the SID approach is "the golden standard" in the analysis of organometals.

The main drawback of the method is that when the number of compounds increases, the equations used in the calculations become increasingly complicated, in order to cover all possible interconversion pathways. This is exemplified by the analysis of butyltin compounds with triple spike methodology (39). In order to simplify the equations, some unlikely pathways are preferably ignored. Another downside of the method is that due to the limited commercial availability of some of the isotopically enriched compounds, the compounds of interest often need to be synthetised in-house from those elements that are available. For these reasons, multiple spiking has not been adopted widely by routine laboratories, despite its indisputable benefits.

Extraction

Due to the structural and behavioural variation among organometal compounds, quantitative release of all compounds of interest from solid sample matrices to the liquid phase is challenging. Thus, a large number of different extraction techniques have been developed and compared even for a same type of sample matrix (40–42).

Typically, biological samples are first liquefied by addition of a base, e.g. KOH (43) or tetramethylammoniumhydroxide (34). The extraction conditions for biological samples

and particularly sediment samples are most often acidic, using HCl (44), HNO_3 (33) or acetic acid (34). The physical means used in the extraction include simple mechanical shaking, ultrasound extraction, microwave assisted extraction (45) and pressurised solvent extraction (46). With regard to methylmercury analysis from certain samples, alkaline and acidic leaching as well as atmospheric pressure distillation can lead to the artificial formation of methylmercury, which can only be adequately accounted for by SID (32). With regard to the organotin compounds, microwave assisted extraction can result in significant debutylation of butyltins (45).

After the release of compounds from the sample matrix, the actual extraction is typically performed with an organic solvent, followed by the necessary cleanup steps and instrumental analysis. Many organometallic compounds are very non-polar and readily volatile after derivatization. Therefore, headspace extraction from the gas phase e.g. using either liquid-phase microextraction (47) or solid phase microextraction (SPME) are commonly used in the analysis of organotin compounds (48–50) and methylmercury in particular (43, 51, 52).

Derivatization

Since most organometallic compounds are charged, their derivatization to neutral volatile species is a necessary step prior to instrumental analysis. Derivatization may be performed by hydride generation, alkylation with Grignard reagents or alkylation/phenylation with sodium tetra alkyl/phenylborates.

Alkylation by the Grignard reaction was previously the most widely used derivatization technique for many organometals, and many reagents with varying lengths of the alkyl chain are commercially available. The actual derivatization is performed in organic extracts, and complete removal of water is required before the reaction. The excess of Grignard reagent is destroyed by a quenching solution, which may be acidic (sulfuric or hydrochloric) or saline (ammonium chloride). In order to avoid violent reactions, it is advisable to add a few drops of water and shake the sample gently before the addition of the quenching solution. Finally, the derivatives are back-extracted into an organic solvent and purifed prior to GC determination. The use of Grignard reagent requires careful control of reaction conditions, but has proven feasible (53).

Volatile organometal hydrides are formed during the reaction of organometal compounds and an aqueous solution of sodium borohydride ($NaBH_4$). The concentration of the reductant solution, pH, and the types of acid used must be selected according to the element considered and the nature of the matrix. In the case of aqueous matrices, the hydride generation method is easy to apply, and allows high preconcentration factors, and the separation of the analytes of interest from potential matrix interferences as well as high hydridization yields. This is a major benefit of hydride generation as compared to the Grignard reaction. However, hydride generation is also very sensitive to the reaction conditions, and reaction yields and volatility are poor for some compounds. Additionally, the presence of humic substances may cause problems with reproducibility, due to foam formation during the stripping step prior to the addition of $NaBH_4$ solution. These problems with foam formation have also been observed with biota samples containing high amounts of fat (53).

Due to the problems related with the derivatization methods described above, sodium boroalkyls and -phenyls have been developed as alternative derivatization agents. The major advantage of these agents is that they enable direct derivatization in the aqueous phase. For example, sodium tetrapropylborate is suitable for the determination of tin, mercury and lead compounds in water samples (54, 55). Some early studies using sodium tetraethylborate obtained unsatisfactory results with biological and sediment samples, because of matrix effects, and the Grignard reaction was favoured instead (56, 57). Nowadays, the reaction conditions for many different extraction methods and matrices have been optimised, and sodium tetraalkylborates have almost completely replaced the use of Grignard reagents and sodium tetraborohydride. However, there are cases where severe analytical obstacles are present. For example, with regards to organotin analysis, derivatization of sediments rich in inorganic and organic sulphur compounds results in the formation of volatile sulphur compounds that co-elute with the alkylated organotins. Different ways to overcome these obstacles have been proposed, such as cleanup using silver nitrate -coated silica gel as an adsorbent (58), application of solid phase microextraction (49) and optimisation of pressurised liquid extraction (59). Additionally, natural organic matter and certain metal ions (Ag^{+}, Cd^{2+}, Cu^{2+} and Pb^{2+}) have been found to interfere with the determination of butyltins in environmental samples, but a masking reagent has been developed to overcome these interferences (60).

Separation and detection

Gas chromatography (GC) is the method of choice for the separation of organometals, due to its high separation power, wide range of connectable detectors, and in most cases, good sensitivity. However, there are a few applications using liquid chromatography, e.g. for organotin compounds (61–63).

In the GC analysis of organometals, different element selective detectors, such as atomic emission detector (AED) (55, 64), atomic absorption detector (AAS) (65), atomic fluorescence spectrometry (AFS) (66), flame photometric detection (FPD) (48) and pulsed flame photometric detection (PFPD) (49) have been used. These detectors are relatively cheap to purchase and are in routine use in many laboratories. Furthermore, modified versions of these detectors, such as cold vapour AFS (CVAFS) for mercury analysis (67), have shown excellent sensitivity. The main drawback of these techniques is that they are not able to differentiate between different isotopes and the use of metals with isotopically labelled organic ligands or enriched elements as internal standards is not possible. Therefore, these detectors have to rely on structurally similar organometals as internal standards, with all of the limitations mentioned above.

During the last decade, the use of inductively coupled plasma mass spectrometry (ICP-MS) has become popular in the analysis of certain organometals, e.g. mercury (33, 54, 68, 69) and tin (39, 45, 46). The costs of purchase and operation of ICP-MS instruments are relatively high, but the high sensitivity and specificity of the method and the ability to distinguish between isotopes of different metals enables highly accurate speciated isotope dilution (SID) analysis and means that ICP-MS is the best technique available. However, the main limitation of ICP-MS and other plasma techniques is inability to measure molecular ions. Therefore, the use ICP-MS is limited to the analysis

of metals, and isotopically labelled organic ligands cannot be used as internal standards.

The cost of ICP-MS-based methods may be high for routine testing laboratories, and only a few GC-ICP-MS interfaces are commercially available from ICP-MS manufacturers. Since GC-MS instruments with electron impact ionisation are cheaper and widespread workhorses used for analysis of a large variety of organic compounds, SID GC-MS could also be regarded as an attractive alternative to SID GC-ICP-MS. Conventional MS (36, 37) and high resolution MS (HRMS) (26, 27) with deuterated internal standards have been applied in the determination of organic tin compounds from different matrices. Although quite rare, MS detectors have also been successfully used in the SID-analysis of organotins (28, 70) and methylmercury (43, 44). However, the spectra generated by GC–MS is complicated due to ^{13}C and 2 D contributions from organic ligands. These contributions make SID equations even more complex for MS than for ICP-MS. This may be one of the reasons why SID has rarely been linked to GC-MS despite its advantages in terms of price. ●

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Language revision

This book is dedicated to celebrate the 60th birthday of Professor Rainer Huopalahti. He has had, and in fact is still enjoying a distinguished career in the analysis of food and food related flavor compounds. One will find it hard to make any progress in this particular field without a valid and innovative sample handling technique. This is a field in which Professor Huopalahti has made great contributions. The title and the front cover of this book honors Professor Huopahti's early steps in science. His PhD thesis which was published on 1985 is entitled "Composition and content of aroma compounds in the dill herb, Anethum graveolens L., affected by different factors". At that time, the thesis introduced new technology being applied to sample handling and analysis of flavoring compounds of dill.

The contributors of the book are leading Finnish scientists on the field of organic instrumental analytical chemistry. Some of them are also Repe's personal friends and former students from the University of Turku, Department of Biochemistry and Food Chemistry.

The editorial team thanks all the authors for their willingness to contribute to this book – and to adhere to the very strict schedule. We also want to thank the various individuals and enterprises who financially supported the book project. Without that support, it would not have been possible to publish the book.

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