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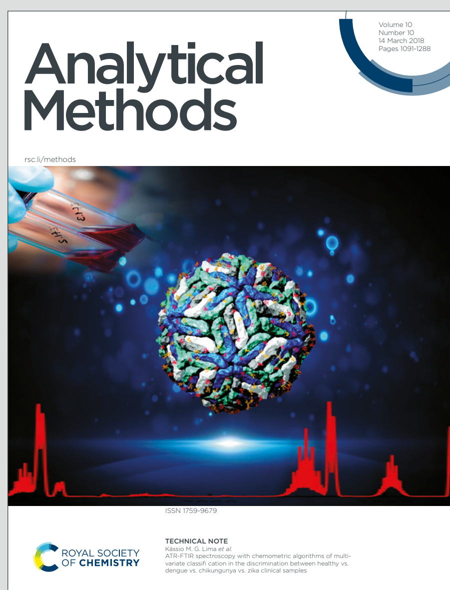

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1 **Sensitive lanthanide label array method for rapid fingerprint analysis of plant polyphenols**
2 **based on time-resolved luminescence**

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26 **Abstract**

27 Polyphenols are the most commonly available secondary metabolites with diverse bioactivities that
28 vary with their chemical composition. Herein, fingerprint analysis of plant polyphenol composition
29 is very crucial to reveal overall bioactivities. In our current investigation, a generic and simplified
30 method based on the time-resolved luminometric lanthanide label array technology has been
31 developed for the rapid fingerprint analysis of plant polyphenols. This method works on the
32 detection of the luminescence signal profiles specific to polyphenol compositions resulting from the
33 nonspecific interaction of long lifetime unstable lanthanide chelates with polyphenols of plant
34 samples. It is much simpler and cost-effective method in comparison to the many existing methods
35 of polyphenol fingerprint analysis. This method allowed us to distinguish plant polyphenols based
36 on their quenching effects on the luminescence in a time-dependent manner. Different samples
37 provided different signal profiles based on their inherent polyphenol compositions. Principal
38 component analysis (PCA) clearly clustered and distinguished oligomeric hydrolyzable tannins
39 (HTs) containing samples from the monomeric HTs containing ones. UHPLC-DAD-ESI-MS
40 identification and quantification of the plant polyphenols was used as a reference for the validation
41 of the method. Quantification results significantly correlated with the clustering of plant samples
42 based on their polyphenol composition.

43 **Keywords**

44 Time resolved fluorometry, time-delayed, chemiluminescence, europium, lanthanide chelates,
45 hydrolyzable tannins, ellagitannins, UHPLC.

46 **Introduction**

47 Plants produce a wide variety of secondary metabolites with numerous bioactivities, among them,
48 polyphenols are the most commonly distributed with potential antiherbivore, anthelmintic, and
49 other health-promoting activities.[1-4] Hydrolyzable tannins (HTs) are one of the complex class of

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polyphenols with a wide variation of structures starting from simple monomers to large oligomers, even up to undecamer. HTs are classified into three major classes, such as simple gallic acid derivatives, gallotannins (GTs) and ellagitannins (ETs). Gallic acid derivatives and gallotannins are rarely found in nature in comparison to the abundance of ETs. Gallic acid derivatives contain five or less galloyl groups, while GTs contain six or more galloyl groups. The diverse ETs, around 600 so far isolated and identified, can be further classified into several classes based on their structural units and linkage between units, such as, dehydro ET, modified dehydro ET, simple HHDP esters, gallagyl ester, *C*-glycosidic ET, *m*-DOG-oligomeric ET, *m*-GOG-oligomeric ET, *m*-DOG-oligomeric macrocyclic ET, *m*-GOD-oligomeric ET, glucopyranose *C*-glycosidic *m*-DOG-oligomeric ET, and *C*-glycosidic m-DOG-oligomeric ET.[5] Oligomers are simply produced by adding two or more simple monomeric units with different types of linkage (**Figure 1**). Typically, HTs are composed of hexahydroxydiphenic acid (HHDP) or nonahydroxyterphenoyl (NHTP) unit or both attached to the sugar moiety. Oligomerization usually occurs via *C-O* oxidative coupling, while *C-C* oxidative coupling only takes place in *C*-glycosidic ellagitannins, such as vescalagin, castalagin, etc.[6]

Polyphenolic fingerprint analysis is very beneficial to reveal the overall polyphenol composition of plant samples which can facilitate the process of screening active and inactive plant species, ultimately leading to a better understanding of bioactivities of polyphenols. To date, fingerprint analysis is being carried out by UHPLC-DAD-MS/MS, which requires expensive instrumentation, maintenance, pure polyphenol standards, and technical skills for interpreting results. However, many efforts have been made so far to develop a simple and sensitive method to reveal HTs composition in large sample sets in the high-throughput fashion. [1,7-9]

To address this issue, we have developed an alternative fingerprinting tool for plant polyphenols, which will allow us to analyze a large set of samples in a more simplified way. It is a generic and sensitive method utilizing the nonspecific interaction between unstable lanthanide chelates and

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chemistry of plant samples. This interaction has a detrimental effect on the luminescence of the lanthanide chelates in a time-dependent manner, which ultimately provides the basis of separation between different samples, because the luminescence signal levels are unique to specific chemical profiles and their ability to quench the signals. However, lanthanide chelates based time-resolved luminescence assay has been utilized successfully in wide variety of areas, mostly but not limited in diagnostics, drug discovery, detection of metals, adulteration, microbes, and biomarkers discovery, etc.[10-13] Earlier, label array methods have been developed by utilizing unstable lanthanide chelates for the detection of adulteration in honey and cacao brands, and metal ion detection and quantification in drinking water.[10,13] Though specific method provides more promising results in detection, but it requires much more optimization, and finally, narrow down the application area because of the specificity. Therefore, in our study, we have developed a nonspecific method which will be applicable to wide application areas, and still giving satisfactory results to solve our key questions without targeting any specific molecules. In the developed lanthanide label array method, we have utilized unstable lanthanide chelates, different modulators with a wide variety of chemistries, and time-resolved fluorescence (TRF) to reveal the holistic picture of HTs composition in plant samples.

The lanthanide label array method was designed on microtiter well plates in high throughput format, where plant samples were introduced to mixtures of unstable lanthanide chelates and modulating ligands. Typically, lanthanide chelates are surrounded by the modulating ligands through coordinate covalent bonds which known as antenna effect, and ensure efficient light absorption, energy transfer, and relatively intense luminescence signals from the chelates, otherwise, the chelates absorb energy poorly in the absence of ligands.[14] The fundamental mechanism of energy transfer and generation of optimum luminescence has been illustrated in **Figure 2**. Moreover, unstable lanthanide chelates are simple in structures, and easily available or can be synthesized easily in comparison to the stable chelates which are costly and often have less

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100 sensitivity to chemistries.[10] Ordinarily the lanthanide chelates have narrow excitation range of 300-
101 340 nm, but unstable chelates have the much wider excitation band of 300-470 nm which extends
102 towards the UV-VIS region with similar emission profile.[10] In addition, the combination of
103 lanthanide chelates and modulating ligands provides the possibility of introducing a large number of
104 chemistries in the experimental development. Coordination of appropriate ligands can enhance the
105 fluorescence dramatically and many essential properties like excitation wavelength and emission
106 lifetime, quantum yield and molar extinction coefficient are highly dependent on the structure of
107 ligands.[15-18]

108 Besides that, lanthanide chelates have some unique properties which give advantages in the
109 development of assays in comparison to the conventional luminescence assays. It is very prevalent
110 in conventional assays to notice messy background because of the sample auto-fluorescence, which
111 ultimately distorts the vital signals from the samples resulting unsuccessful methods. In this
112 scenario, luminescent lanthanide complexes have exceedingly long-lived luminescence,
113 empowering the removal of short-lived background interferences using the time-gated acquisition
114 of signals which ensures better sensitivity and wider dynamic range.

115 Recent advances, wide applications, and unique properties of time-resolved lanthanide label array
116 assays inspired us to develop such methodology in polyphenol research to enable rapid fingerprint
117 analysis and separation of plant samples based on their HTs composition. Therefore, in our current
118 study, we have developed very rapid, sensitive, relatively simple, and cost-effective lanthanide label
119 array method for rapid fingerprint analysis of plant polyphenols in a high-throughput setup. The
120 method also enabled us to distinguish between monomer and oligomer HTs containing samples.
121 This method was not specific to a compound but provided a simplified way of separation based on
122 their chemistries in a holistic way.

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Experimental

Plant samples

Total 12 plant samples (**Table 1**) were collected from the Botanical Garden of the University of Turku and nearby forests, except *Terminalia chebula* retz., which was obtained from the commercial source (Banyan Botanicals, USA). All the samples were properly identified with the herbarium. Samples were HTs producing covering different classes of HTs from simple monomers to large oligomers. After the collection, plant samples were freeze-dried, grounded in powder, and finally stored in the freezer for the further process.

Chemicals and reagents

The lanthanide chelates, europium (III) chloride ($EuCl_3$) and terbium (III) chloride ($TbCl_3$) were from Alfa Aesar (Ward Hill, MA) and Sigma-Aldrich (St. Louis, MO), respectively. NTA (4,4,4-trifluoro-1-(2-naphthyl)-1,3-butanedione) was procured from Acros Organics (Geel, Belgium). TOPO (trioctylphosphine oxide), TTA (2-thenoyltrifluoroacetone), Michler's ketone, and Triton X-100 were from Sigma-Aldrich (St. Louis, MO, USA). TPPO (Triphenylphosphine oxide) and Phen (1,10-phenanthroline monohydrate) were from Fluka, Sigma-Aldrich (St. Louis, MO, USA). DMSO (dimethyl sulfoxide) and microtiter plates were obtained from Thermo Scientific (Waltham, MA).

Extraction of plant samples

Finely ground dried plant material of 20 mg of each plant samples was macerated in 1400 μL of extraction solvent, acetone/water (80/20, V/V) through proper mixing by the vortex mixer. Maceration was carried out overnight in cold room at 4 °C. Samples were then shaken with a planetary shaker for 3 hours at speed of 280/min. Insoluble plant debris was separated from the clear supernatant by centrifugation for 10 minutes at 14000 rpm. Second successive extraction was conducted by adding 1400 μL of extraction solvent to the debris and followed by shaking for 3

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148 hours, centrifugation and separation of the clear supernatant. Clear supernatant from the two
149 successive extractions was combined, concentrated under reduced pressure to remove acetone, and
150 then freeze-dried for storage in the freezer.

151 **Preparation of plant samples**

152 Freeze-dried sample was dissolved in ultrapure Milli-Q water to achieve the concentration of 0.02
153 gL^{-1}. The solution was then filtered using 0.2 μm PTFE filter to remove insoluble debris and
154 lipophilic materials. The experiment was conducted on the same day as the sample preparation to
155 avoid probable precipitation in the refrigeration.

156 **Preparation of modulators**

157 Modulators were prepared by adding the lanthanide chelates to the ligands. At first, different
158 solutions with different chemicals were prepared and added to each other to make final chelate
159 solutions or modulators. The ratio of the chemicals was modified many times during the
160 development phase to obtain optimum emission signals and efficient separation of samples.
161 Composition of modulators was changed keeping the sample concentration same. Each modulator
162 was the combination of chemicals to introduce unique chemistry. Total 8 different modulators, 1 to
163 8 were formulated using different chemical solutions prepared with mixing different chemicals at a
164 certain ratio. Initially, 11 different chemical solutions (A to K) were formulated by mixing different
165 chemicals. Chemical A was formulated by mixing 10 µl 0.2 M $Eucl_3$, 90 µl 0.6 M NTA and 90 µl
166 0.6 M TOPO in 810 µl DMSO. Chemical B was formulated by adding 100 µl chemical A to 2900
167 µl DMSO. Chemical C was formulated by adding 20 µl chemical A to 1980 µl DMSO. Chemical
168 D was formulated by mixing 10 µl 0.2 M $Eucl_3$, 90 µl 0.6 M TTA and 90 µl 0.24 M TPPO in 810 µl
169 DMSO. Chemical E was formulated by adding 500 µl chemical D to 1000 µl DMSO. Chemical F
170 was formulated by mixing 10 µl 0.2 M $Eucl_3$, 10 µl 0.6 M NTA and 30 µl 0.2 M Michler's Ketone
171 in 950 µl DMSO. Chemical G was formulated by adding 10 µl chemical F to 1660 µl DMSO.

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Chemical H was formulated by mixing 10 µl 0.2 M $Eucl_3$, 10 µl 0.6 M NTA and 10 µl 0.6 M Phen in 970 µl DMSO. Chemical I was formulated by adding 10 µl chemical H to 1660 µl DMSO. Chemical J was formulated by mixing 10 µl 0.2 M $Eucl_3$, 10 µl 0.6 M NTA and 25 µl 0.24 M TPPO in 955 µl DMSO. Chemical K was formulated by adding 10 µl chemical J to 1660 µl DMSO. Then modulator 1 was prepared by adding 10 µl chemical B to 3320 µl 0.1 M Na_2CO_3. Modulator 2 was prepared by adding 10 µl chemical B to 990 µl 0.1 M Glycine. Modulator 3 was prepared by adding 10 µl chemical C and 100 µl 250g/L Triton X-100 to 2920 µl Milli-Q H_2O. Modulator 4 was prepared by adding 1 µl chemical B to 1503 µl Milli-Q H_2O. Modulator 5 was prepared by adding 10 µl chemical E to 3320 µl Milli-Q H_2O. Modulator 6 was prepared by adding 100 µl chemical G to 233 µl Milli-Q H_2O. Modulator 7 was prepared by adding 100 µl chemical I to 900 µl Milli-Q H_2O. Modulator 8 was prepared by adding 100 µl chemical K to 900 µl Milli-Q H_2O.

Time-resolved luminescence assay

The modulator of 10 μL was added with the sample volume of 100 μL in microtiter wells. It was then shaken for 10 seconds to allow proper mixing of the chemicals. The time-resolved fluorescence signals were measured with the Perkin Elmer Wallac VICTOR plate reader. The filters were 340 nm excitation and 616 nm emission for europium (Eu) chelates, and the delay and window times were 400 and 400 μs, respectively. Delay time was used for the removal of background interferences. Each sample was analyzed in three separate experiments with three replicates in each experiment. Data recording was started at 3 min and continued until 36 minutes with 3 minutes interval.

UHPLC-DAD-QqQ-MS/MS analysis

Each sample was analyzed by UHPLC-DAD-QqQ-MS/MS for the quantification of total phenolics, monomeric HTs, oligomeric HTs, and total HTs. The system was Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a Xevo TQ triple-quadrupole mass spectrometer (Waters

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Corp.). The UPLC system had an automatic sample manager, a binary solvent manager, an analytical grade column, and a photodiode array detector (DAD). The column was 1.7 µm Acquity UPLC BEH Phenyl column (Waters Corp., Wexford, Ireland) with 100 mm × 2.1 mm i.d. The photodiode array detector was operated between 190-500 nm wavelength, but the quantification was done at λ=280 nm, which is suitable for most of the phenolic compounds. Two solvents were used, acetonitrile (A) and 0.1% aqueous formic acid (B) at the flow rate of 0.5 mL min^{-1} with the following elution events: 0−0.5 min, 0.1% A in B; 0.5−5.0 min, 0.1−30% A in B (linear gradient); 5.0−6.0 min, 30−35% A in B (linear gradient); 6.0−9.5 min, column wash and stabilization. UV–vis (190–500 nm) and MS data (*m/z* 150 to 1200) were recorded from 0 to 7 min. Electrospray ionization (ESI) was used in negative mode with the following ionization condition: capillary voltage, 2.4 kV; source temperature, 150 °C; desolvation temperature, 650 °C; desolvation and cone gas (N_2), a flow rate of 1000 and 100 Lh^{-1}, respectively; and collision gas, argon. To monitor the ionization efficiency and the performance of the system, catechin at 1 μgmL^{-1} was analyzed before and after the experiment. Different mixtures of flavonoids at 4 μgmL^{-1} was analyzed to monitor the variations in retention time and *m/z* values. Samples were dissolved in Milli-Q water and filtered with VWR® 0.2 µm PTFE filter to remove insoluble plant particles. The injection volume of the sample was 5 μL.

Statistical analysis

In all figures, data have been presented with error bar as standard error for mean (SEM) of replicates (n=3). Microsoft Excel and GraphPad Prism were used for data analysis and plotting the figures.

Results and discussion

Nonspecific lanthanide label array method was developed systematically in several phages for the rapid fingerprint analysis of plant polyphenols. The experiment was designed on microtiter well

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220 plate, in which plant samples were added to the unstable lanthanide chelates encircled by
221 modulating agents, and generated luminescence was recorded by well plate reader through time-
222 gated emission measurements to avoid the interference of background signals. Time-gating signal
223 measurement is possible because of the long-lived luminescence (μs to ms range) of the unstable
224 lanthanide chelates, and the rapid background signal usually decays at the early stage before the
225 actual measurement starts. In our study, we used 400 μs of delay time before the recording of
226 luminescence signals to avoid background interference. This unique property also increases the
227 sensitivity of the method by increasing the signal to background ratio, practically, eliminating high
228 background signal or autofluorescence of the sample matrix or blank solution. Moreover, lanthanide
229 chelates are free from the common problems of conventional fluorophores, such as narrow emission
230 bands, an overlap between excitation and emission spectra, and Stokes shifts.[14] There were two
231 filters in the well-plate reader, one was excitation filter to supply energy to the chelates by antenna
232 effect using modulating ligands, and another was emission filter to record the luminescence
233 generated from the shifting of chelates from high energy state to ground state. Antenna effect of the
234 ligand was desirable to enable high luminescence signal as we noticed low absorption of light in the
235 absence of the antenna ligands in earlier studies.[13]

236 In the development phase, a selection of europium (Eu) and terbium (Tb) chelates was used to
237 ensure variable luminescence fingerprints between plants samples with variable polyphenol
238 composition. Europium and terbium chelate were selected based on their readiness, known
239 properties, and wide applications in different areas. Furthermore, a wide variety of chemistries were
240 introduced to make different chelating complexes with wide varieties of modulating agents. Also,
241 the reaction kinetics were observed to find out the optimum time point for the separation of
242 samples. As we know sugars present as a major portion of the crude plant samples which warranted
243 to assess the effect of sugars separately. Sugars were separated from the plant samples by solid
244 phase extraction using Sep-Pak C18 cartridges (Waters Corporation, USA) to assess their

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quenching effects on the luminescence of lanthanide chelates. We analyzed separately both sugar and polyphenol fractions for all the plant samples using the same protocol. Interestingly, we did not observe any quenching effect of sugar fractions on the luminescence of the chelates, moreover, signal level was similar to control (Milli-Q water) and reaction kinetics were unchanged throughout the observation. Stability of modulating ligands and plant samples were also tested to see if same solution or sample can be used many days for the experiment. Modulating ligands are quite stable can be used at least two weeks after preparation without any compromise of signal intensity but plant samples are not stable to be used for later because of the possible precipitation while refrigeration. Besides that, after refrigeration plant samples need to be filtered again which removes the precipitate particles resulting in the distortion of the actual concentration of the samples. Modulating ligand solutions are stable at room temperature and no refrigeration is needed unless any enzyme is used as a ligand.

In the optimization phase, samples were tested in different concentrations to know the best concentration of the sample to get optimum signals as well satisfactory quenching effects on the luminescence of the chelates to separate samples from each other. There were many parameters to be modified to ensure optimum signal levels as well as subtle separation. Therefore, we fixed a suitable concentration of sample based on our preliminary experiments, in this case, it was 0.02 gL^{-1}, and later modified the composition of modulating agents to get optimum signal levels within the detection limit. This approach allowed us to use the same sample of uniform concentration for all the modulators with the low coefficient of variation, repeatability, high modulation, and optimum signal level. In the experimental phase, we have utilized the fine-tuned information gathered through the development and optimization phages. Finally, we decided to use Eu chelates which showed better separation and optimum luminescence signals in comparison to Tb chelates, and among the Eu chelates containing modulators, 8 of them showed significant signals, quenching effects, proper separation, and clustering of alike samples. With this experimental set up we did

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270 final experiments with all the model plant samples selected for the method development, and the
271 kinetics of the experiments were recorded to observe the progression of the reaction which in fact
272 also a means of separation. Method development approach has been discussed in detail in the
273 supplementary file.

274 **Time-resolved luminescence assay**

275 Plant samples showed quenching effect on the luminescence signal of the lanthanide chelates in a
276 time-dependent manner based on their chemical composition. With the progress of the reaction,
277 quenching effect was increased as the luminescence signal was decreasing. Average luminescence
278 signal levels with the standard error for mean (SEM) for all samples with all modulators of different
279 chemistries have been mentioned in **Figure 3(A)**. Data showed the significant repeatability in three
280 experiments for all samples with considerable SEM values. In **Figure 3(A)**, only data recorded at 3
281 minutes have been mentioned, but data showed significant repeatability at all the time points of the
282 reading. Quenching effect increased similarly with the progress of the reaction in all three separate
283 experiments, which ensures robustness, and repeatability of the method. For instance, in **Figure**
284 **3(B)**, average luminescence signals for all the samples for modulator 1 and 2 at 3 min have been
285 compared. This type of comparison or separation can be done with every modulator at the different
286 time points that we have optimized so far in our current study. Therefore, there are many ways to
287 separate them, such as average luminescence, reaction kinetics, and a combination of multiple assay
288 parameters in multivariate tools such as principal component analysis (PCA). PCA analysis gives
289 the privilege of incorporating multiple parameters and their associated data to separate and cluster
290 similar samples.

291

292 **Reaction kinetics**

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Reaction kinetics were observed for each sample throughout the experiment, starting from 3 min to 36 min to observe the dynamic range and plateau phase of the reaction. In **Figure 4A**, reaction kinetics for all the sample with modulator 1 have been illustrated. Similar reaction kinetics were observed for all other tested modulators. The reaction was faster at the initial phase, which is the typical dynamic range, but at the later stage, the progress of the reaction was slowed down as we noticed from the minimal change of the luminescence signal levels. Reaction kinetics data were further analyzed to find the reaction order through the linear regression analysis. Reaction was found to be following first order kinetics. At the beginning from 3 min to 18 min, reaction was faster and followed 1st order kinetics with negative slope and R^2 value of 0.97 in linear regression analysis. Later half of the time points from 21 min to 36 min, reaction became slower and reached plateau phase and did not show significant R^2 value. Based on the kinetics results, measurement between 3 to 18 min would be significant for separating plant chemistry using this methodology. **Figure 4B** shows the curve fitting of the kinetics data of sample 1 with modulator 1 by linear regression analysis.

UHPLC-DAD-QqQ-MS/MS analysis

Quantitative determination of phenolic contents was carried out by diode array detector (DAD) at λ=280 nm by UHPLC-DAD analysis.[19] After the initial determination of the peak area of total phenolic content, it was further classified and determined the peak area of oligomeric HTs, monomeric HTs, and totals HTs separately. The percentage of oligomeric ETs, monomeric HTs, and totals HTs of total phenolic were determined as the percentage of the total peak area, which provided the picture of overall dominance by categories in a simplified way. Phenolic content and percentage of each class have been mentioned in **Figure 5**. Phenolic content can be calculated by the peak area as mg g^{-1} of the crude plant material using external calibration curve but the percentage of each class of HTs of the total peak area of phenolics was easier to understand the chemistry of samples rather than specific mg g^{-1} values which eventually distorts the overall

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glimpse of the composition. Phenolic compounds were classified and identified by known UV spectra and MS analysis through mining published literature.[5,7,19-21] Sample 4,5,7,8,9,10,11, and 12 were dominated by oligomeric HTs ranging from 7 to 40% of the total phenolic content (**Figure 5**). Sample **5** had the lowest percentage of oligomeric HTs among the sample set with the same percentage of monomeric HTs, and also the total HTs percentage (14%) was lowest in comparison to other samples, therefore, it was more dominated by other types of phenolic compounds rather than monomeric or oligomeric HTs. The composition of sample 5 was also reflected in the PCA analysis, where it did not cluster with other oligomers because of the dominance of other phenolic compounds. In **Figure 5**, the percentage of oligomeric HTs, monomeric HTs and total HTs of all samples have been compared. Sample 9 had the highest percentage of oligomeric HTs (40%), while sample 3 had the highest percentage of monomeric HTs (66%). Overall, sample 3 had the highest percentage of total HTs (69%) of total phenolic content, while sample 5 had the lowest (14%). Overall, oligomeric HTs ranged from 7% to 40%, monomeric HTs ranged from 7% to 66%, and total HTs ranged from 14% to 69% in the sample set. Major phenolic compounds identification and characterization data have been discussed in detail with relevant UHPLC-DAD chromatograms and MS spectrums in the supporting information file (**Fig. S1 to S42**).

Principal component analysis (PCA)

In our current investigations, multiple assay parameters were utilized; therefore, data were analyzed with the multivariate tools such as principal component analysis (PCA) to allow the representation of data in simplified two-dimensional format. PCA analysis showed the potential clustering of the sample 4, 7, 8, 9, 10, 11, and 12, while considering the reaction kinetics of the samples from 3 to 36 minutes with chemistries of modulators 4, 5, 6, 7 and 8, which strongly supports their inherent HTs composition (**Figure 6**). Similar clustering was achieved with the consideration of any specific chemistry of any single modulator or the combination of two or three modulators (**Figure 6**). Modulator 1, 2 and 3 were not able to cluster the samples exactly in the similar fashion but did a

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343 cluster of oligomeric samples 8, 9, 10, 11, and 12, and separated others in the good marginal
344 distance. Judging the nature of clustering and separation, modulator 4, 5, 6, 7, and 8, were the best
345 for separating oligomeric HTs from the monomeric ones. These plant samples contained oligomeric
346 HTs, namely, gemin A, salicarinin A to D, oenothein A and B, cocciferin D_2, sanguiin H-6,
347 lambertianin C, etc., which were identified and quantified by UHPLC-DAD-MS/MS analysis. Other
348 samples containing monomeric HTs and simple phenolic compounds were clearly separated from
349 the cluster of oligomeric HTs containing samples. In these samples, major monomeric HTs were
350 tellimagrandin I, pedunculagin, punicalagin, gallotannins, geraniin, ascorgeraniin, vescalagin,
351 castalagin, vescavaloninic acid, castavaloninic acid, etc.

352 Overall, the plant samples showed different levels of quenching effect on the luminescence
353 correlating their overall dominance of the polyphenol subclass. Mostly, oligomers dominated
354 samples showed a similar pattern, which allowed us to cluster and separate them from monomers in
355 PCA analysis. This is the very first time in polyphenol research we have utilized array based time-
356 resolved luminometric assay after the successful earlier application of this methodology in natural
357 product and food chemistry research mostly for the identification and quantification of adulteration
358 in commercial products.[10,22,23] In the current study, we did further progress to use this technique to
359 introduce a holistic way of revealing polyphenol composition of crude plant samples through rapid
360 finger-print analysis. This method will facilitate the process of rapid screening of crude plant
361 samples in a high-throughput format. In our study, we utilized 18 different types of modulators or
362 chelating solutions, among them 8 modulators were successful, but the unsuccessful ones could be
363 modified further to introduce new chemistries. Therefore, there are enormous opportunities of
364 further development and serendipity could play a role, for instance, if we can come up with a
365 unique chemistry which is very specific to compounds then we can even do the quantification from
366 the ability to quench the luminescence signal. While analyzing the crude samples, other chemicals
367 in the sample matrixes could affect the signal, which is a very common problem in the context,

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that's why we separated sugars from the crude sample to examine their effects on the signal, but luckily we did not find any effect. Besides that, we designed the extraction protocol in such a way to accumulate mostly polyphenols, and lipophilic materials were filtered out before the analysis as they were not soluble in water. Thus the quenching effect mostly came from the plant polyphenols and other smaller phenolic compounds but still there might be some other chemicals which are definitely in very less quantity. Furthermore, we utilized two lanthanide probes and found Eu (III) as best in comparison to Tb (III), but there are many other chelates such as Sm (III), Dy (III), etc. which can be tested and utilized. Additionally, a wide variety of coordination ligands can be utilized to introduce new chemistries. However, this method has to be improved further to make it faster and suitable for separation of each class of polyphenols in the more meaningful way and accurate quantification of HTs which are the objectives of our future investigations.

Conclusions

We have successfully developed rapid fingerprint analysis tool based on time-resolved lanthanide label array technique for the very first time in plant polyphenol research. The developed method allowed us to do a high-throughput screening of plant samples to reveal their overall polyphenol composition. It also efficiently clustered oligomeric HTs and separated from the monomeric ones. Overall, the method is also user-friendly which requires the simple luminescence plate reader with time-gated detection feature and mixing of relatively cheap chemicals in the experimental setup. The scope of further development is not limited moreover beyond less because of the privileges of modification of many parameters and institution of a new type of modulating agents. In future, this method should be tested for many different types of samples, not only limited to polyphenol-rich samples, furthermore, could be utilized for other compound classes.

Funding

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Conflicts of interest

There are no conflicts to declare.

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Abbreviations

DAD, diode array detector; DMSO, dimethyl sulfoxide; ET, ellagitannin; Eu, Europium; GT, gallotannin; HHDP, hexahydroxydiphenic acid; HT, hydrolyzable tannin; NHTP, nonahydroxytriphenoyl; NTA, 4,4,4-trifluoro-1-(2-naphthyl)-1,3-butanedione; PCA, principal component analysis; Phen, 1,10-phenanthroline monohydrate; SEM, standard error for mean, Tb, Terbium; TOPO, trioctylphosphine oxide; TPPO, Triphenylphosphine oxide; TRF, time-resolved fluorescence; TTA, 2-thenoyltrifluoroacetone; UHPLC, Ultra-high performance liquid chromatography.

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Table 1: Model plant species with their typical HTs.

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ID	Plant Species	Typical HTs
1	*Acer platanoides* L.	Hexagalloyl glucose Heptagalloyl glucose Octagalloyl glucose
2	*Geranium sylvaticum* L.	Geraniin Askorgeraniin
3	*Terminalia chebula* Retz.	Chebulagic acid Chebulanin Chebulinic acid
4	*Filipendula ulmaria* (L.) Maxim.	Pedunculagin Tellimagrandin I Tellimagrandin II Strictinin Isostrictinin Casuarictin
5	*Punica granatum* L.	Punicalagin Punicalin
6	*Hippophaë rhamnoides* L.	Vescalagin Castalagin Vescavaloninic acid Castavaloninic acid Stachyurin Casuarinin
7	*Geum rivale* L.	Rugosin D Rugosin G Rugosin E
8	*Fragaria vesca* L.	Agrimoniin Gemin A
9	*Epilobium angustifolium* (L.) Scop.	Oenothein A Oenothein B Tellimagrandin I Tellimagrandin II
10	*Rubus idaeus* L.	Sanguiin H-6 Lambertianin A Lambertianin C Rubusuavin C
11	*Quercus robur* L.	Vescalagin Castalagin Cocciferin D_2
12	*Lythrum salicaria* L.	Salicarinin A Salicarinin B Salicarinin C

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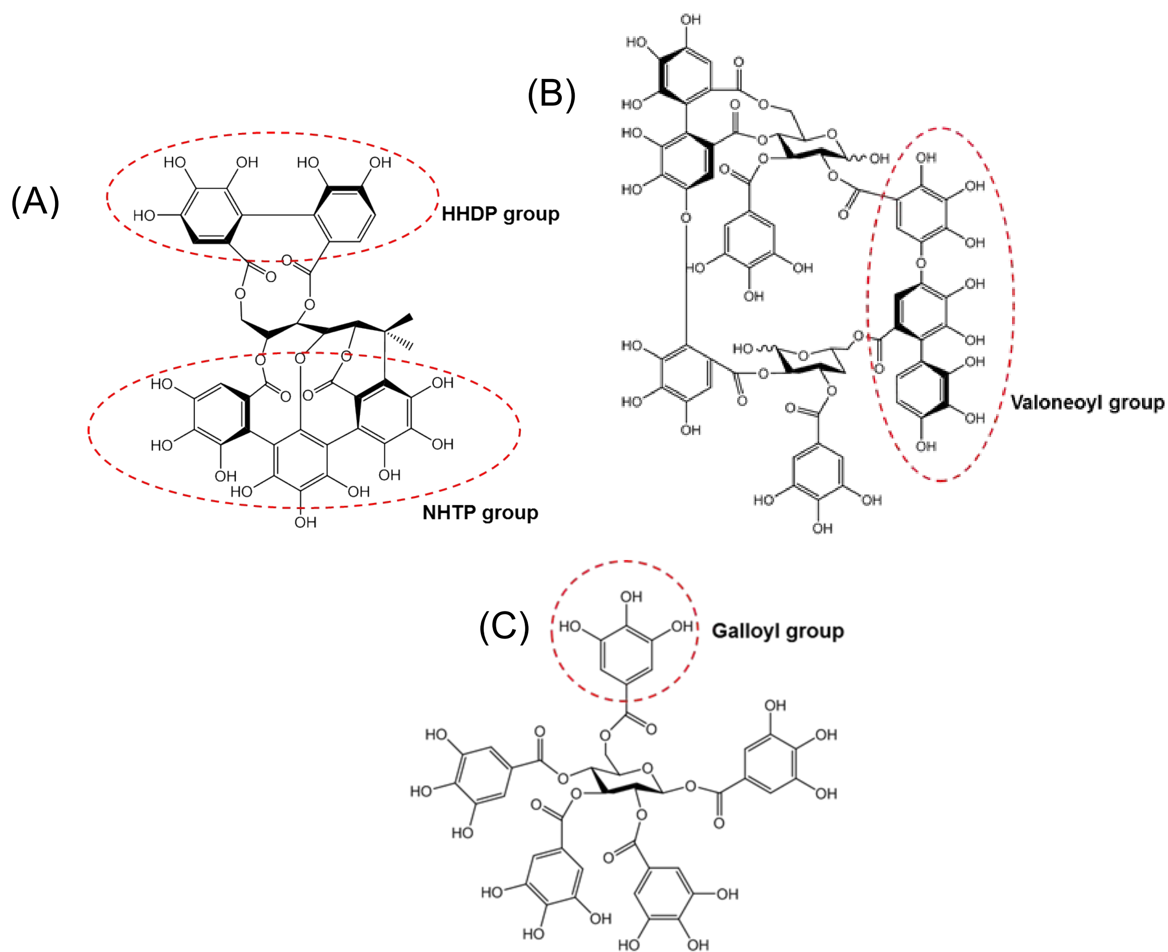

Figure 1: Typical examples of polyphenols. **(A)**: Vescalagin, a monomeric HT with HHDP and NHTP group; **(B)**: Oenothein B, a dimeric HT with valoneoyl group; **(C)**: Pentagalloyl glucose, gallic acid ester with simple galloyl groups, which is the precursor of all monomeric and oligomeric HTs in the biosynthetic pathway.

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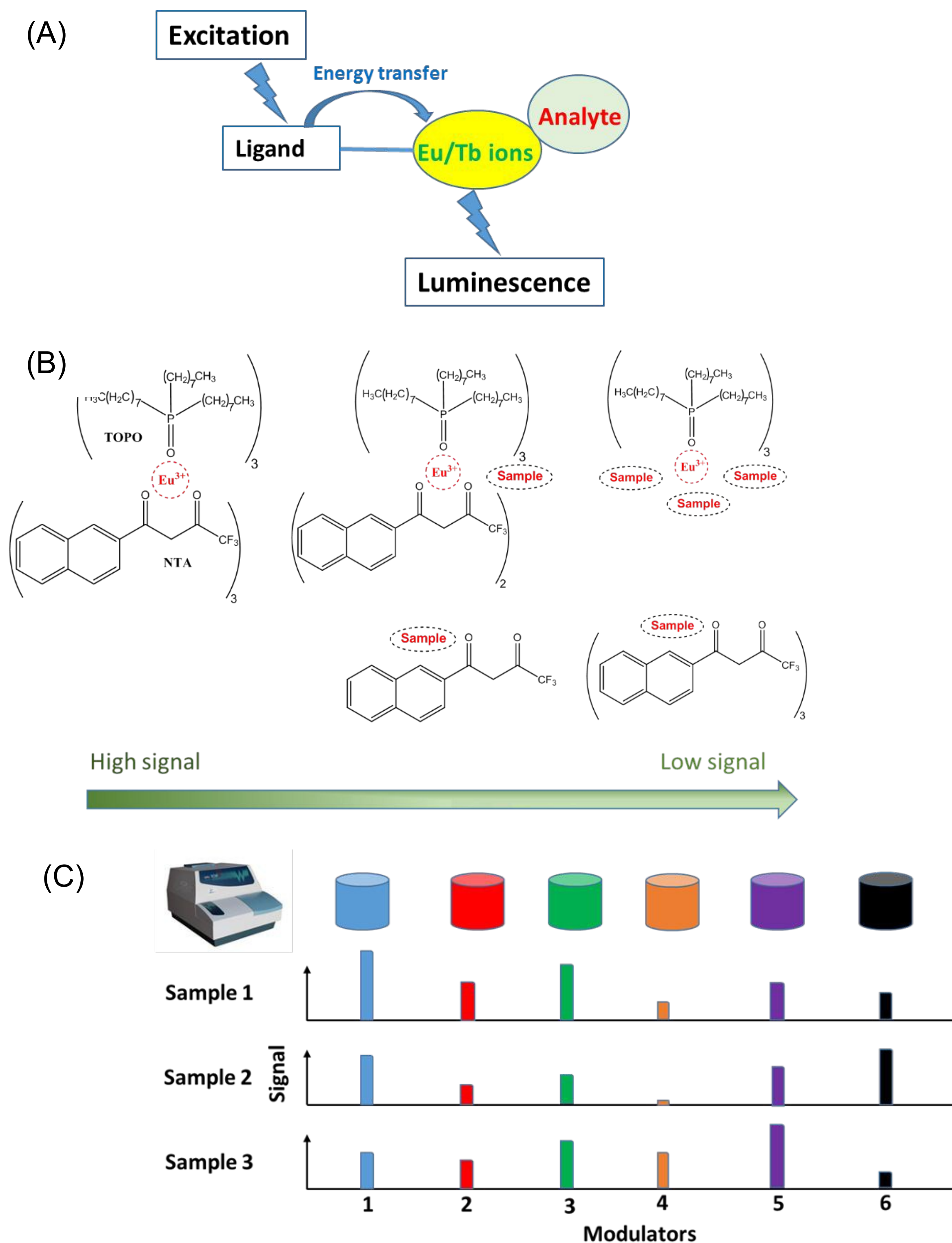

Figure 2: Principle of solution-based time-resolved lanthanide label array method. **(A)**: Simplified mechanism of luminescence spectroscopy where ligands are attached with the chelates to produce antenna effect to ensure strong luminescence; **(B)**: Example of modulators where ligands are attached with unstable chelates, and typically, samples interact with the ligands, which ultimately produces quenching effect on luminescence and way of separation; **(C)**: Example of fingerprints of samples with different modulators.

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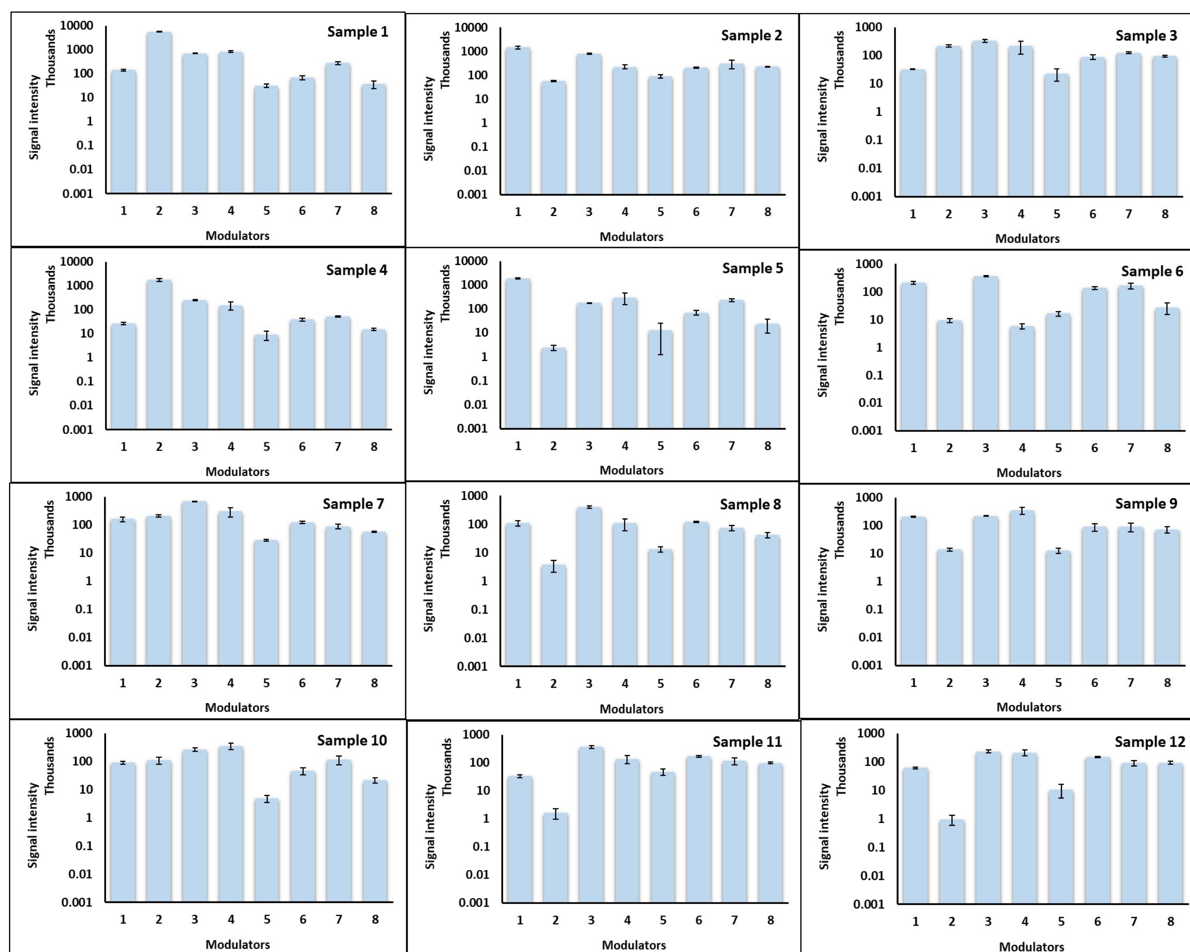

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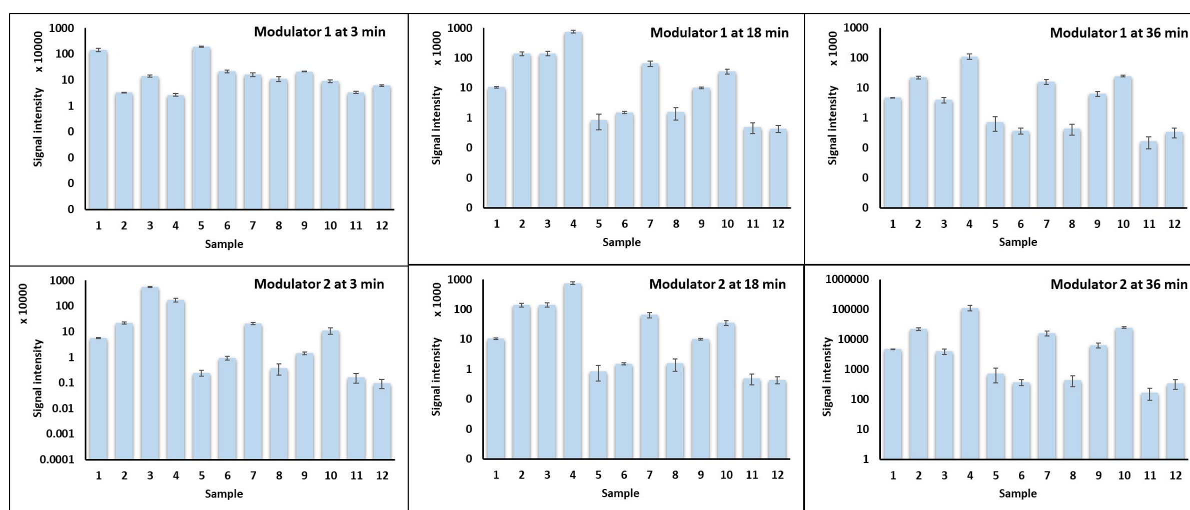

Figure 3: **(A):** Luminescence fingerprints of samples with all modulators recorded at 3 min in three separate replicative experiments. **(B):** Average luminescence signal of all 12 samples with modulator 1 and 2 recorded at 3, 18 and 36 min. Error bar represents standard error for mean (SEM) of replicates (n = 3).

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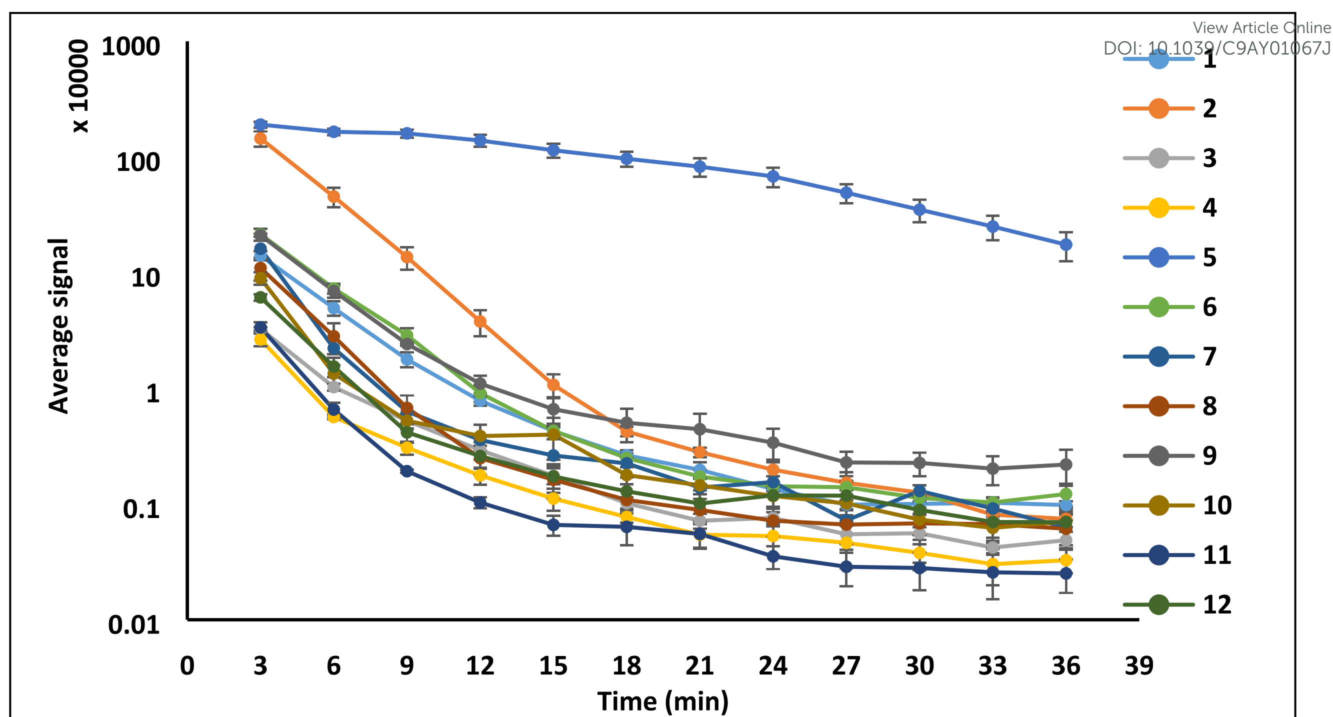

Figure 4A: Reaction kinetics of all the samples with modulator 1 recorded from 3 to 36 min in 3 min interval. Error bar represents standard error for mean (SEM) of replicates (n = 3).

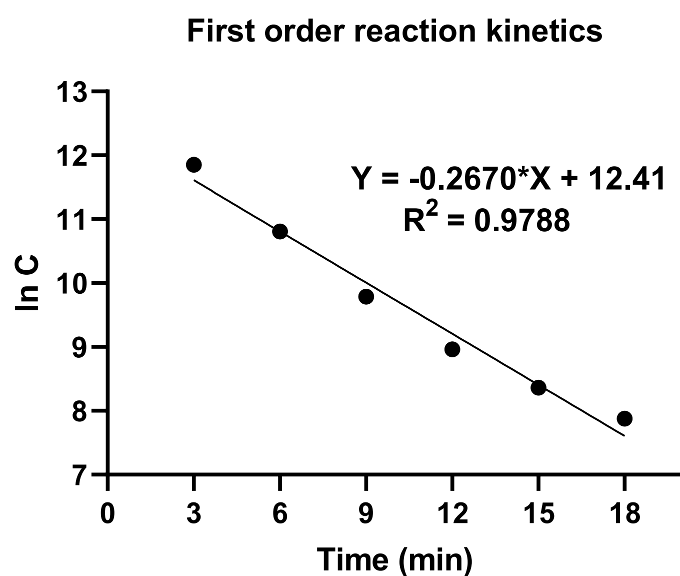

Figure 4B: Curve fitting of the kinetics data of sample 1 with modulator 1 by linear regression analysis. Reaction followed 1st order kinetics.

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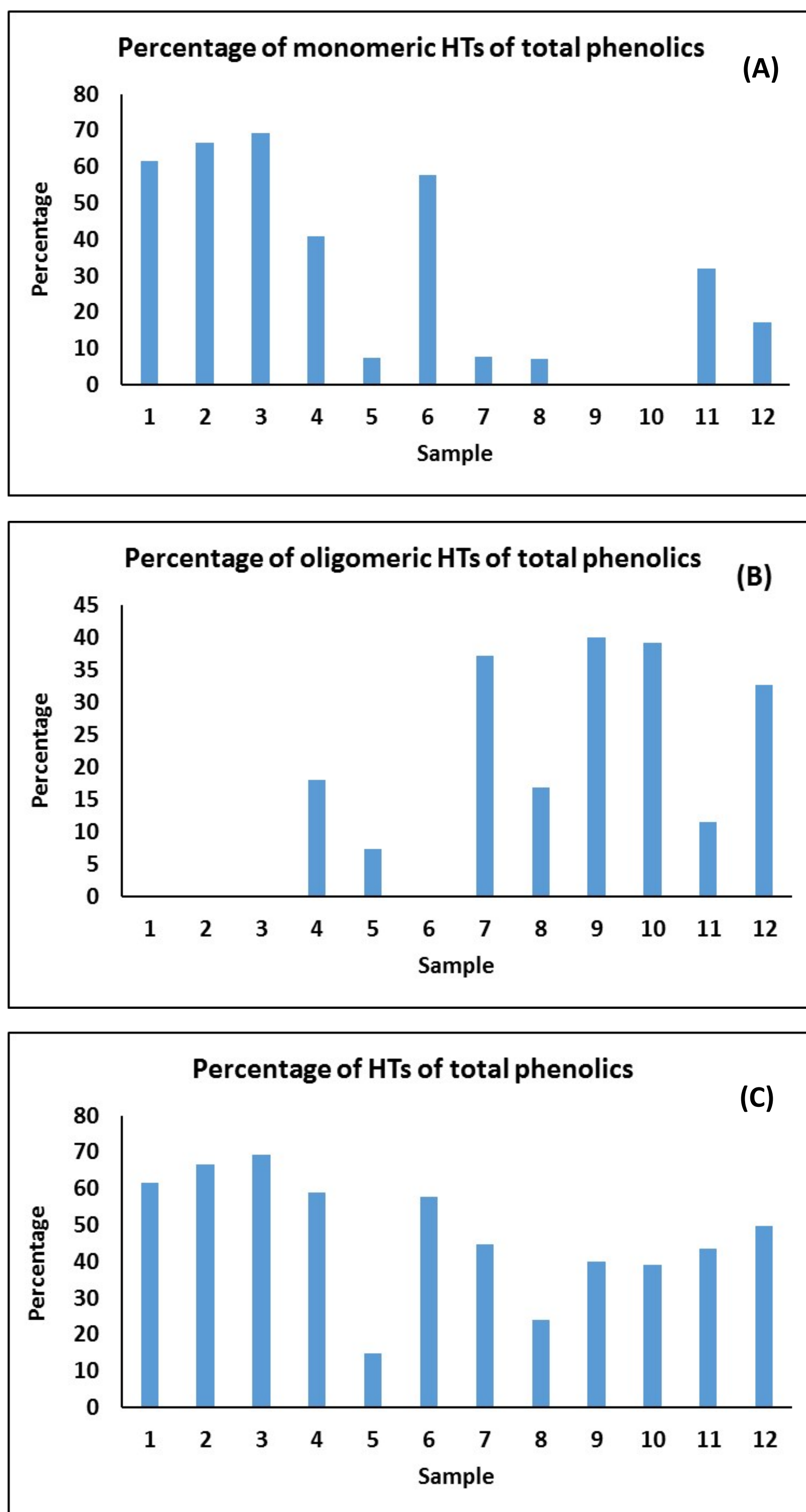

Figure 5: UHPLC quantification by Diode-array detector at λ= 280 nm of different class of HTs as percentage of total phenolic content of each sample. **(A)**: Percentage of monomeric HTs of total phenolics; **(B)**: Percentage of oligomeric HTs of total phenolics; **(C)**: Percentage of HTs of total phenolics.

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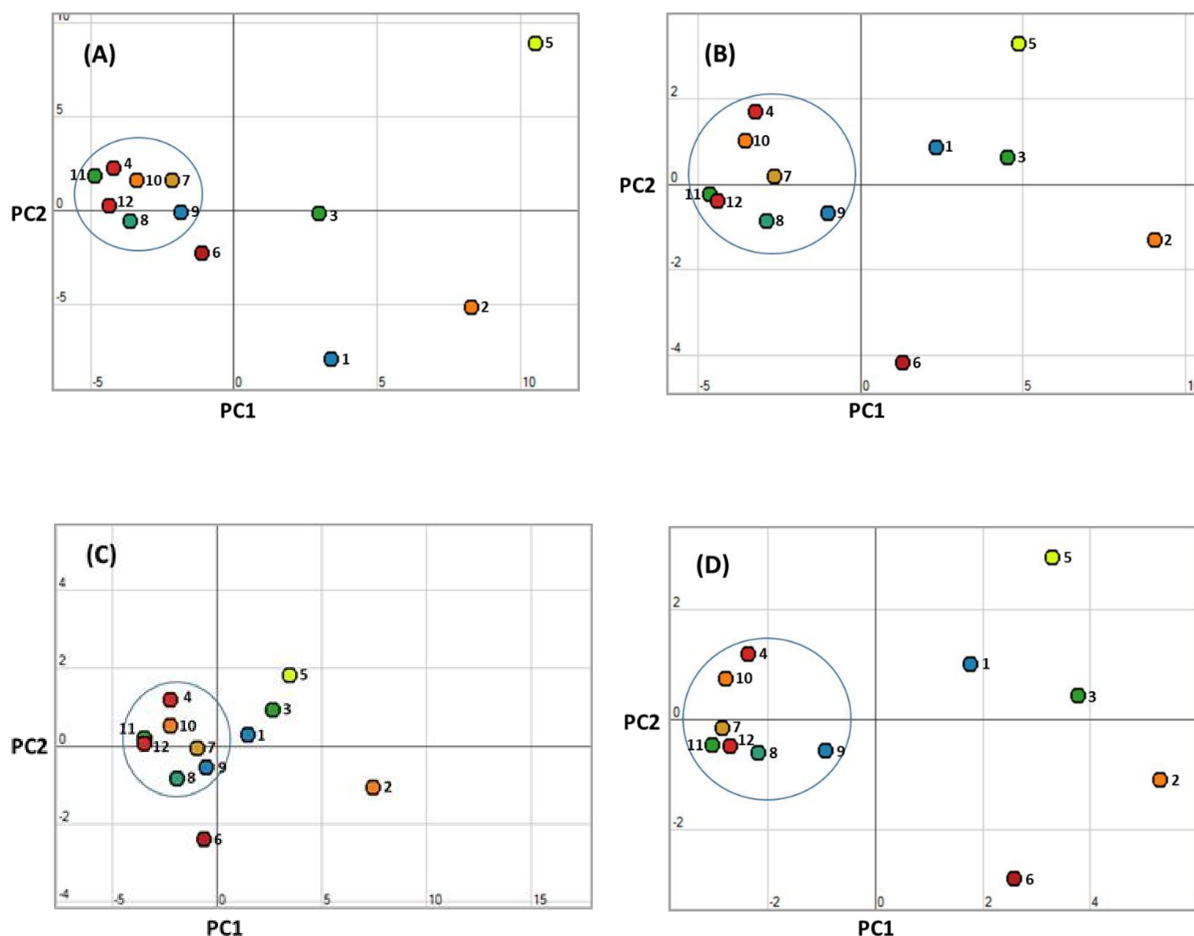

Figure 6: PCA analysis of the samples distinguished by lanthanide label array technique using europium chelates. **(A)**: cluster of oligomeric HTs (blue circle) by the chemistry of modulators 4,5,6,7 and 8; **(B)**: cluster by modulators 7 and 8; **(C)**: cluster by modulator 7; **(D)**: cluster by modulator 8.

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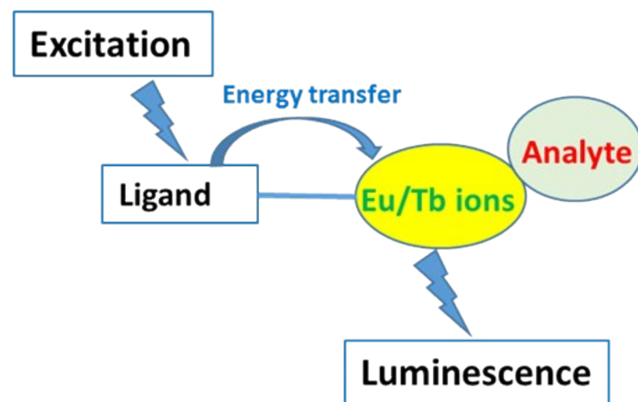
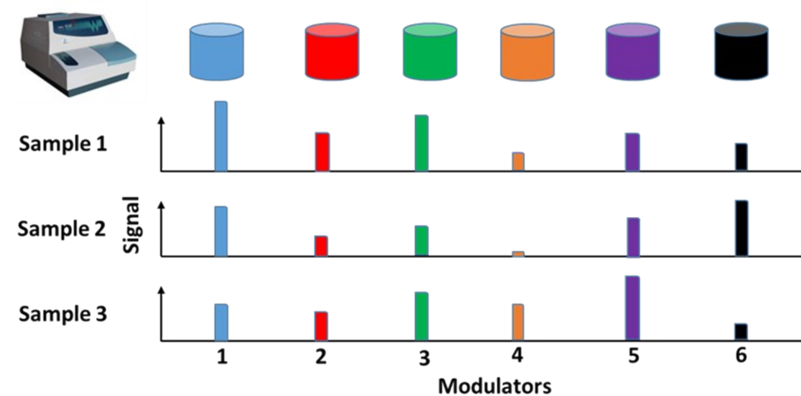
For the first time, a novel lanthanide label array method was developed for rapid fingerprint an of plant polyphenols in high throughput format.





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