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Isolation, detection, and quantification of hydrolyzable tannins of the biosynthetic pathway by liquid chromatography coupled with tandem mass spectrometry

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Abstract

RATIONALE: Hydrolyzable tannins (HTs) are widely distributed complex secondary metabolites with potential bioactivities and health-promoting benefits. A highly sensitive compound-specific UHPLC/MS/MS method is required for their successful detection and quantification in order to advance the study of HTs.

METHODS: In this study, 36 HTs belonging to the HT biosynthetic pathway covering 13 major branches were extracted by cold extraction and fractioned by Sephadex LH-20 size exclusion chromatography. Followed fractionation, the HTs were purified by semipreparative HPLC so that they could be used for the development of a UHPLC/QqQ-MS/MS multiple reaction monitoring (MRM) method for their characterization. The cone voltage and collision energy for each HT were extensively optimized during the development of the MRM method.

RESULTS: The developed method was very useful for the detection and quantification of marker tannins with a low limit of detection (LOD) and limit of quantification (LOQ), depending on the size and complexity of the structures of HTs. Each isolated compound was successfully identified and characterized by UHPLC/ESI-Orbitrap-MS/MS analysis. In addition, a new methodology for cold extraction and fractionation by Sephadex LH-20 chromatography has been developed for the targeted extraction of HTs.

CONCLUSIONS: This study has provided a compound-specific MRM method for the detection and quantification of representative HTs from the diverse phytochemical samples, with higher sensitivity than the existing group-specific MRM method.

Keywords

Ellagitannin, hydrolyzable tannins, multiple reaction monitoring (MRM), anthelmintics, size exclusion chromatography, fractionation.

1. INTRODUCTION

Intestinal parasitic infestation is an alarming complex disease that affects ruminant all over the world, causing serious health hazards and life-threatening infectious diseases.[1-3] Synthetic anthelmintic drugs are widely used against intestinal parasites, although that ultimately has led to a situation where resistance to the available anthelmintics is widespread.[4] In addition, the applicability of commercially available anthelmintics is decreasing rapidly because of the alarming increase of resistant strains all over the world, especially in the developing countries. Furthermore, the amount of drug residues in food products and in the environment is increasing because of the higher doses of synthetic anthelmintics being used. As a result, there is an urgent need to find an alternative solution for the control of gastrointestinal nematodes. Previous studies have shown that the consumption of tannin-rich forage can significantly reduce the intestinal parasites of ruminants.[2,5,6] Moreover, tannin-rich forage can reduce methane emission which is another problem associated with the animal farming.[6] However, the active types of tannin structures are not equally distributed in the plant kingdom and the verification of their presence in plants is not a trivial task; every plant produces tens and even hundreds of tannins e.g. ellagitannin, a single class of HTs, can be segmented into more than ten branches. Each of these branches produces several tannins with various structures and typically, each branch has variable average bioactivities. However, there is a lack of chemical analysis methods for the specific detection and quantification of the most active tannin types e.g. HTs. Such a specific method would significantly aid in the discovery of the most active plants, plant products, bred plant varieties or plant-derived industrial side-stream to be used. It is clearly important to explore those features in order to speed up the process of searching for new natural anthelmintics with lower side effects and environmental effects. To facilitate the screening process, we need highly sensitive, specific, and fast analytical methods, which can reliably detect possible drug candidates from diverse phytochemical sources.

Hydrolyzable tannins (HTs) are the most diverse elite class of polyphenols, with numerous types of bioactivities and health benefits, among which anthelmintic activity is the most crucial.[7-9] Plants produce HTs through the biosynthetic pathway, and they can be divided into at least 13 major branches with varying chemical and structural features.[10] For a long time, it was unknown which chemical and structural features of the tannins correlate with the observed medicinal effects. However, recent developments in detailed structure-activity relationship studies using purified hydrolyzable tannins have shed light on this problem and it is now possible to estimate directly from the chemical structures of hydrolyzable tannins if they are potential anthelmintics.[9] It has been always a challenging task for the phytochemists to detect what types of HTs are produced by which plant species and from which biosynthetic branches they are derived because of their relatively diversified complex chemical structures, extremely high molecular weights and also their low abundance in the plant material. Rapid, specific, and sensitive analytical methods are required to screen plant samples for the bioactive tannins.

HTs are generally assigned to three major classes: gallic acid derivatives, gallotannins (GTs) and ellagitannins (ETs). GTs contain six or more galloyl units whereas simple gallic acid derivatives contain five galloyl units or fewer. ETs are more diverse and around 600 of them have been identified and characterized - from simple monomers to complex oligomers.[11] Typically, two major units, namely, hexahydroxydiphenic acid (HHDP) and nonahydroxyterphenoyl (NHTP), either one of twhich or both cam attach to the sugar moiety to build the basic structure of HTs.

The number of studies on polyphenols is increasing rapidly because of the availability of the LC/MS/MS technique. To date, tandem mass spectrometry in multiple reaction monitoring (MRM) QqQ-MS/MS mode is the most selective analytical tool to have been utilized for the identification and quantification of the tannins.[12] The group-specific MRM method was utilized widely in earlier development of the tannin analysis method.[13] In addition, our research group

has developed compound-specific MRM methods for a few selected HTs covering only one or two branches of the biosynthetic pathway.[14] Unfortunately, compound-specific MRM methods have not been used widely in tannins research. Such methods would enable all QqQ users to quantify the chosen tannins rapidly and selectively. The lack of reference standards makes HTs analysis a laborious job because of the necessity to isolate the compounds from the natural sources before compound-specific MRM methods can be developed.[15]

In this study, all the above necessities led us to develop a compound-specific MRM method for the 36 isolated marker tannins covering all 13 major branches of the HT biosynthetic pathway. The method was developed successfully for the identification and quantification of the HTs biosynthetic branches where a specific precursor ion was selected in the first quadrupole followed by fragmentation in the collision cell and finally filtering the desired ions in the third quadrupole. The MRM method development by QqQ-MS/MS is an extensive process because of the optimization of cone voltage for the precursor ion and subsequent optimization of the collision energy for product ions. Each compound was also characterized by the high-resolution Orbitrap mass spectrometry. We have shown for the first time very small-scale extraction and Sephadex LH-20 fractionation of the plant polyphenols which is comparatively fast and provides a high yield, and provides simple monomeric to larger oligomeric HTs. Our current study advances the analytical analysis of HTs through the development of a compound-specific MRM method for all the major chemical classes from simple monomers to oligomers.

2. EXPERIMENTAL

2.1 Chemicals and Reagents

HPLC grade HiPerSolv CHROMANORM acetone and methanol from VWR (Fontenay-sous-Bois, France) were used for extraction and Sephadex fractionation. HPLC grade HiPerSolv

CHROMANORM acetonitrile and analR NORMAPUR formic acid from VWR were used for semi-preparative HPLC. LC-MS grade HiPerSolv CHROMANORM acetonitrile from VWR and formic acid from Fluka Analytical (Sigma Aldrich Chemi, Steinheim, Germany) were used in the UHPLC/ESI-QqQ MS and UHPLC/ESI-Orbitrap MS analyses. Water was purified with a Millipore (Merck KGaA, Darmstadt, Germany) Synergy UV water purification system. Sephadex LH-20 was procured from GE Healthcare (Uppsala, Sweden). Catechin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Flavonoids were from ExtraSynthese (Genay, France), and ascorbic acid from Sigma-Aldrich.

2.2 Plant Materials

Thirteen HT-producing plant species from the 13 different genera were selected covering the 13 major branches of the HT biosynthetic pathway from which 36 model compounds were isolated for the development of the method. Details of the plant species are presented in **Table 1**. These compounds were selected to represent different classes of HTs from the different branches of the biosynthetic pathway (**Figure 1**). All the plant materials were collected from the botanical garden of the University of Turku in Turku, Finland with the proper identification and voucher specimens except for *Terminalia chebula* Retz., which was procured from Banyan Botanicals, Albuquerque, NM, USA. After collection, the plant samples were freeze-dried, ground into powder, and stored in a freezer (-20 °C) until further use. A list of the isolated HTs from these plant species is provided in **Table 2**.

2.3 Isolation, Detection, and Quantification of Marker Tannins

2.3.1 Small Scale Extraction

Dried plant material, 200 mg for each plant, was macerated overnight in 10 mL of extraction solvent (80% acetone and 20% water, v/v) in test tubes at 4°C. Ascorbic acid, 0.1% solution, was added to the solvent to prevent oxidation during the extraction process. The samples were

shaken in a planar shaker for 3 hours to enhance the extraction and then centrifuged. Insoluble debris was discarded after the centrifugation. The clear supernatant was transferred into a Falcon tube and 10 mL of extraction solvent was added initially to each tube, which was shaken for 3 hours followed by centrifugation and then separation of the supernatant. Then, 5 mL of extraction solvent was added to the remaining debris followed by a final shaking by hand, centrifugation, and separation of the supernatant. After combining the extracts, acetone was evaporated under a nitrogen flow in a water bath (maximum temperature 40°C). A concentrator under reduced pressure was also used to confirm the evaporation of acetone. The extracts were then centrifuged twice to remove insoluble lipophilic impurities from the aqueous phase. A flow diagram describing the entire experimental process from extraction to quantification is shown in **Figure 2**.

2.3.2 Gel Chromatography and Fractionation

Sephadex LH-20 gel, 1 g, was added to the test tube as the stationary phase in water, and kept overnight to allow the gel particle to swell. Plant extract was then added to the gel followed by shaking by hand to ensure proper mixing with the gel. The tube was shaken in the planar shaker for 72 hours in a cold room at 4°C, followed by centrifugation for 10 min. The supernatant, named as the 1st water fraction, was transferred to a different test tube. Then, 6 mL of water was added to the original test tube which was shaken in the planar shaker for 30 min, followed by centrifugation for 10 min. The collected supernatant was then collected and named as the 2nd water fraction. The fractionation was performed one more time with water followed by three times with methanol and three times with acetone/water mixture (80/20, v/v). In total nine fractions were obtained from the fractionation (**Table 3**). The fractions were stored, frozen and lyophilized as required.

2.3.3 Analysis of Sephadex LH-20 Fractions by UHPLC-DAD-ESI-QqQ MS/MS

To evaluate the efficiency of small-scale extraction and fractionation, samples were analyzed using an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a Xevo TQ triple-quadrupole mass spectrometer (Waters Corp.) equipped with an electrospray ionization (ESI) source. The UHPLC system consisted of an automatic sample manager, a binary solvent manager, and a photodiode array detector (DAD). A reversed phase 100 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH Phenyl column (Waters Corp., Wexford, Ireland) was employed. The chromatographic and tandem mass spectrometric (MS/MS) operating conditions were the same as reported in our previously published UHPLC-DAD-ESI-QqQ MS/MS method.[11] Samples were diluted with water as needed to keep them within the detection limit and filtered using VWR® 0.2 μm PTFE syringe filters before the analysis.

2.3.4 Isolation of HTs by Semi-preparative HPLC

The HPLC system consisted of a Waters 2535 Quaternary Gradient module coupled with a Waters 2998 Photodiode Array Detector (DAD). A Waters Fraction Collector III was used as automatic sample collector. The semi-preparative column was a reversed phase Gemini® 10 μm C18 110 Å AXIA™ (150 mm × 21.2 mm i.d.) from Phenomenex (Torrance, CA, USA). The stationary phase of the column was C18 with TMS end-capping. The elution was carried out with two solvents, acetonitrile (A) and 0.1% aqueous formic acid (B). The flow rate of eluent was 8 mL min^{-1}. The lyophilized sample was dissolved in water with a maximum of 10% ethanol and filtered using 0.2 μm PTFE syringe filters before analysis to remove any insoluble debris. We utilized the full scan data from the UHPLC/MS/MS analysis to decide the elution profile and strength of the starting solvent to ensure the optimum time at which to collect all the desired compounds from the fraction. We selected the starting strength of acetonitrile from UHPLC and HPLC gradient correlation from our experimental experience. For example, if our target compound eluted at 4 min in the initial UHPLC/MS/MS scan of the

Sephadex fraction, from the correlation standpoint we selected 17% acetonitrile as the starting strength of the eluent and the solvent gradient as follows: 0-5.0 min, 17% A in B; 5.0-51.0 min, 17−42% A in B (linear gradient); 51.0-55.0 min, 42−70% A in B (linear gradient); 55.0-85.0 min, 70% A in B for washing, and 85.0-120.0 min for stabilization. The elution events were modified for each compound based on their elution time in the UHPLC-MS/MS scan of the Sephadex fraction. An aliquot of each fraction was analyzed by UHPLC-DAD–ESI-MS/MS with necessary dilutions to avoid saturation of the detector and the fractions with a similar composition were combined. If the quantity of the fraction was enough, it was frozen and lyophilized; otherwise, it was stored in the freezer for further analysis.

2.3.5 Screening of HPLC fractions by UHPLC-DAD-ESI-MS/MS analysis

Fractions obtained from semi-preparative HPLC were analyzed by the Acquity UPLC system coupled with the Xevo TQ triple-quadrupole mass spectrometer. The sample was diluted with acetonitrile/Milli-Q water before analysis. The acetonitrile/Milli-Q water composition was adjusted based on the retention time and the dilution factor was decided from their UV absorbance. The chromatographic and column condition was same as described earlier and negative ion mode ESI was employed. A 5-µL sample was injected into the system. The elution was carried out with two solvents, acetonitrile (A) and 0.1% aqueous formic acid (B) with the same elution events and chromatographic condition as described in a previous publication from our group.[14] UV–vis (190–500 nm) and MS data (*m/z* 150 to 1200) were recorded from 0 to 5 min. The mass spectrometer settings were also the same as previously published.[14] The cone voltages was increased from 20 to 100 V in 20 V increments. Identification of the compound was confirmed by its UV spectrum, MS spectrum and retention time.

2.3.6 Compound characterization by UHPLC-DAD-Orbitrap-HRMS

Each compound was further characterized by high resolution UHPLC-DAD-Orbitrap high-resolution mass spectrometry (HRMS) analysis. The UHPLC-DAD-Orbitrap-HRMS system used the same column as the QqQ system, but it was coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany). Samples for UHPLC-DAD-QqQ-MS/MS analysis were prepared similarly to those for UHPLC-DAD-Orbitrap-HRMS analysis. The elution events were also same as for the QqQ analysis. A heated electrospray ionization probe (H-ESI II, Thermo Fisher Scientific GmbH) was used for ionization in negative ion mode under the following condition: spray voltage, -3.0 kV; sheath gas (N_2) flow rate, 60 (arbitrary units); aux gas (N_2) flow rate, 20 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); capillary temperature, 380 ˚C. A high resolution of 70,000 was used in the detector and an automatic gain of 3 × 106 was used. For MS-MS fragmentation studies full MS/dd-MS^2 (TopN) experiments were conducted using a loop count and TopN value of 5 with a resolution of 17,500 and an automatic gain of 1 × 105. The detector was calibrated using Pierce ESI Negative Ion Calibration Solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). The data were analyzed and processed with Xcalibur Qual Browser software (Version 3.0.63, Thermo Fisher Scientific Inc.).

2.3.7 Multiple reaction monitoring (MRM) mode optimization

Multiple reaction monitoring (MRM) mode was used for the quantitative analysis of the isolated compounds. The main precursor ion and the highest intensity product ion were selected for each hydrolyzable tannin and used to create the MRM transition that was used for the quantification (MRM1). Another transition (MRM2) was employed for each compound to ensure the specificity of the detection and to avoid false positive results, where the transition involved the same precursor ion and the second most intense product ion.

The cone voltage was optimized for the $[M\text{-}nH]^{n-}$ (n = 1 or 2) precursor ion of each selected compound by UHPLC-ESI-QqQ-MS/MS (see **Table 4**). Two different UHPLC gradients were used based on the screening information of previous analyses. If there was only one pure compound present in the fraction, a 1-min rapid UHPLC gradient was used; otherwise a 6-min gradient program was utilized for the mixtures of multiple compounds. The elution was carried out with two solvents, acetonitrile (A) and 0.1% aqueous formic acid (B). The short 1-min gradient was: 0−0.5 min, 30% A in B; 0.5−1.0 min, 30% A in B (isocratic). The longer 6-min gradient was: 0−0.5 min, 0.1% A in B; 0.5−5.0 min, 0.1−30% A in B (linear gradient); 5.0−5.10 min, 30−0.1% A in B (linear gradient); 5.10−6.40 min, column wash, and stabilization. UV–vis (190–500 nm) and MS data (*m/z* 150 to 1200) were recorded from 0 to 5 min, except for tetra gallyol glucose, for which date were recorded up to 6 min because it elutes later. The ionization condition was same as described earlier. The eluent flow rate was 0.5 $mLmin^{-1}$. The cone voltage was increased from 10 to 100 V in 10 V increments to find the voltage that would produce the most efficient ionization of the precursor ion.

The $[M\text{-}nH]^{n-}$ precursor ion was fragmented with collision energies varying from 10 to 50 eV in 5 eV increments to find the most efficient collision energy at which to obtain the highest intensity product ions.. If this collision energy range was not sufficient to produce product ions or an optimum collision energy curve, the CE was increased from 10 to 100 eV in 10 eV increments. The same 1-min and 6-min gradient methods were used as in the optimization of cone voltages.

2.4 Method Performance Evaluation

Previously, our research group has published similar compound-specific MRM methods utilizing the same UHPLC-MS/MS instrumentation, with proper validation including extraction efficiency, linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and matrix effect, but only for oligomeric macrocyclic ellagitannins.[14] Our

current study is the continuation of that work as a comprehensive MRM study of 36 HTs covering all 13 different major classes of HTs. We only investigated the LOD, LOQ and linear range for each HT to evaluate the efficiency of the method for the detection and quantification of HTs. The LOD is the lowest concentration at which the analyte can be reliably detected, and the LOQ is the concentration at which the analyte can be reliably quantified. The LOD and LOQ were determined from the calibration curves according to the guidelines recommended by the International Conference of Harmonization (ICH). In the calibration plot method, the following formulae were used:

LOD = 3.3*(P/Q) and LOQ = 10*(P/Q), where P is the standard deviation of the y-intercept and, Q is the slope of the linear regression.[15] The MRM transition for quantification was used for the preparation of the calibration curves. The linear range was measured for each compound from the calibration curves. The range was considered to be linear if the value of $R^2 > 0.99$.

3. RESULTS AND DISCUSSION

3.1 Identification of HTs

The HTs were identified by comparing the *m/z* values of the precursors and typical product ions, exact retention times and UV spectra with the published literature. Most of the plant species used in the current investigations have been investigated and detailed MS spectra have been published in our recent paper with the identification of major monomeric and oligomeric HTs.[11] All the major HTs have been extensively characterized previously by researchers from our group and others.[15,17-24] The compound features and identification strategies are discussed in the Supporting Information.

3.2 Purity of Compounds

The purity of the isolated HTs (**Table 2**) was determined by UHPLC-DAD-ESI-MS/MS at 280 nm. All the HTs were pure compounds except for cocciferin D2, which had a purity of less than 64% because of the low extraction yield of the plant materials. In total, 28 of the 36 HTs investigated in the present study had a purity greater than 90% . Some HTs had less than 90% purity because large oligomers are very difficult and complex to isolate and purify in comparison with monomeric HTs.[14] Depending on the complexity of the process, having some HTs at relatively low purity is acceptable.

3.3 Extraction and Fractionation by Sephadex LH-20 Gel Chromatography

Small-scale extraction of crude plant powder successfully extracted all the sugars, phenolic compounds and hydrolyzable tannins including both monomers and oligomers. Utilization of this method enabled us to carry out a targeted polyphenol extraction and accumulation of HTs. The plant extracts werewell separated by Sephadex LH-20 gel chromatography and analyzed by UHPLC-DAD-ESI-QqQ MS/MS. Three fractions were obtained after the fractionation, containing three different major classes of compounds (**Table 3**). Water eluted all the sugars, methanol eluted low molecular weight phenolic compounds such as flavonoids and caffeic acid derivatives, and 80/20, v/v: acetone/water eluted all the HTs. The UHPLC-DAD-ESI-QqQ MS/MS analysis showed excellent separation of the compounds compared with the raw plant extract (**Figure 3**). As a prototype, the results of the extraction and separation of *Filipendula ulmaria* (L.) Maxim. (sample 5 in Table 1) are shown in **Figure 3**. Figures 3A, 3B, 3C, and 3D represent the UV chromatogram of the raw extract, water, methanol, and acetone/water fractions, respectively, at λ = 280 nm. The water fraction did not contain any UV-active compounds so there were no polyphenols except sugars in that fraction, as was confirmed by the MS/MS analysis. The ethanol fractions contained small phenolic compounds like flavonoids, caffeic acid derivatives, etc. The acetone/water fractions contained our targeted

polyphenols, specifically HTs. From the UV and full scan MS data we were able to confirm the presence of monomeric and oligomeric HTs, namely, Tellimagrandin I *m/z* 785 $[M–H]^–$, Tellimagrandin II *m/z* 937 $[M–H]^–$, Rugosin E *m/z* 860 $[M–2H]^{2–}$, Rugosin A *m/z* 1105 $[M–H]^–$, Rugoisn D *m/z* 936 $[M–2H]^{2–}$, etc. in the acetone/water fraction (**Figure 3D**).

3.4 Multiple reaction monitoring (MRM) mode optimization

Ultra-high-performance liquid chromatography coupled with Multiple Reaction Monitoring (MRM) tandem mass spectrometry can perform the detection and quantification of multiple compounds in a single run, and it is a very powerful analytical tool for the study of secondary metabolites from diverse phytochemicals sources.[14] Our research group has previously used the same triple quadrupole mass spectrometer set-up for the UHPLC/MS/MS detection and quantification of oligomeric macrocyclic ellagitannins from *Epilobium angustifolium*.[14] To develop the method reported herein, the cone voltage used to generate the precursor ion, obtained in negative ion mode, from each compound was optimized. Because of the nature of electrospray ionization, multiple charged $[M\text{-}nH]^{n-}$ ions were often more intense than the singly charged $[M\text{-}H]^-$ ions. As the mass range of the instrument was from *m/z* 40 to 2048, we were able to detect only multiple charged ions for higher mass oligomeric HTs. For lower mass oligomer, if the $[M\text{-}H]^-$ ion was sufficiently intense, it was selected as the precursor ion; otherwise, a multiply charged ion was chosen. As an example, the $[M\text{-}H]^-$ ion (*m/z* 1567) of oenothein B was visible within the mass range of the instrument but the $[M\text{-}2H]^{2-}$ ion at *m/z* 783 was much more intense that ion was therefore selected as the precursor. Of the 36 HTs studied, $[M\text{-}nH]^{n-}$ ions were selected as precursors for 12 compounds and the $[M\text{-}H]^-$ ions for the remainder. The cone voltage employed to generate the highest intensity precursor ions of the HTs varied between 28 V and 79 V (**Table 5**). Initially, the intensity of the precursor ion increased with increased cone voltage but after reaching a maximum, it started to decline (**Figure 4**). This was because further increase of the voltage caused in-source fragmentation of

the precursor ion and formation of fragment ions at *m/z* 301 and 275 for the HTs, which were the result of cleavage and rearrangement of the HHDP moiety.[16]

A wide range of collision energies, from 10 eV to 100 eV, was utilized to maximize the fragmentation and to observe common fragmentation pathways. The selectivity and sensitivity of the method were ensured by optimizing the collision energies to maximize the formation of high intensity product ions of specific precursors. The highest intensity product ion was used for the MRM quantification transition and the second highest intensity ion for the qualitative confirmation transition. It can be ascertained clearly from **Figure 5 and Table 5** that *m/z* 301 and 275 were the most common product ions for HTs and typically they became more intense at higher collision energy. In most cases, the *m/z* 301 ion was more abundant than *m/z* 275, except for oenothein A, where the opposite was found (**Figure 5**). In addition, for salicarinin A, the product ion at *m/z* 249 was selected for the qualitative transition as it was more intense than *m/z* 275. Some other exceptions were as follows: the *m/z* 633 ion for casuarictin as the secondary product ion; the *m/z* 915 ion for vescalagin as the primary product ion; the *m/z* 425 ion for castalagin as the secondary product ion; the *m/z* 633 ion for casuarinin as the secondary product ion; the *m/z* 765 ion for oenothein B as the secondary product ion; the *m/z* 315 ion for sanguiin H-6 as the secondary product ion, the *m/z* 974 ion for ascorgeraniin as the secondary product ion; the *m/z* 933 ion for geraniin as the secondary product ion; and the *m/z* 249 ion for salicarinin B as the secondary product ion (**Figure 5**). For both chebulanin and chebulinic acid, *m/z* 169 was the most intense product, making it the lowest mass product ion used for a MRM transition in this study.

The peak areas of the MRM1 and MRM2 transitions were measured for each compound (**Table 5**), and the ratios (MRM2/MRM1) of these areas were found to be constant throughout the dilution series, ensuring accurate detection. For some compounds, namely, tetragallyol glucose, chebulanin, casuarictin, casuarinin and cocciferin D_2, the ratio was very low because

of the low intensity of the MRM2 transition in comparison with the high intensity of the MRM1 transition. This is because for some HTs we only observed one very high intensity product ion such as *m/z* 169, *m/z* 275 or *m/z* 301 while the other product ions were minor in intensity. Clearly HTs do not fragment as readily as other low mass phenolic compounds in the MS/MS process. Having only a few product ions from large oligomeric HTs also makes development of the MRM method a cumbersome process.

3.7 Method Performance Evaluation

In the UHPLC-DAD-QqQ-MS/MS analysis, the LOD ranged from 3.61 to 1193.41 $ngmL^{-1}$ and the LOQ from 10.93 to 3616.39 $ngmL^{-1}$ (**Table 6**). Overall, the LOD and LOQ obtained for each compound with the developed method were low and would enable us to carry out the quantification at the very low concentration level of 3 ng mL^{-1}. The lowest LOD and LOQ were detected for cocciferin D2 as 3.61 and 10.93 $ngmL^{-1}$, respectively and the highest for chebulanin as 1193.41 and 3616.39 $ngmL^{-1}$, respectively. The results for our study as in good agreement with those from previously reported quantification methods for HTs, and, in most cases, we achieved much lower levels of detection and quantification.[12,16,24] A few oligomeric compounds had high LOD and LOQ values mainly because they had very few product ions even at very high cone voltage and collision energy. These compounds have relatively large structures and are more difficult to fragment.

The calibration curves were analyzed by linear regression and showed excellent correlation (R^2 > 0.99) (**supporting information, Figure 1**), and thus in the perfect linear range. The lower level of the linear range for most of the compounds was identified successfully; however, for some compounds, the dilution series was not sufficient to reach the lower limit. In all cases, the higher limit of the linear range was not identified because the curve was linear throughout the dilution series used for all the compounds. Therefore, in the experimental setup we were not able to reach the saturation level of the detector. The higher limit of the linear range

corresponds to much higher concentration of the compounds. The linear ranges for each compound are given in **Table 6**.

4. CONCLUSIONS

This study reports the development of a compound-specific MRM method for the detection and quantification of 36 HTs covering the 13 major branches of the hydrolyzable tannin biosynthetic pathway. These compound-specific methods would allow us to screen HTs with varying structures from diverse phytochemical samples with higher sensitivity than the existing group-specific method. The method performed will in detecting and quantifying small monomers to large oligomers. The method suffered in having high LOD and LOQ for a few complex oligomers, as they are very difficult to fragment even at high CE. However, despite these shortcomings, the method is still a significant advance given that no other compound-specific MRM method is available for these complex HTs. Small scale extraction, size exclusion fractionation and semipreparative HPLC allowed us to perform targeted extraction of HTs and the subsequent isolation of pure HTs from the initial fractions. This is the first time that such a targeted small-scale extraction method for HTs has been reported. The method parameters such as optimum CVs and CEs will provide invaluable information to the scientific community for rapidly analyzing wide varieties of HTs by MRM. Similar compound-specific methods can be developed for other HTs in future work.

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CONFLICTS OF INTEREST

There are no conflicts to declare.

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ABBREVIATIONS

DAD, diode array detector; ESI, electrospray ionization; ET, ellagitannin; GT, gallotannin; GG, galloyl glucose; HHDP, hexahydroxydiphenic acid; HT, hydrolyzable tannin; LOD, limit of detection; LOQ, limit of quantification, NHTP, nonahydroxytriphenoyl; UHPLC, Ultra-high-performance liquid chromatography.

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Table 1: Plant species used in the present study to isolate marker tannins.

No.	Genus	Compound class	Possible compounds	Plant name
1	Betula	Galloyl glucoses	Monogalloyl glucose Digalloyl glucose Trigalloyl glucose Tetragalloyl glucose Pentagalloyl glucose	*Betula nana* L.
2	Acer	Gallotannins	Hexagalloyl glucose Heptagalloyl glucose Octagalloyl glucose	*Acer platanoides* L.
3	Geranium	Dehydro-ET	Geraniin Askorgeraniin	*Geranium sylvaticum* L.
4	Terminalia	Modified dehydro-ET	Chebulagic acid Chebulanin Chebulinic acid	*Terminalia chebula* Retz.
5	Filipendula	Simple HHDP esters	Pedunculagin Tellimagrandin I Tellimagrandin II Strictinin Isostrictinin Casuarictin	*Filipendula ulmaria* (L.) Maxim.
6	Punica	Gallagyl ester	Punicalagin	*Punica granatum* L.
7	Hippophae	C-glycosidic ET	Vescalagin Castalagin Vescavaloninic acid Castavaloninic acid Stachyurin Casuarinin Hippophaenin B	*Hippophaë rhamnoides* L.
8	Geum	m-DOG-oligomeric ET	Rugosin D Rugosin G Rugosin E	*Geum rivale* L.
9	Fragaria	m-GOG-oligomeric ET	Agrimoniin Gemin A	*Fragaria vesca* L.
10	Epilobium	m-DOG-oligomeric macrocyclic ET	Oenothein A Oenothein B	*Oenothera biennis* L.
11	Rubus	m-GOD-oligomeric ET	Sanguiin H-6 Lambertianin A Lambertianin C Rubusuavin C	*Rubus idaeus* L.
12	Quercus	Glucopyranose C-glycosidic m-DOG-oligomeric ET	Cocciferin D2	*Quercus robur* L.
13	Lythrum	C-glycosidic m-DOG-oligomeric ET	Salicarinin A Salicarinin B Salicarinin C	*Lythrum salicaria* L.

Table 2: Compounds used in the present study with UHPLC purity determined at λ = 280 nm.

Compound class	No.	Compounds	MW	Purity
Galloyl glucoses	1	Monogalloyl glucose	332.3	99.74
	2	Digalloyl glucose	484.4	97.15
	3	Trigalloyl glucose	636.5	84.91
	4	Tetragalloyl glucose	788.6	99.43
	5	Pentagalloyl glucose	940.7	97.63
Gallotannins	6	Hexagalloyl glucose	1092.8	91.49
	7	Heptagalloyl glucose	1244.9	96.60
	8	Octagalloyl glucose	1397.0	93.31
Dehydro-ET	9	Geraniin	952.6	99.27
	10	Ascorgeraniin	1110.7	93.08
Modified dehydro-ET	11	Chebulagic acid	954.7	98.68
	12	Chebulanin	652.5	99.33
	13	Chebulinic acid	956.6	65.29
Simple HHDP esters	14	Pedunculagin	784.5	87.36
	15	Tellimagrandin I	786.6	99.13
	16	Strictinin	634.4	99.05
	17	Casuarictin	936.6	95.51
Gallagyl ester	18	Punicalagin	1084.7	93.53
C-glycosidic ET	19	Vescalagin	1102.7	88.53
	20	Castalagin	1102.7	91.47
	21	Vescavaloninic acid	936.6	89.04
	22	Castavaloninic acid	936.6	98.52
	23	Casuarinin	936.6	98.58
	24	Hippophaenin B	1104.7	94.58
m-DOG-oligomeric ET	25	Rugosin D	1875.3	99.50
	26	Rugosin E	1723.2	98.83
m-GOG-oligomeric ET	27	Agrimoniin	1871.3	98.83
	28	Gemin A	1873.3	98.58
m-DOG-oligomeric macrocyclic ET	29	Oenothein A	2353.6	76.63
	30	Oenothein B	1569.1	92.57
m-GOD-oligomeric ET	31	Sanguiin H-6	1871.3	94.85
	32	Lambertianin C	2805.9	92.90
	33	Rubusuaviin C	2805.9	99.86
Glucopyranose C-glycosidic m-DOG-oligomeric ET	34	Cocciferin D2	1869.3	64.05
C-glycosidic m-DOG-oligomeric ET	35	Salicarinin A	1869.3	93.25
	36	Salicarinin B	1869.3	85.51

Table 3: Sephadex LH-20 gel chromatography and fractionation

Fraction no.	Solvent	Target compounds
1	Water	Sugars
2	Water	Sugars
3	Water	Sugars
4	Methanol	Flavonoids, caffeic acid derivatives, etc.
5	Methanol	Flavonoids, caffeic acid derivatives, etc.
6	Methanol	Flavonoids, caffeic acid derivatives, etc.
7	80/20 acetone/water	Hydrolyzable tannins
8	80/20 acetone/water	Hydrolyzable tannins
9	80/20 acetone/water	Hydrolyzable tannins

Table 4: Characterization profile of the compounds by UHPLC/ESI-Orbitrap-HRMS analysis.

Compound	Precursor ion (*m/z*)	Chemical formula	Exact mass (*m/z*)		Error (ppm)
			Measured	Calculated	
Monogalloyl glucose	331.0663 $[M–H]^-$	$C_{13}H_{16}O_{10}$	332.0735	332.0743	– 2.41
Digalloyl glucose	483.0773 $[M–H]^-$	$C_{20}H_{20}O_{14}$	484.0845	484.0853	– 1.65
Trigalloyl glucose	635.0882 $[M–H]^-$	$C_{27}H_{24}O_{18}$	636.0955	636.0963	– 1.26
Tetragalloyl glucose	787.0987 $[M–H]^-$	$C_{34}H_{28}O_{22}$	788.1060	788.1072	– 1.52
Pentagalloyl glucose	939.1094 [M-H]-	$C_{41}H_{32}O_{26}$	940.1166	940.1182	– 1.70
Hexagalloyl glucose	1091.1190 $[M–H]^-$	$C_{48}H_{36}O_{30}$	1092.1263	1092.1291	– 2.56
Heptagalloyl glucose	1243.1285 $[M–H]^-$	$C_{55}H_{40}O_{34}$	1244.1358	1244.1401	– 3.46
Octagalloyl glucose	1395.1388 $[M–H]^-$	$C_{62}H_{44}O_{38}$	1396.1461	1396.151	– 3.51
Geraniin	951.0712 $[M–H]^-$	$C_{41}H_{28}O_{27}$	952.0789	952.0818	– 3.04
Ascorgeraniin	1109.0938 $[M–H]^-$	$C_{47}H_{34}O_{32}$	1110.1010	1110.1033	– 2.07
Chebulagic acid	953.0874 $[M–H]^-$	$C_{41}H_{30}O_{27}$	954.0947	954.0974	– 2.83
Chebulanin	651.0831 $[M–H]^-$	$C_{27}H_{24}O_{19}$	652.0904	652.0912	– 1.23
Chebulinic acid	955.1038 $[M–H]^-$	$C_{41}H_{32}O_{27}$	956.1111	956.1131	– 2.09
Pedunclagin	783.0684 $[M–H]^-$	$C_{34}H_{24}O_{22}$	784.0756	784.0759	– 0.38
Tellimagrandin I	785.0841 $[M–H]^-$	$C_{34}H_{26}O_{22}$	786.0913	786.0916	– 0.38
Strictinin	633.0727 $[M–H]^-$	$C_{27}H_{22}O_{18}$	634.0800	634.0806	– 0.94
Casuarictin	935.0784 $[M–H]^-$	$C_{41}H_{28}O_{26}$	936.0857	936.0869	– 1.28
Punicalagin	1083.0606 [M-H]-	$C_{48}H_{28}O_{30}$	1084.0678	1084.0665	1.20
Vescalagin	933.0624 $[M–H]^-$	$C_{41}H_{26}O_{26}$	934.0696	934.0712	– 1.71
Castalagin	933.0623 $[M–H]^-$	$C_{41}H_{26}O_{26}$	934.0695	934.0712	– 1.82
Vescavaloninic acid	1101.0664 $[M–H]^-$	$C_{48}H_{30}O_{31}$	1102.0737	1102.0771	– 3.08
Castavaloninic acid	1101.0667 $[M–H]^-$	$C_{48}H_{30}O_{31}$	1102.0734	1102.0771	– 3.35
Casuarinin	935.0784 $[M–H]^-$	$C_{41}H_{28}O_{26}$	936.0866	936.0856	1.07
Hippophaenin B	1103.0848 $[M–H]^-$	$C_{48}H_{32}O_{31}$	1104.0921	1104.0927	– 0.54
Rugosin D	936.0856 $[M–2H]^{2-}$	$C_{82}H_{58}O_{52}$	1874.1858	1874.1894	– 1.92
Rugosin E	860.0807 $[M–2H]^{2-}$	$C_{75}H_{54}O_{48}$	1722.1759	1722.1785	– 1.50
Agrimoniin	934.0697 $[M–2H]^{2-}$	$C_{82}H_{54}O_{52}$	1870.1539	1870.1581	– 2.24
Gemin A	935.0787 $[M–2H]^{2-}$	$C_{82}H_{56}O_{52}$	1872.1719	1872.1738	– 1.01
Oenothein A	1175.1082 $[M–2H]^{2-}$	$C_{102}H_{72}O_{66}$	2352.2308	2352.2278	1.27
Oenothein B	783.0668 $[M–2H]^{2-}$	$C_{68}H_{48}O_{44}$	1568.1482	1568.1518	– 2.29

Sanguin H-6	934.0704	[M–2H]$^{2-}$	$C_{82}H_{54}O_{52}$	1870.1553	1870.1581	– 1.50
Lambertianin C	933.7364	[M–3H]$^{3-}$	$C_{123}H_{80}O_{78}$	2804.2310	2804.2294	0.57
Rubusuaviin C	933.7336	[M–3H]$^{23-}$	$C_{123}H_{80}O_{78}$	2804.2227	2804.2293	–2.35
Cocciferin D2	933.0621	[M–2H]$^{2-}$	$C_{82}H_{52}O_{52}$	1868.1388	1868.1425	– 1.98
Salicarinin A	933.0626	[M–H]$^{-}$	$C_{82}H_{52}O_{52}$	1868.1398	1868.1425	– 1.44
Salicarinin B	933.0624	[M–H]$^{-}$	$C_{82}H_{52}O_{52}$	1868.1393	1868.1425	– 1.71

Table 5: Multiple reaction monitoring (MRM) parameters for UHPLC/ESI-QqQ MS/MS analysis.

No.	Compounds	Precursor ion (*m/z*)	MRM retention time window (min)	Cone voltage (V)	MRM1 (*m/z*)	Collision energy (eV)	MRM2 (*m/z*)	Collision energy (eV)	MRM2/MRM1 (%)
1	Monogalloyl glucose	331.1	0.20–0.29	40	168.9	20	270.9	16	30.6
2	Digalloyl glucose	483.0	0.73–0.82	40	168.9	35	210.9	40	58.6
3	Trigalloyl glucose	635.2	1.01–1.07	32	465.0	20	169.1	37	41.1
4	Tetragalloyl glucose	787.30	1.10–1.42	30	483.0	31	634.7	18	0.7
5	Pentagalloyl glucose	939.10	1.44–1.54	68	769.0	30	617.1	40	57.7
6	Hexagalloyl glucose	1091.3	1.54–1.64	38	939.1	28	769.1	36	33.9
7	Heptagalloyl glucose	1243.10	1.66–1.78	49	939.0	35	1091.3	30	29.7
8	Octagalloyl glucose	1395.1	1.70–2.00	50	939.3	50	769.0	60	30.6
9	Geraniin	951.20	1.02–1.12	49	301.0	45	933.0	23	24.3
10	Ascorgeraniin	1109.0	1.01–1.17	60	301.2	45	973.3	30	19.1
11	Chebulagic acid	953.10	1.25–1.35	59	301.0	40	275.1	45	27.0
12	Chebulanin	651.1	0.91–1.00	52	168.9	30	481.1	21	0.2
13	Chebulinic acid	955.0	1.37–1.46	55	168.9	56	205	68	70.6
14	Pedunculagin	783.1	0.60–0.82	50	301.0	40	274.7	36	8.8
15	Tellimagrandin I	785.20	0.98–1.16	50	300.9	40	275.0	41	64.3
16	Strictinin	633.1	0.80–0.98	44	300.8	36	274.9	35	10.2
17	Casuarictin	935.20	1.21–1.27	50	300.9	41	632.8	30	1.3
18	Punicalagin	1083.1	0.67–0.95	70	601.1	44	575.3	45	29.7
19	Vescalagin	933.4	0.50–0.58	42.0	915.1	20	301.0	40	40.4
20	Castalagin	933.2	0.45–0.80	50.0	301.0	35	424.9	29	7.5
21	Vescavaloninic acid	1101.0	0.35–0.50	31.0	1082.9	24	569.2	40	79.3
22	Castavaloninic acid	1101.0	0.45–0.70	60.0	1056.9	30	425.0	45	40.8
23	Casuarinin	935.20	0.93–1.00	44	274.8	31	632.7	42	0.39
24	Hippophaenin B	1103.3	0.94–1.02	48	1059.3	26	633.0	30	17.7
25	Rugosin D	935.4	1.35–1.41	40	301.2	58	274.8	59	15.3
26	Rugosin E	860.2	1.25–1.33	31	301	40	275.2	48	51.5
27	Agrimoniin	934.5	1.33–1.50	79	301.1	30	274.6	58	6.0

28	Gemin A	934.8	1.40–1.47	39	300.9	55	275.0	51	12.3
29	Oenothein A	1175.4	0.97–1.04	46	275.4	72	300.9	51	71.9
30	Oenothein B	783.5	0.50–1.25	78	300.8	40	765.2	25	23.5
31	Sanguiin H-6	934.4	1.25–1.33	40	301	41	314.8	48	18.6
32	Lambertianin C	935.0	1.21–1.28	79	300.9	58	275.1	59	18.9
33	Rubusuaviin C	934.2	1.24–1.31	39	300.8	52	275.1	41	6.9
34	Cocciferin D2	933.3	1.00–1.07	45	301.1	41	275.0	36	3.2
35	Salicarinin A	933.3	0.47–0.60	30	249.0	39	301.2	60	71.1
36	Salicarinin B	933.0	0.62–0.85	76	301.0	60	249.1	59	46.5

Table 6: Method parameters and quantification results of hydrolyzable tannins by QqQ-MS/MS analysis.

No.	Compounds	Quantification method (*m/z*)	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
1	Monogalloyl glucose	MRM 331.1>168.9	6.4-Nf	7.01	21.26
2	Digalloyl glucose	MRM 483.0>168.9	2-Nf	17.45	52.89
3	Trigalloyl glucose	MRM 635.2>465.0	18.6-Nf	66.61	201.85
4	Tetragalloyl glucose	MRM 787.30>483.0	51.2-Nf	79.91	242.16
5	Pentagalloyl glucose	MRM 939.10>769.0	35.2-Nf	173.67	526.26
6	Hexagalloyl glucose	MRM 1091.3>939.1	16.7-Nf	241.76	732.61
7	Heptagalloyl glucose	MRM 1243.10>939.0	4.8-Nf	31.83	96.47
8	Octagalloyl glucose	MRM 1395.1>939.3	6.6-Nf	95.23	288.56
9	Geraniin	MRM 951.20>301.0	91.2-Nf	669.80	2029.70
10	Ascorgeraniin	MRM 1109.0>301.2	23.3-Nf	356.92	1081.58
11	Chebulagic acid	MRM 955.10>301.0	59.1-Nf	458.64	1389.81
12	Chebulanin	MRM 651.1>168.9	428-Nf	1193.41	3616.39
13	Chebulinic acid	MRM 955.0>168.9	19.5-Nf	208.22	630.95
14	Pedunculagin	MRM 783.1>301.0	2.6-Nf	5.23	15.85
15	Tellimagrandin I	MRM 785.20>300.9	168.1-Nf	245.31	743.36
16	Strictinin	MRM 633.1> 300.8	2-Nf	12.17	36.88
17	Casuarictin	MRM 935.20>300.9	20.3-Nf	48.69	147.53
18	Punicalagin	MRM 1083.1>601.1	136.1-Nf	387.22	1173.4
19	Vescalagin	MRM 783.1>301.0	36.4-Nf	365.84	1108.62
20	Castalagin	MRM 933.4>915.1	Nf	787.87	2387.48
21	Vescavaloninic acid	MRM 933.2>301.0	Nf	402.52	1219.76
22	Castavaloninic acid	MRM 1101>1082.9	Nf	747.73	2265.84
23	Casuarinin	MRM 935.3>298.9	Nf	40.80	123.65
24	Hippophaenin B	MRM 1103.3>1041.9	Nf	125.75	381.06
25	Rugosin D	MRM 936.8>300.9	401.5-Nf	564.81	1711.56
26	Rugosin E	MRM 860.2>301	90.6-Nf	219.02	663.7
27	Agrimoniin	MRM 934.5>301.1	93.8-Nf	361.86	1096.55
28	Gemin A	MRM 934.8>300.9	21.9-Nf	62.26	188.67
29	Oenothein A	MRM 1175.4>275.4	75.7-Nf	137.79	417.55
30	Oenothein B	MRM 783.5>300.8	263.9-Nf	431.95	1308.92
31	Sanguiin H-6	MRM 934.4>301	37.5-Nf	53.20	161.20
32	Lambertianin C	MRM 935>300.9	37.5-Nf	324.15	982.28
33	Rubusuaviin C	MRM 934.2>300.8	106.1-Nf	238.55	722.87
34	Cocciferin D_2	MRM 933.3>301.1	1.5-Nf	3.61	10.93
35	Salicarinin A	MRM 933.3>249.0	13.6-Nf	27.92	84.62
36	Salicarinin B	MRM 933>301.0	Nf	226.52	686.42

Note: Nf: not found in the current experimental set up as the curve was linear throughout the dilution series

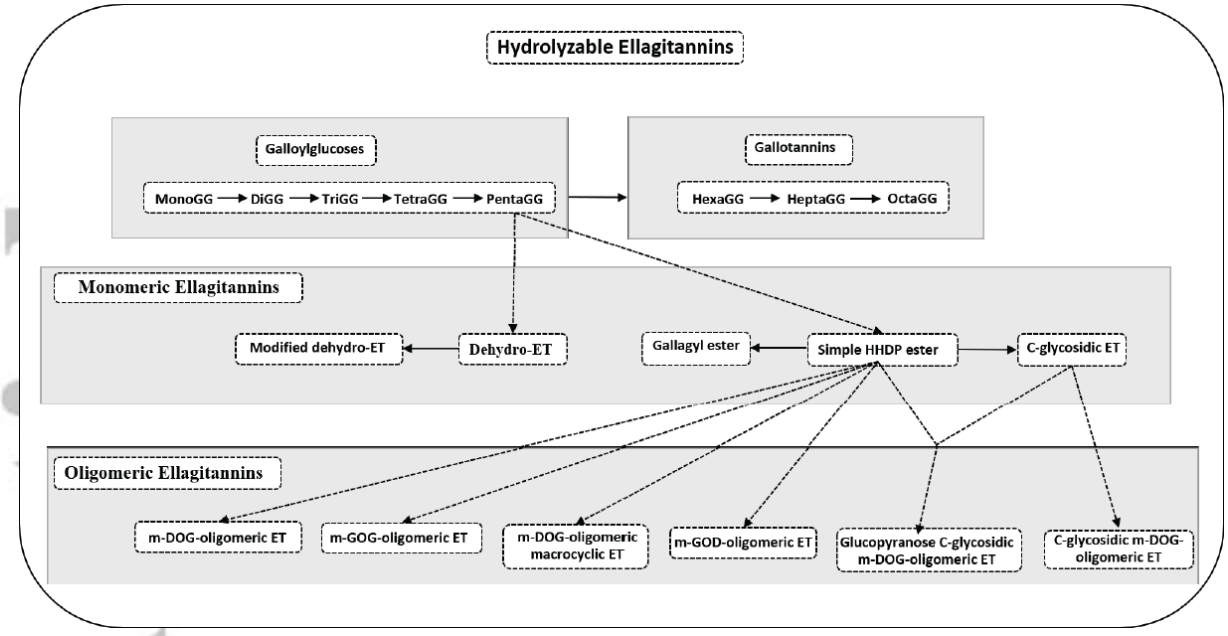

Figure 1

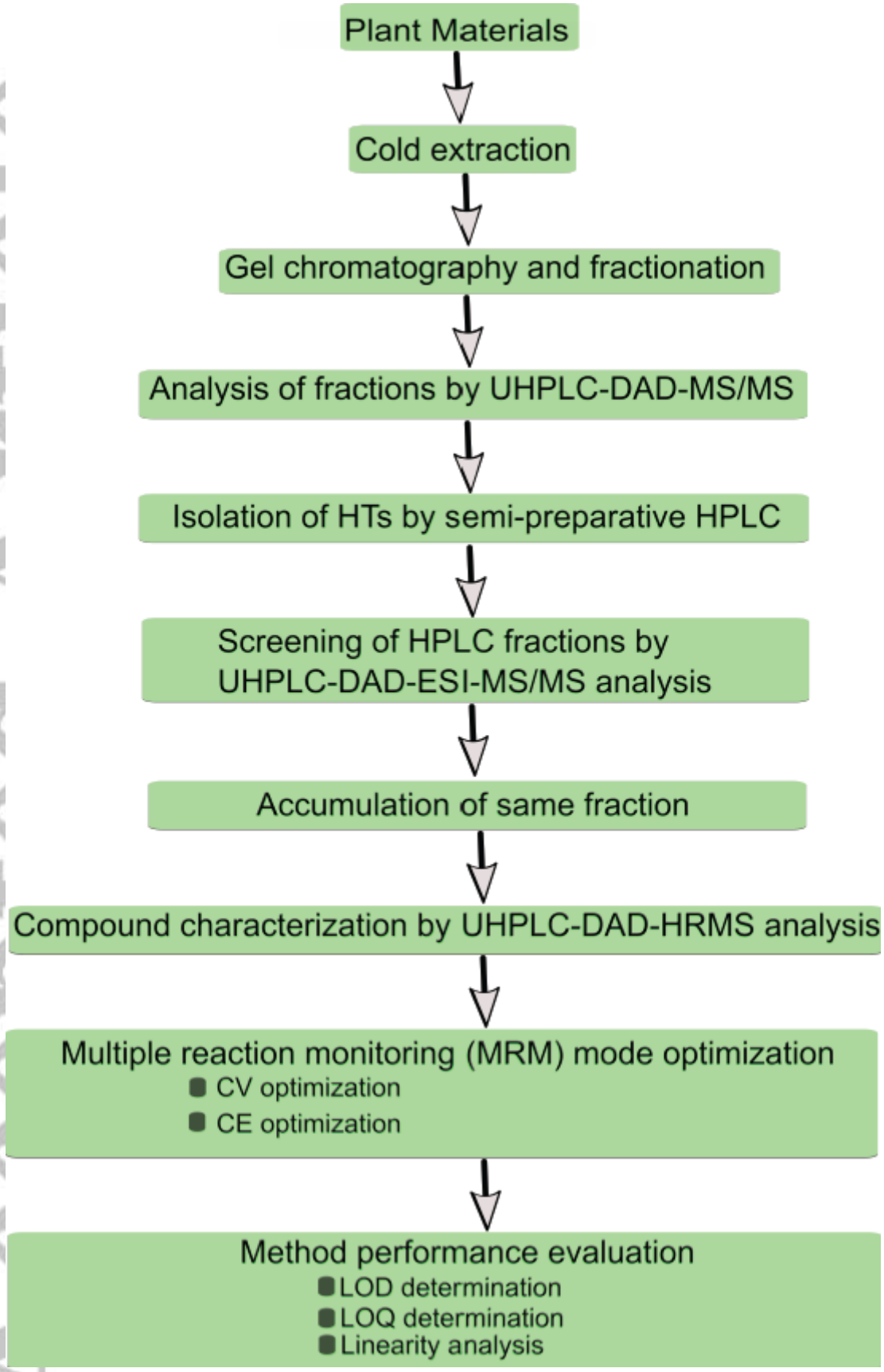

Figure 2

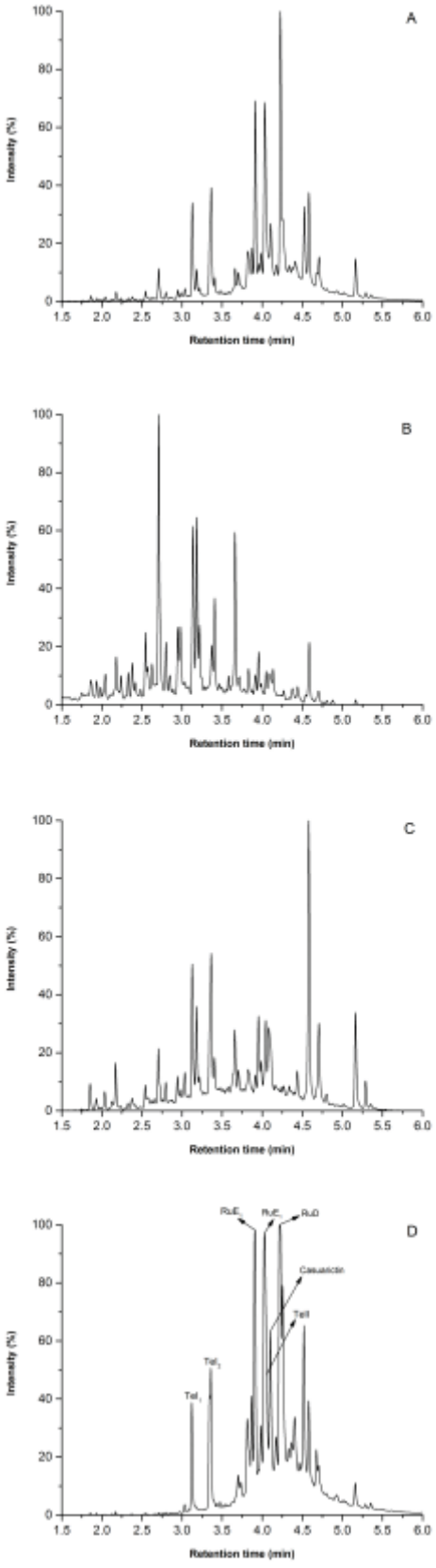

Figure 3

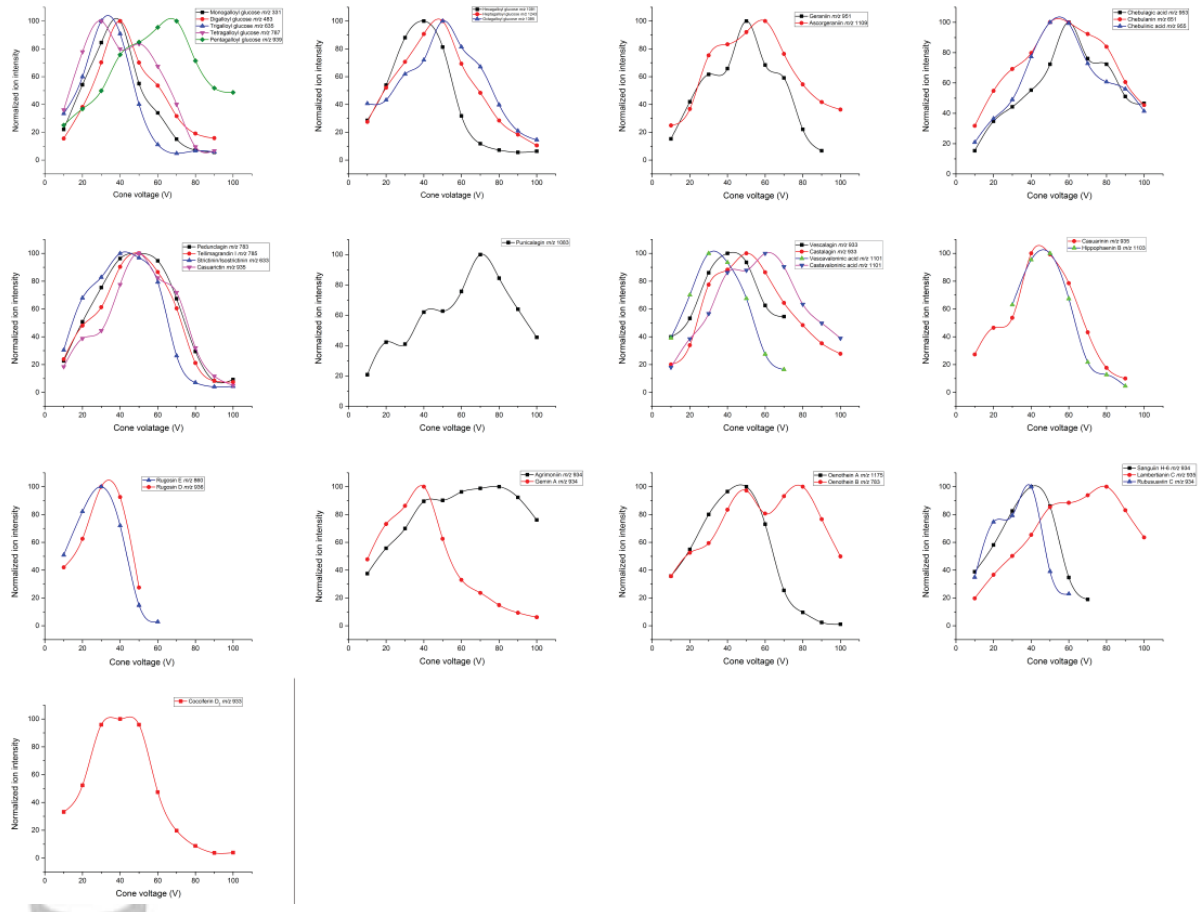

Figure 4

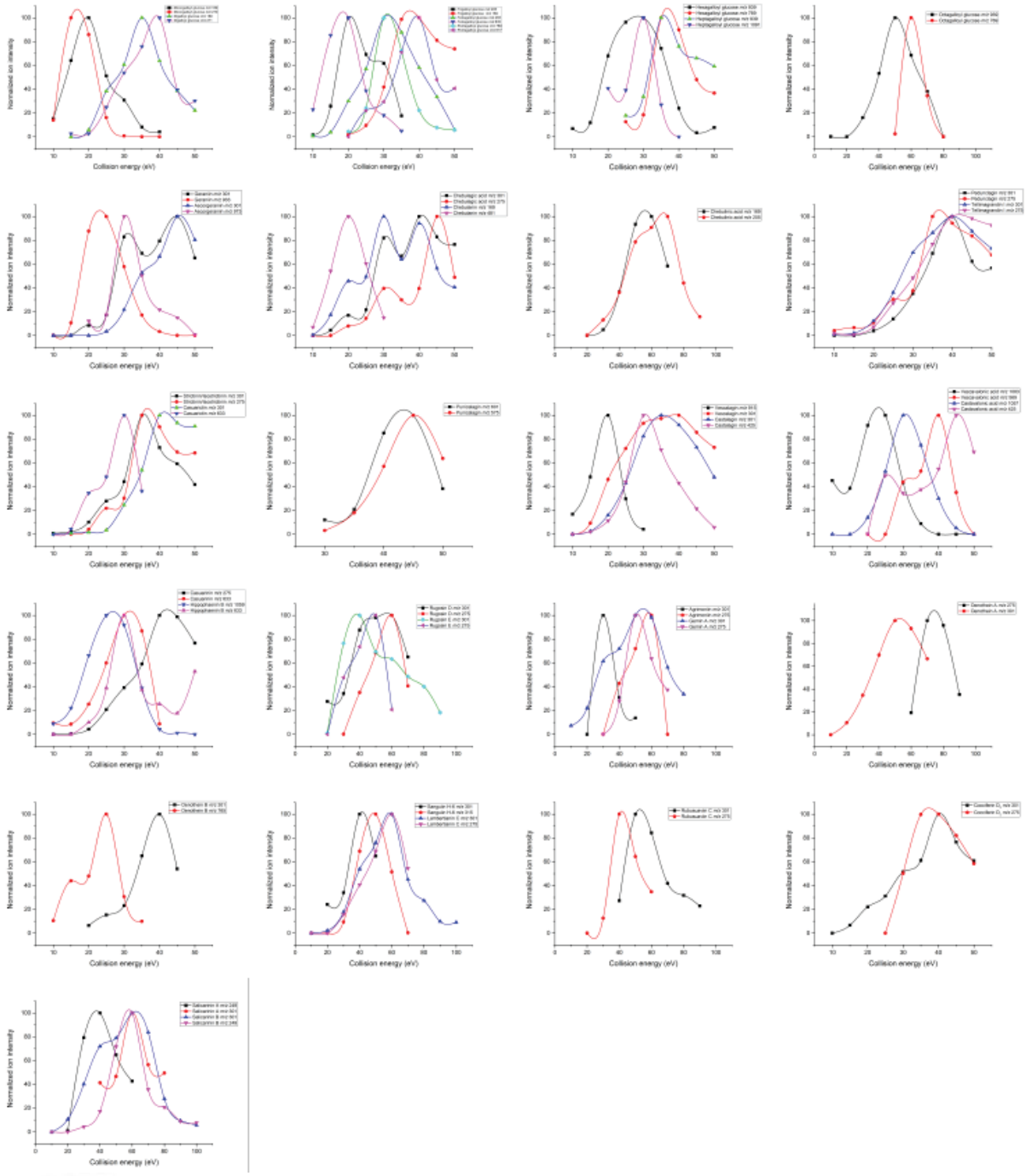

Figure 5