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Comprehensive characterization of C-glycosyl flavones in wheat (*Triticum aestivum* L.) germ using UPLC-PDA-ESI/HRMSⁿ and mass defect filtering

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Abstract

A comprehensive characterization of C-glycosyl flavones in wheat germ has been conducted using multi-stage high resolution mass spectrometry (HRMSⁿ) in combination with a mass defect filtering (MDF) technique. MDF performed the initial search of raw data with defined C-glycosyl flavone mass windows and mass defect windows to generate the noise-reduced data focusing on targeted flavonoids. The high specificity of the exact mass measurement permits the unambiguous discrimination of acyl groups (nominal masses of 146, 162 and 176.) from sugar moieties (rhamnose, glucose or galactose and glucuronic acid). A total of 72 flavone C-glycosyl derivatives, including 2 mono-C-glycosides, 34 di-C-glycosides, 14 acyl di-C-glycosides and 7 acyl tri-C-glycosides, were characterized in wheat germ, some of which were considered to be important marker compounds for differentiation of whole grain and refined wheat products. The 7 acylated mono-O-glycosyl-di-C-glycosyl flavones and some acylated di-C-glycosyl flavones are reported in wheat for the first time. The frequent occurrence of numerous isomers is a remarkable feature of wheat germ flavones. Both UV and mass spectra are needed to maximize the structure information obtained for data interpretation.

Keywords

di-C-glycosyl flavones; O-acylated-C-glycosyl flavones; mass defect filter; wheat germ; high resolution mass spectrometry

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Supporting Information

Supporting information may be found in the on line version of this article.

Notes

The authors declare no competing financial interest.

Introduction

Wheat has been a basic staple food in Europe, West Asia, and North Africa for over 8,000 years. Today, wheat is grown on more land area than any other commercial crop and continues to be the most important food grain source for humans.^[1] Whole grain wheat contains various health benefiting phytochemicals in addition to the basic and essential nutrients (carbohydrates, proteins, and dietary fiber). Among them, flavonoids, a large group of polyphenolic compounds, have gained attention due to their diverse health-promoting properties.^[2, 3] Flavonoids normally accumulate in plants as *O*-glycosylated derivatives, but the major cereal crops, predominantly synthesize flavone *C*-glycosides. In wheat, the glycosidic forms are mainly apigenin and luteolin 6-*C* and/or 8-*C*-glycosidic conjugates.^[4–7]

C-glycosyl flavonoids differ from *O*-glycosyl flavonoids in that their glycosyl moiety is attached via the anomeric carbon directly to the flavonoids backbone, almost exclusively at the *C*-6 or *C*-8 position of the A ring (Fig. 1A). In general, flavonoid *C*-glycosides are further divided into four classes: mono-*C*-glycosylflavonoids, di-*C*-glycosylflavonoids, *O*-glycosyl-*C*-glycosylflavonoids, (where *O*-glycosylation may be on the phenolic hydroxyl or on the sugar moiety from *C*-glycosylation, mainly at 2'' position followed by 6'' position), and *O*-acylated-*C*-glycosylflavonoids (in which different acyl groups are attached to the sugar moiety and/or to the flavonoid skeleton).^[8, 9]

Mass spectrometry has been extensively applied in the structure elucidation of flavonoid *O*-glycosides. The aglycone moiety, saccharide sequence, glycosylation position, and interglycosidic linkage position can be defined by a variety of MS methods.^[10–14] Their cleavage occurs mainly at the glycosidic *O*-linkages, leading to the elimination of sugar moiety. Analysis of *C*-glycosyl flavonoids is more challenging than *O*-glycosyl flavonoids due to the following two facts: 1) internal cleavage in the sugar is favored over breakage of the carbon-carbon bond between the flavonoids and sugar moiety in the MS/MS fragmentation of protonated or deprotonated molecules for *C*-glycosyl flavonoids, and 2) the lack of standards, in particular for di-*C*-glycosides or more complex subtypes. Although MS/MS has been successfully used to characterize some di-*C*-glycosyl and *O*-*C*-diglycosyl flavonoids,^[9, 15–18] complete structure elucidation by MS/MS is not routinely performed because authentic reference compounds with structural diversity are scarce and the relative intensities of characteristic fragments vary between instrument platforms and experimental conditions.

Profiling of flavonoid glycosides with LC-MS methods from wheat (*Triticum aestivum*) leaves^[7] or grains^[19, 20], and durum (*Triticum durum*) plant^[4] or grains^[21] was reported earlier. The wheat flavonoids are found to be mainly derivatives of only four flavones: apigenin, luteolin, chrysoeriol, and triclin. Tricin derivatives occur exclusively as *O*-glycosides, whereas apigenin and luteolin are always present as *C*-glycosides (Table 1). No special effort was dedicated to the study of *C*-glycosyl flavonoids of wheat germ. Our previous work has shown that apigenin 6-*C*-pentosyl-8-*C*-hexoside and its isomers from the germ fraction are the prominent discriminatory compounds to differentiate between whole grain and refined wheat flour.^[22] Asenstorfer et al. also reported that apigenin *C*-diglycosides which are responsible for the yellow color of alkaline noodle are located only

in the germ of wheat grains.^[23] Hence, in the current study, closer attention was paid to wheat germ regarding its flavonoids constituents owing to their nutritional value and biological activities.

However, profiling flavonoids in foods is a very challenging and laborious task. Usually advanced expertise in both polyphenols and MS are required. To perform comprehensive profiling on a routine basis put tremendous demands on the knowledge and time on the analysts due to the sample complexity and the resulting large and complex datasets collected during HR-MS, HR-MS/MS, and MSⁿ acquisitions. Using mass defect filtering (MDF) helps simplify the complex process by highlighting the candidate peaks of interest in raw data and save valuable time on data analysis.

The term “mass defect” originates from the fact that only the mono isotopic element ¹²C, has an integer value for atomic weight, i.e. 12.00000. Mass defect is defined as the difference between the exact mass of an element (or a compound) and its closest integer value. It may be positive (larger than the nominal mass) or negative (smaller than the nominal mass). Mass defect filtering (MDF) is a data filtering technique based on the mass defect of the core structure and its structural analogues that requires and takes advantage of HRMS. Initially, the MDF technique was developed for metabolite detection purposes based on a narrow and well-defined mass-defect window between the parent drug and its metabolites.^[24] It is available as a post-acquisition data processing tool for various mass spectrometry vendor software packages, including MetWorks (Thermo Fisher Scientific).

Similar to parent drug and its metabolites, a series of natural product analogs, like *C*-glycosyl flavones, share a common biosynthesis pathway and core-structure and differ in modification via oxidation, reduction, methylation, alkylation, rearrangement, etc. Therefore, they often possess similar mass defects.^[6, 25] Based on this concept, MDF could be applied to detect natural constituents in a similar way as in drug metabolite screening. When a well-defined filter was applied to LC-HRMS data, the majority of interference ions were automatically removed, and the resulting simplified data made data analysis more straightforward. The following interpretation of candidate peaks relies on the understanding of nature and fragmentation behaviors of target compounds. The new strategy employing MDF was complementary to the traditional molecular mass/MS/MS fragmentation-based LC/MS approaches and greatly facilitated the identification process.

The present work applies the MDF as a screening technique for singling out potential *C*-glycosyl flavones of the four different types from wheat germ extract in a rapid manner. Then traditional molecular mass/MS/MS fragmentation-based LC/MS methodology is employed for mass spectral interpretation for structural information on the sugar classes and sizes and their substitution pattern on an aglycone. HRMS and MS/MS scan make it possible to differentiate caffeoyl (162.0317 Da) and *p*-coumaroyl (146.0368 Da) moieties from hexosyl (162.0528 Da) and deoxyhexosyl (146.0579 Da) residues. The recognition of acylated flavones glycosides with phenolic acids is achieved without performing alkaline hydrolysis to remove acyl residues.^[26] To the best of our knowledge, this is the first report on systematic MS characterization of various *C*-glycosylated flavonoids in wheat germ.

Experimental

Materials and chemicals

Raw wheat germ was purchased from local grocery stores. Standard samples of vitexin, isovitexin, orientin, isoorientin, and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were purchased from VWR International, Inc. (Clarksburg, MD, USA). HPLC water was prepared from distilled water using a Milli-Q system (Millipore Laboratories, Bedford, MA, USA).

Sample preparation

The germ flakes were ground into a fine powder and then passed through a 60 mesh sieve. A 500 mg sample of the germ powder was extracted with 20.0 mL of methanol-water (7:3, v/v) using a sonicator (Advanced Sonic Processing Systems, Oxford, CT, USA) at 16 kHz with 300W power for 40 min. The slurry mixture was centrifuged at 13000 g for 15 min in a Contifuge 28RS centrifuge (Heraeus, South Plainfield, NJ) at 4°C. The supernatant was evaporated to dryness on a rotary evaporator (Buchi RotaVapor, R-200, Buchi, Switzerland) at 50 °C. The dried sample was dissolved in 2 ml water and loaded onto a C18 SPE cartridge (500 mg, JT Baker, Phillipsburg, NJ, USA). The loaded cartridge was washed with 5 mL of water, and the target compounds were eluted with 2 mL of methanol-water (4:6, v/v). The SPE elution was filtered through a 17 mm (0.20 µm) PVDF syringe filter (VWR Scientific, Seattle, WA) and stored at -80 °C until analysis.

The UPLC-HRMSⁿ conditions

The UPLC-HRMSⁿ system consists of a LTQ Orbitrap XL MS with an Acquity UPLC (Waters, Elstree, UK). The operating conditions of the ESI source are as follows: sheath gas at 80 (arbitrary units), auxiliary and sweep gas at 10 (arbitrary units), spray voltage at -4.0 kV; capillary temperature, 300 °C; capillary voltage, -50 V; tube lens offset, -150 V. The scan event cycle uses a FT full scan mass spectrum at a resolving power of 30,000 and three data-dependent MS², MS³, MS⁴ events acquired by LTQ triggered by the most abundant ion from each previous scan event. The automated gain control (AGC) target value was set to 200,000 for FT full scan, and 10,000 for IT MSⁿ scan. MSⁿ activation parameters used an isolation width of 1.0 amu, max ion injection time of 200 ms, normalized collision energy at 25% (unless otherwise stated), and an activation time of 30 ms. To obtain accurate mass MS² spectra, a dependent FT MS² scan at a resolving power of 15,000 after an FT full scan was used when needed.

The UPLC separation was carried out on a Waters Acquity UPLC BEH 1.7 µm-C₁₈ column (2.1 × 100 mm) with a flow rate of 0.25 mL/min. The mobile phase consist of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A gradient elution program was employed as follows: 10% B at 0–10.0 min, 15% B at 25.0–40.0min, 15% – 25% B over 40.0 –50.0 min, 25% –50% B over 50.0 –55.0 min, 50% –90% B over 55.0 – 60.0 min, and held at 90% B till 65.0 min. The column temperature was set at 40 °C. UV spectra were recorded between 200 and 500 nm.

Nomenclature

The fragmentation pattern of the sugar unit of *C*- and *O*-glycosyl flavones are present in Fig. 1. The cross-ring fragments of carbohydrates on flavonoid glycosides are denoted according to Domon and Costello.^[27] The symbols kX^- , Y^- , and Z^- represent the product ion still containing the intact aglycone, in which the superscripts k and l refer to the cleaved bonds within the carbohydrate rings. In some cases, a subscript, H, D or P, is added to labels referring to hexose, deoxyhexose, or pentose, respectively.

Results and discussion

MDF strategy to capture C-glycosyl flavones

Phytochemical investigations have shown that *C*-glycosides based on apigenin and luteolin are widespread in the genus *Triticum*.^[23, 28–32] The MDF setting was defined using these published information. Glycosylation and acylation are the predominant modifications of wheat flavonoids. The sugars involved in the glycosylated flavonoids could be grouped into hexose (glucose and galactose), deoxyhexose (rhamnose), and pentose (arabinose and xylose). Triticum species contain *O*-glycosyl-*C*-glycosyl flavones acylated with a phenolic acid, i.e. ferulic acid, sinapic acid, *p*-coumaric acid, and caffeic acid.^[9, 33] The mass value and mass defect of mono-, di-, tri-glycoside candidates, in free form or acylated, are predictable according to the core structure and possible substituents. Table 2 lists the mono-, di-, tri-, and feruloyl/sinapoyl di-glycoside candidates of apigenin and luteolin according to the literature cited previously. More predicted candidates are shown in supporting material Table S1.

A good mass defect filter of *C*-glycosyl flavones depends strongly on defining two windows: the “mass defect window” (MDW) and the “*C*-glycosyl flavone mass window” (CFW), according to predicted substituents. An appropriate MDW will filter out those ions that fall into the CFW but out of the MDW, and vice versa. Five mass defect filters were constructed for screening *C*-glycosyl flavones in the crude extract of wheat germ with apigenin and luteolin as template compounds. Since these two aglycones are structurally similar (luteolin is 3'-OH apigenin), the mass defect profile calculated based on them are partly overlapped, as shown in Fig. 2. Each symbol represents a flavone glycoside candidate plotted against the accurate mass and mass defect values. The mass defect filters (shown in Fig 2 as dotted rectangles) with CFW/MDF setting slightly wider (± 0.1 – 0.2 Da/ ± 1.0 – 2.0 mDa) but covered all potential target compounds are: 1) 400.8–447.3 Da/80.0–104.0 mDa for flavone mono-glycosides; 2) 533.0–609.3 Da/122.0–161.5 mDa for di-glycosides; 3) 665.0–771.5 Da/164.0–219.0 mDa for tri-glycosides; 4) 709.0–815.5 Da/169.0–219.0 mDa for acyl-diglycosides, and 5) 841.0–977.5 Da/211.0–276.5 mDa for acyl-triglycosides. The unfiltered total ion chromatogram (TIC) and five filtered MS chromatograms are compared in Fig. 3, wherein different peaks were picked out from the TIC by each filter. It is easy to see that the MDF allows the analyst to quickly zero in on the respective groups of compounds, thus, greatly facilitating the identification process. The peak profiles in Fig. 3D and 3E are very similar, it is reasonable because the filter settings for tri-glycosides and acyl-diglycosides mostly overlap, as seen in Fig. 2. The structural characterization of the candidates relies on retention times, UV spectra, MS spectra (HRMS, HR-MS/MS, MS³, MS⁴), and the use of

standards when available (stacked chromatograms of the UV traces and BPC are shown in Fig. S1). A total of 72 *C*-glycosyl flavones, including 2 mono-, 34 di-, 15 tri-, 14 acyl di-, and 7 acyl tri-glycosides, were characterized (Table 3).

Mono-*C*- and di-*C*-glycosyl flavones

In contrast to *O*-glycosides, which can be easily characterized by the loss of sugar moiety, the fragmentations of *C*-glycosides occur preferentially on the glycosidic moiety. The product ions are usually aglycones with sugar moieties that remain linked. Thus, the ions $[\text{Ag}+41]^-/[\text{Ag}+71]^-$ in mono-*C* and $[\text{Ag}+83]^-/[\text{Ag}+113]^-$ in di-*C* are indicative of the nature of the aglycones (Table 1).^[9, 16] Since the vast majority of *C*-linked sugars have been found at *C*-6- and/or *C*-8- positions,^[8] the major challenge here is the differentiation between 6-*C*- and 8-*C*-glycosyl flavones and the determination of the type of sugar substituted at the *C*-6 and *C*-8 positions in 6,8-di-*C*-glycosyl flavones.

Four authentic mono *C*-glucosides (vitexin, isovitexin, orientin, and isoorientin) were compared by collision induced dissociation (CID) analysis in the LTQ Orbitrap platform described in the instrument section. The CID behaviors of the two groups of isomers at varying collision energy are shown in Fig. 4. The fragmentation characteristics are similar to those reported by other researchers.^[10, 16, 26, 34, 35] Loss of water in the deprotonated ion $[\text{M}-\text{H}]^-$ are much more pronounced for 6-*C*- than 8-*C*-glycosyl flavonoids.^[16, 36] 6-*C*-isomers can easily lose water between the 2''-hydroxyl group of the sugar and the 5- or 7-hydroxyl groups of the aglycone, which is facilitated by hydrogen bonding between either the oxygen atom of the sugar ring and the 5- or 7-hydroxyl group of the aglycone part; whereas 8-*C*-glycosyl flavonoids lose water only with the 7-hydroxyl group, a process that is counteracted by hydrogen bonding between either the oxygen atom of the sugar and the 7-hydroxyl group of the aglycone.^[10, 37] The other distinctive difference between 6-*C*- and 8-*C*-isomers lies in the relative intensities of ion $^{0,3}\text{X}$, which are higher in 6-*C*-isomers in comparison to 8-*C*-isomers (Fig. 4B vs. 4A; 4D vs. 4C). Collision energy at 25% selected in this study achieved reliable and distinguishable spectra.

Peaks M1 and M2 (Fig. 3B) were identified as vitexin (apigenin 8-*C*-glucoside) and isovitexin (apigenin 6-*C*-glucoside), respectively, by chromatographic and mass spectral analysis with reference standards. Vitexin elutes ahead of isovitexin on our reverse phase LC system. The result is in agreement with previous reports that *C*-8-glycoside elutes before its *C*-6 isomer.^[38–40]

In di-*C*-glycosides, preferential fragmentation of the sugar moiety is at the *C*-6 rather than the *C*-8 position. However, it is difficult to establish the position of *C*-glycosylation if there is no co-existence of both isomers. Wheat flavonoids are featured with multiple isomers, as shown in Table 3 and other reports.^[19, 23] It is not possible to determine whether these two compounds are a pair of Wessely-Moser isomers or have isomeric sugars by MS alone. The factors that influence the relative intensities of cross-ring cleavage fragments (including $[\text{M}-\text{H}-120]^-$, $[\text{M}-\text{H}-90]^-$, and $[\text{M}-\text{H}-60]^-$) are complicated. Cao et al. found that the relative intensities of these structure-diagnostic ions are highly dependent on the nature and anomeric configuration of the sugars at *C*-6 and *C*-8.^[18] Based on the above considerations,

the precise placement of pentose, hexose, and deoxyhexose, at the *C*-6 or *C*-8 was not determined in this study.

Compounds D6, D8, D11, D12, D14, D15, D16, D17, D18, D20, D21, D23, and D30 present the same deprotonated molecule ion at m/z 563.1406 ($C_{26}H_{27}O_{14}$). They share some major product ions in the MS^2 spectra as shown in Fig. 5. They all produce the fragment ions at m/z 353 (**Ag**+83) and 383 (**Ag**+113), which are characteristic of the di-*C*-glycosylflavone fragmentation, suggesting the trihydroxylated nature of the aglycone (Table 1, apigenin, MW 270). The UV spectra of these peaks presented λ_{max} at 270–272 and 335–337 nm, providing additional evidence of di-*C*-glycosyl apigenin.^[39, 41, 42] The ion at m/z 473 $[M-H-90]^-$ could be produced by $^{0,2}X$ cleavage of a pentose or $^{0,3}X$ cleavage of a hexose. The $[M-H-60]^-$ ion at m/z 503 and the $[M-H-120]^-$ ion at m/z 443 can only be produced by cleavage of the 0,3 bond of *C*-pentosyl ($^{0,3}X_P$) and the 0,2 bond of *C*-hexosyl ($^{0,2}X_H$), respectively. (Fig. 1). The ion at m/z 545 was produced by losing one molecule of H_2O . Therefore the compounds are concluded to be isomers of 6,8-di-*C*-hexosyl/pentosyl apigenin.

Full scan spectra of compounds D3, D4, D7, D19, D22, D25, and D27 had the deprotonated molecular ion at m/z 593.1512 ($C_{27}H_{29}O_{15}$). The MS^2 and MS^3 analyses of these compounds provided a typical fragmentation of di-*C*-glycosyl flavones, and also indicate two different patterns of aglycone-sugar combination. The multi-stage mass spectra of representative compounds D7 and D27 are shown in Fig. 6. In Fig. 6A, simultaneous neutral loss of 120 and 90 Da from $[M-H]^-$ ion at m/z 593 and successive cross-ring cleavage from ions at m/z 473 and 503 suggest the presence of two hexoses; and the ions at m/z 353 (**Ag**+83) and 383 (**Ag**+113) indicate the aglycone as a trihydroxyflavone. However, in Fig. 6B, the ion at m/z 533 ($[M-H-60]^-$) in the MS^2 spectrum and ions at m/z 413 ($[M-H-120-60]^-$)/383 ($[M-H-120-90]^-$) in the MS^3 spectrum of $[593 \rightarrow 473]$ reveal the pentose nature of the saccharide; and the ions at m/z 383 (**Ag**+83) and 413 (**Ag**+113) indicated the aglycone to be chrysoeriol (MW 300, Table 1). The UV spectra show two maxima at 270 and 334–335 nm for D3, D4 and D7; and at 270 and 348 nm for D19, D22, D25 and D27. These UV data are in accordance to *C*-glycosyl apigenin and chrysoeriol, respectively.^[41, 42] Taking into account the UV and MS information, D3, D4, and D7 were putatively assigned as apigenin 6,8-di-*C*-hexosides, whereas D19, D22, D25, and D27 were assigned as 6,8-di-*C*-hexosyl/pentosyl chrysoeriol.

In a similar way, more 6,8-di-*C*-glycosyl (hexosyl/pentosyl/deoxyhexosyl) flavones (apigenin/luteolin/chrysoeriol) were characterized and listed in Table 3.

O-glycosyl-C-glycosyl flavone

Ferreres and co-workers^[9, 26] investigated the fragmentation behaviors of *O*-glycosyl-*C*-glycosyl flavones with *O*-glycosylation on phenolic hydroxyl or on the *C*-glycosyl residue or combination of both forms by ion trap in the negative mode, and revealed some useful features to characterize them. These features are: the occurrence of an abundant ion Y^- ($[M-H-132/-146/-162]^-$) (Fig. 1A) after MS^2 fragmentation characterizes the *O*-glycosylation on phenolic hydroxyls; the preferential fragmentation leading to a relevant Z^- ($[Y-18]^-$) (Fig. 1B) fragment is characteristic of 2''-*O*-glycosyl-*C*-glycosyl derivatives; and the *O*-

glycosylation on the hydroxyl at 6'' of the sugar moiety from *C*-glycosylation leads to $^{0,2}\text{X}^-$ that implies a global loss of the sugar moiety from *O*-glycosylation plus the fraction of the sugar moiety from *C*-glycosylation which contains the carbons 6''–3'' ($[\text{M}-\text{H}-162-120]^-$, in the case of 6''-*O*-hexosyl-*C*-hexosyl derivatives) (Fig. 1C).

The *O*-glycosyl-*C*-glycosyl flavones encountered in wheat germ are more complicated than the model compounds used in the study of Ferreres et al. The identified *O*-glycosyl-*C*-glycosyl flavones in this work are all *O*-glycosylated *C*-*C*-di-glycosyl flavones. The diversity and similarity of these spectra indicate that the differences of fragmentation are derived from not only the position of *O*- and *C*-glycosylation, but also the sugar type and even the anomeric configuration of the sugars (Fig. S2, Table 3). Moreover, spectral evidence implies that 3'' or 4''-*O*-glycosylation may be present as well.

A total of 11 compounds possess the same deprotonated molecular ion at m/z 725.1935 ($\text{C}_{32}\text{H}_{37}\text{O}_{19}$) with an additional 162.0528 mass shift (compared to compounds D11, D16, and D17 etc.), suggesting an additional *O*-hexosyl moiety (not caffeoyl, 162.0317). This hypothesis is confirmed by the fragmentation pattern shown in Fig. 7, and more spectra of the representative isomers are shown in Fig. S2. The spectra of T2, T5, and T6 are quite different but have one common feature; the fragment ions at m/z 353 ($\text{Ag}+83$) and 383 ($\text{Ag}+113$) indicate derivatives of 6, 8-di-*C*-glycosyl apigenin as mentioned above. In Fig. 7A, a global loss of the sugar moiety from *O*-glycosylation and a fraction of the sugar moiety from *C*-glycosylation provide $^{0,2}\text{X}^-_{\text{H}}$ (m/z 443, $[\text{M}-\text{H}-162-120]^-$) and $^{0,3}\text{X}^-_{\text{H}}$ (m/z 473, $[\text{M}-\text{H}-162-90]^-$), suggesting a 6''-*O*-hexosyl-*C*-hexosyl structure. The successive loss of 60 and 90 Da resulted from ions at m/z 443 and 473 in the MS^3 spectra implies the other *C*-glycosylated sugar is pentose. Therefore, T2 could be considered as *C*-(6''-*O*-hexosyl) hexosyl-*C*-pentosyl apigenin. The base peak observed in MS^2 spectrum of T5 (Fig. 7B) at m/z 563 corresponded to the loss of a hexosyl moiety, in agreement with the *O*-glycosylation on phenolic hydroxyls; other fragments produced by losing 60, 90, and 120 Da suggest the presence of *C*-hexosyl-*C*-pentosyl apigenin. Thus, it was putatively assigned as *O*-hexosyl-*C*-hexosyl-*C*-pentosyl apigenin. The 7-OH in flavones is favored to be glycosylated,^[4] but no sufficient evidence was obtained to exclude other sites (e.g. 4'-OH). The predominant ion at m/z 545 (Z ion, $[\text{M}-\text{H}-162-18]^-$) led T6 to be more likely a 2''-*O*-hexosylated than 6''-*O*-hexosylated compound (Fig. 7C). A global loss of the hexosyl moiety and a fraction of *C*-glycosylation generated a fragment ion at m/z 473 ($[\text{M}-\text{H}-162-90]^-$) with absence or very scarce presence of an ion at m/z 443 ($[\text{M}-\text{H}-162-120]^-$). In the MS^3 spectra of $[725 \rightarrow 545]$ and $[725 \rightarrow 473]$ and MS^4 spectra of $[725 \rightarrow 545 \rightarrow 527]$, major fragments generated from the internal cleavage of a hexose by losing 90 and 120 Da are observed. All the information indicates that the *O*-hexosyl is linked to *C*-pentosyl, not *C*-hexosyl, and the *C*-hexose is unsubstituted. Hence, the ion at m/z 473 ($[\text{M}-\text{H}-162-90]^-$) was $^{0,2}\text{X}^-_{\text{P}}$, not $^{0,3}\text{X}^-_{\text{H}}$. The presence of this ion demonstrated the *O*-hexosyl was linked to position 3'' or 4'' instead of 2'' of the *C*-pentosyl moiety. Compound T6 was putatively assigned as *C*-(3'' or 4''-*O*-hexosyl) pentosyl-*C*-hexosyl apigenin, which is reported in wheat for the first time.

More mono-*O*-glycosyl-di-*C*-glycosyl flavones characterized from wheat germ are listed in Table 3.

O-acylated-C-glycosyl flavone

Some plant secondary metabolites occur in the form of glycosides acylated by phenolic acids such as *p*-coumaric, caffeic, and ferulic acid, etc. The main problem in interpreting the mass spectra of these compounds arises from the fact that the neutral loss of these acyl groups (nominal masses of 146, 162, and 176, respectively) is equal to that of common glycosidic moieties (rhamnose, glucose or galactose and glucuronic acid). For this reason, high resolution MS/MS spectra are necessary for acyl group identification. UV spectra can also help to recognize acylated *C*-glycosyl flavone. The majority of them show a UV spectrum with an intense band I (approximately 330 nm) and a small band II (approximately 270 nm) as a result of the overlapped UV spectra of flavonoid and cinnamoyl acid.^[17] Sinapoyl, feruloyl, *p*-coumaroyl, syringoyl, and vanilloyl were identified from 21 acylated di- or tri- glycosides (Table 3). Fourteen of them were acylated flavone di-*C*-glycosides; the remaining seven are acylated mono- *O*-glycosyl-di-*C*-glycosyl flavones.

In the MS² spectra of compounds AD1-AD14, the base peak was produced by loss of the radical acyl and a molecule of water ([M-H-acyl-18]⁻), with [M-H-acyl]⁻ also detected (Table 3, Fig. 8A). This fragmentation behavior was similar to that observed in *C*-glycosyl flavones *O*-glycosylated in position 2'', indicating that the acylation was probably in the 2''-OH of the *C*-glycosylated sugar. Compound AD7 is illustrated in Fig. 8A. Apart from the [M-H-sinapoyl-18]⁻ and [M-H-sinapoyl]⁻ ions that show up in MS², cross-ring cleavage ions of the *C*-glycosyl moiety were observed in the following MS³ and MS⁴ spectra, indicating a *C*-hexosyl (-90, -120 Da) and *C*-pentosyl (-60, -90 Da). The aglycone indicative ions appear as [A+83-18]⁻ and [A+113-18]⁻ instead of [A+83]⁻ and [A+113]⁻ in this category. Previous reports have tried to determine the attached position of acyl group in *C*-6 or *C*-8-glycosyl by comparing the relative intensity of cross-ring cleavage ions from a pair of isomers^[17] or depending on whether the [M-H-acyl-18]⁻ or [M-H-acyl]⁻ ion was detected as the base peak,^[18] But there is not enough evidence to support these conclusions. Therefore, AD7 was determined to be 2''-*O*-sinapoyl, *C*-pentosyl-*C*-hexosyl apigenin.

Acylated tri-glycosides (AT1, AT4, and AT7) exhibited large differences in fragmentation patterns as shown in Fig. 8B, 8C, and 8D. The base peak at *m/z* 739 in AT1 (Fig. 8B) is due to the loss of 162.0528 Da (*O*-hexosylation on the phenolic hydroxyl), another major ion in the full MS spectrum was *m/z* 545 (loss of feruloyl+18 and of a hexosyl moiety). It was also the major product ion in MS³ spectrum (901→739), indicating that the acylation had a great chance to be on the 2''-OH of *C*-glycosylation; neutral loss of 120 Da (901→545→MS³) and 90/60 Da (901→545→425→MS⁴) typified a *C*-glycosylated hexose and pentose, respectively. The acyl group of AT4 and AT7 was assigned to be on the 2''-OH of *C*-glycosylation for their base peak at [M-H-acyl-18]⁻ in their MS² spectra (Fig. 8C, Fig. 8D), just as AD7; but the following fragmentation of ion [M-H-acyl-18]⁻ at *m/z* 707 demonstrated different glycan structure in these two compounds. For AT4, the product ion at *m/z* 587 in MS³ of [931→707] generated by cleavage of 120 Da suggested a *C*-hexosylation; further MS⁴ fragmentation of *m/z* 587 gave two major product ions at *m/z* 425 (loss of hexosyl) and 335 (loss of hexosyl and ^{0,2}X_P cross-ring cleavage), which indicated a *C*-(3'' or 4''-*O*-hexosyl) pentosyl moiety. In the case of AT7, ions produced from the internal cleavage of the sugars from *C*-glycosylation plus hexosyl (-162-120 and -162-90)

were detected, providing the evidence of a *C*-(4'' or 6''-*O*-hexosyl) hexosyl moiety. Ion [A +83–18][–] (*m/z* 335) and/or [A+113–18][–] (*m/z* 365) in their respective MS⁴ spectra indicate AT1, AT4, and AT7 have the same aglycone apigenin. Consequently, they were assigned as 2''-*O*-feruloyl, *O*-hexosyl-*C*-pentosyl-*C*-hexosyl apigenin, 2''-*O*-sinapoyl, *C*-(3'' or 4''-*O*-hexosyl) pentosyl-*C*-hexosyl apigenin, and 2''-*O*-feruloyl, *C*-(4'' or 6''-*O*-hexosyl) hexosyl-*C*-pentosyl apigenin.

Conclusions

The combination of UPLC-PDA-ESI/HRMSⁿ and MDF proves to be a powerful and efficient technique in the structural elucidation of four different types of *C*-glycosyl flavones. MDF based on precisely defined mass defect windows and mass windows removes noise and highlights the targets, enabling MS profiling work more versatile and routine. By using the MSⁿ fragmentation rules of flavone mono-*C*-glycosides and di-*C*-glycosides from the standards and previous reports, 72 *C*-glycosyl flavones were putatively identified in the extract of wheat germ. Accurate mass measurement of MS² spectra differentiated flavone diglycosides acylated with hydroxycinnamic acid from their isobaric flavone triglycosides. Among the characterized compounds, fourteen were acylated di-*C*-glycosyl flavones and seven were acylated mono-*O*-glycosyl-di-*C*-glycosyl flavones. It should be noted that no MS fragmentation rules of these acylated di-*C*-glycosyl or mono-*O*-glycosyl-di-*C*-glycosyl flavones based on authentic compounds have been previously reported. Our study provides useful information for their structure elucidation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fragmentation nomenclature commonly used for flavonoid glycosides. A: 6-*C*-hexosyl-8-*C*-pentosyl-7-*O*-hexosyl apigenin; B: 6-*C*-(2''-*O*-hexosyl)-hexosyl flavonoid; C: 6-*C*-(6''-*O*-hexosyl)-hexosyl Flavonoid

Figure 2.

Mass defect profiles of predicted flavone glycoside candidates. Symbols: ○ apigenin as template, △ luteolin as template. A: non-acylated mono-, di-, and tri-glycosides; B: acylated di- and tri-glycosides.

Figure 3.

The unfiltered TIC of raw data (A) and filtered chromatograms obtained by different CFW/MDW setting as follows: (B) 400.8–447.3 Da/80.0–104.0 mDa for mono-glycosides; (C) 533.0–609.3 Da/122.0–161.5 mDa for diglycosides; (D) 665.0–771.5 Da/164.0–219.0 mDa for triglycosides; (E) 709.0–815.5 Da/169.0–219.0 mDa for acyl-diglycosides; and (F) 841.0–977.5 Da/211.0–276.5 mDa for acyl-triglycosides.

Figure 4.

High resolution MS/MS spectra of vitexin (A: apigenin 8-*C*-glucoside), isovitexin (B: apigenin 6-*C*-glucoside), orientin (C: luteolin 8-*C*-glucoside) and isoorientin (D: luteolin 6-*C*-glucoside) at different collision energies.

Figure 5. MS/MS spectra of the ions at m/z 563.14 corresponding to compounds D11, D12, D16, D17, and D21.

Figure 6.
MS2 and MS3 spectra of compounds D7 (A) and D27 (B).

Figure 8. MSⁿ spectra of acylated compounds. A: AD7; B: AT1; C: AT4; and D: AT7.

Table 1

Aglycone structure of C-glycosyl flavones identified in wheat

Name	Structure	Formula	MW	[M-H] ⁻	Diagnostic ions of di-C-glycosides
apigenin		C ₁₅ H ₁₀ O ₅	270.0528	269.0455	353(Ag+83), 383(Ag+113), 335(Ag+83-18), 365(Ag+113-18)
luteolin		C ₁₅ H ₁₀ O ₆	286.0477	285.0405	369(Ag+83), 399(Ag+113), 351(Ag+83-18), 381(Ag+113-18)
chrysoeriol		C ₁₆ H ₁₂ O ₆	300.0634	299.0561	383(Ag+83), 413(Ag+113), 365(Ag+83-18), 395(Ag+113-18)

Table 2

Predicted glycoside candidates of apigenin and luteolin

substituents ^d		flavone glycoside candidates			
		Apigenin		Luteolin	
		[M-H] ⁻	mass defect (mDa)	[M-H] ⁻	mass defect (mDa)
mono-glycoside	pen	401.0875	86.7	417.0825	81.6
	dhex	415.1031	102.3	431.0981	97.2
	hex	431.0980	97.2	447.0930	92.1
diglycoside	pen,pen	533.1295	128.7	549.1245	123.6
	pen,dhex	547.1451	144.3	563.1401	139.2
	pen,hex	563.1400	139.2	579.1350	134.1
	dhex,hex	577.1556	154.8	593.1506	149.7
	hex,hex	593.1505	149.7	609.1455	144.6
	dhex,dhex	561.1607	159.9	577.1557	154.8
triglycoside	pen,pen,pen	665.1715	170.7	681.1665	165.6
	pen,pen,dhex	679.1871	186.3	695.1821	181.2
	pen,pen,hex	695.1820	181.2	711.1770	176.1
	dhex,dhex,dhex	707.2183	217.5	723.2133	212.4
	dhex,dhex,pen	693.2027	201.9	709.1977	196.8
	dhex,dhex,hex	723.2132	212.4	739.2082	207.3
	hex,hex,hex	755.2030	202.2	771.1980	197.1
	hex,hex,dhex	739.2081	207.3	755.2031	202.2
	hex,hex,pen	725.1925	191.7	741.1875	186.6
	pen,dhex,hex	709.1976	196.8	725.1926	191.7
feruloyl-diglycoside	feruloyl,pen,pen	709.1766	175.8	725.1716	170.7
	feruloyl,pen,dhex	723.1922	191.4	739.1872	186.3
	feruloyl,pen,hex	739.1871	186.3	755.1821	181.2
	feruloyl,dhex,hex	753.2027	201.9	769.1977	196.8
	feruloyl,hex,hex	769.1976	196.8	785.1926	191.7
	feruloyl,dhex,dhex	737.2078	207.0	753.2028	201.9

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flavone glycoside candidates			
substituents ^a	Apigenin		Luteolin
	[M-H] ⁻	mass defect (mDa)	[M-H] ⁻ mass defect (mDa)
sinapoyl-diglycoside	sinapoyl, pen, pen	739.1871	186.3
	sinapoyl, pen, dhex	753.2027	201.9
	sinapoyl, pen, hex	769.1976	196.8
	sinapoyl, dhex, hex	783.2132	196.8
	sinapoyl, hex, hex	799.2081	212.4
	sinapoyl, dhex, dhex	767.2183	207.3
sinapoyl, hex, hex	sinapoyl, hex, hex	799.2081	207.3
	sinapoyl, dhex, dhex	767.2183	202.2
	sinapoyl, hex, hex	799.2081	202.2
	sinapoyl, dhex, dhex	767.2183	202.2
	sinapoyl, hex, hex	799.2081	202.2
	sinapoyl, dhex, dhex	767.2183	202.2

^a hex, hexosyl; pen, pentosyl; dhex, deoxyhexosyl.

Table 3

The C-glycosyl flavones characterized in wheat germ

Peak No.	ion <i>m/z</i>	RT	error (ppm)	RDB	Formula [M-H] ⁻	MS ⁿ data	partial identification ^d
M1	431.0985	32.27	0.302	12.5	C ₂₁ H ₁₉ O ₁₀	MS2[431.1]: 341.1(7), 311.1(100); MS3[431.1→311.1]: 283.1(100), 268.0(2), 191.0(2)	vitexin
M2	431.0985	33.96	0.302	12.5	C ₂₁ H ₁₉ O ₁₀	MS2[431.1]: 413.1(6), 341.1(29), 311.1(100); MS3[431.1→311.1]: 283.1(100)	isovitexin
D1	609.1451	19.09	-1.655	13.5	C ₂₇ H ₂₉ O ₁₆	MS2[609.2]: 591.4(6), 519.3(27), 489.1(100), 471.4(8), 399.2(15), 369.4(12)	luteolin 6,8-di-C-hex
D2	579.1356	19.48	0.098	13.5	C ₂₆ H ₂₇ O ₁₅	MS2[579.1]: 561.2(8), 519.3(39), 489.2(100); MS3[579.1→489.2]: 471.2(11), 399.2(41), 369.1(100)	luteolin 6-C-pen-8-C-hex
D3	593.1512	19.79	0.011	13.5	C ₂₇ H ₂₉ O ₁₅	MS2[593.2]: 575.2(8), 503.3(40), 473.2(100), 383.1(15), 353.2(28)	apigenin 6,8-di-C-hex
D4	593.1512	20.37	0.011	13.5	C ₂₇ H ₂₉ O ₁₅	MS2[593.2]: 575.2(9), 503.3(44), 473.3(100), 383.2(25), 353.2(27); MS3[593.2→473.2]: 383.1(28), 353.2(100)	apigenin 6,8-di-C-hex
D5	579.1356	21.13	0.098	13.5	C ₂₆ H ₂₇ O ₁₅	MS2[579.1]: 561.1(14), 519.2(13), 489.3(50), 459.1(100), 441.2(8), 399.2(16), 369.1(11)	luteolin 6-C-hex-8-C-pen
D6	563.1406	21.58	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.2(27), 503.1(78), 473.1(90), 443.2(100), 425.2(15), 413.2(11), 383.3(56), 353.2(40); MS3[563.1→443.2]: 425.3(18), 383.1(62), 353.1(100); MS3[563.1→473.1]: 383.3(26), 353.2(100)	apigenin C-hex, C-pen
D7	593.1512	22.58	0.011	13.5	C ₂₇ H ₂₉ O ₁₅	MS2[593.2]: 575.2(12), 503.2(33), 473.2(100), 383.2(19), 353.3(28); MS3[593.2→473.2]: 383.2(20), 353.2(100); MS3[593.2→503.2]: 413.3(18), 383.2(100)	apigenin 6,8-di-C-hex
D8	563.1406	23.47	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.3(15), 503.2(12), 473.2(100), 443.2(80), 383.2(32), 353.2(54); MS3[563.1→473.2]: 413.2(11), 383.2(83), 353.2(100); MS3[563.1→443.2]: 383.2(18), 353.2(100)	apigenin C-hex, C-pen
D9	579.1356	23.69	0.098	13.5	C ₂₆ H ₂₇ O ₁₅	MS2[579.1]: 489.2(25), 459.2(100), 399.2(9); MS3[579.1→459.2]: 441.3(33), 399.1(95), 369.2(100)	luteolin 6-C-hex-8-C-pen
D10	579.1356	24.11	0.098	13.5	C ₂₆ H ₂₇ O ₁₅	MS2[579.1]: 561.2(14), 519.2(15), 489.2(100), 459.3(17), 399.3(17), 369.3(12); MS3[579.1→489.2]: 471.2(10), 429.1(8), 399.2(67), 369.2(100); MS3[579.1→459.3]: 399.2(21), 369.2(100)	luteolin 6-C-pen-8-C-hex
D11	563.1406	25.00	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.4(27), 503.2(41), 473.2(100), 443.3(98), 425.2(9), 383.3(34), 353.3(33); MS3[563.1→473.2]: 413.2(22), 383.2(88), 353.2(100); MS3[563.1→443.3]: 383.1(34), 353.2(100)	apigenin C-hex, C-pen
D12	563.1406	25.73	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.2(19), 503.2(29), 473.2(100), 455.2(12), 443.2(62), 353.2(30), 353.2(34); MS3[563.1→473.2]: 413.2(8), 383.1(62), 353.1(100); MS3[563.1→443.2]: 425.2(8), 383.3(29), 353.2(100)	apigenin C-hex, C-pen

Peak No.	ion m/z	RT	error (ppm)	RDB	Formula [M-H] ⁻	MS ⁿ data	partial identification ^a
D13	579.1356	25.88	0.098	13.5	C ₂₆ H ₂₇ O ₁₅	MS2[579.1]: 561.2(13), 519.1(16), 489.2(100), 459.3(14), 399.2(16), 369.2(14); MS3[579.1]→489.2: 471.2(11), 399.1(68), 369.1(100)	luteolin 6-C-pen-8-C-hex
D14	563.1406	26.20	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.3(11), 503.2(17), 473.2(100), 455.3(14), 443.2(56), 413.2(9), 383.2(32), 353.1(33); MS3[563.1]→473.2: 455.2(8), 413.2(18), 383.2(100), 353.2(86); MS3[563.1]→443.2: 383.2(23), 353.2(100)	apigenin C-hex, C-pen
D15	563.1406	26.54	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.3(32), 503.1(67), 485.3(9), 473.2(88), 443.2(100), 425.3(17), 413.2(11), 383.2(55), 353.2(42); MS3[563.1]→443.2: 425.2(17), 383.3(65), 353.1(100); MS3[563.1]→473.2: 413.3(11), 383.3(28), 353.2(100)	apigenin C-hex, C-pen
D16	563.1406	27.45	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.2(13), 503.2(8), 473.2(60), 443.2(100), 383.3(19), 353.2(26); MS3[563.1]→443.2: 383.2(24), 353.2(100); MS3[563.1]→473.2: 413.2(22), 383.2(100), 353.1(36)	apigenin 6-C-hex-8 C-pen
D17	563.1406	27.76	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.2(34), 503.3(81), 473.2(100), 443.2(88), 425.2(15), 383.2(50), 353.2(40); MS3[563.1]→473.2: 383.1(23), 353.1(100); MS3[563.1]→443.2: 425.1(21), 383.2(63), 353.2(100)	apigenin 6-C-pen-8 C-hex
D18	563.1406	28.87	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.3(12), 503.2(11), 473.2(88), 455.2(12), 443.3(100), 383.2(20), 353.2(31); MS3[563.1]→443.3: 383.2(15), 353.2(100); MS3[563.1]→473.2: 413.1(14), 383.1(100), 353.2(49)	apigenin C-hex, C-pen
D19	593.1512	29.60	0.011	13.5	C ₂₇ H ₂₉ O ₁₅	MS2[593.2]: 575.2(12), 533.2(29), 503.3(46), 473.3(100), 413.2(32), 383.2(33); MS3[593.2]→473.3: 455.1(20), 413.2(59), 383.2(100); MS3[593.2]→503.3: 413.4(15), 383.3(100)	chrysoeriol C-hex, C-pen
D20	563.1406	29.89	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.2(17), 503.3(19), 473.2(100), 455.2(18), 443.2(65), 383.2(34), 353.2(40); MS3[563.1]→473.2: 413.1(15), 383.1(78), 353.2(100); MS3[563.1]→443.2: 383.2(24), 353.1(100)	apigenin C-hex, C-pen
D21	563.1406	31.72	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.2(11), 473.2(53), 443.2(100), 383.2(11), 353.2(21); MS3[563.1]→443.2: 383.2(14), 353.2(100); MS3[563.1]→473.2: 413.2(13), 383.2(100), 353.2(24)	apigenin C-hex, C-pen
D22	593.1512	31.93	0.011	13.5	C ₂₇ H ₂₉ O ₁₅	MS2[593.2]: 575.3(12), 503.2(85), 473.2(100), 413.2(20), 383.2(41); MS3[593.2]→473.2: 413.3(11), 383.2(100); MS3[593.2]→503.2: 443.3(11), 413.2(100), 383.1(70)	chrysoeriol C-hex, C-pen
D23	563.1406	32.81	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.2(16), 503.2(23), 473.2(47), 443.2(100), 425.2(18), 383.2(39), 353.2(43); MS3[563.1]→443.2: 425.2(15), 383.2(47), 353.2(100); MS3[563.1]→473.2: 413.2(26), 383.2(100), 353.2(46), 353.2(100)	apigenin C-hex, C-pen
D24	533.1300	34.07	-0.120	13.5	C ₂₅ H ₂₅ O ₁₃	MS2[533.1]: 515.3(21), 473.2(55), 443.2(100), 383.3(15), 353.3(15); MS3[533.1]→443.2: 383.1(28), 353.2(100); MS3[533.1]→473.2: 413.2(26), 383.2(100)	apigenin 6,8-di-C-pen
D25	593.1512	34.57	0.011	13.5	C ₂₇ H ₂₉ O ₁₅	MS2[593.2]: 575.2(9), 533.3(9), 503.2(100), 485.2(11), 473.3(51), 413.2(18), 383.1(34)	chrysoeriol C-hex, C-pen

Peak No.	ion m/z	RT	error (ppm)	RDB	Formula [M-H] ⁻	MS ⁿ data	partial identification ^a
D26	577.1563	35.08	0.037	13.5	C ₂₇ H ₂₉ O ₁₄	MS2[577.2]: 559.3(32), 503.1(49), 487.3(17), 473.2(73), 457.3(100), 439.3(11), 383.2(38), 353.2(40)	apigenin C ² -hex, C-dhex
D27	593.1512	35.80	0.011	13.5	C ₂₇ H ₂₉ O ₁₅	MS2[593.2]: 575.2(15), 533.2(8), 503.2(99), 473.2(100), 413.2(22), 383.2(49); MS3[593.2->473.2]: 413.2(7), 383.2(100); MS3[593.2->503.2]: 485.3(7), 443.2(8), 413.2(100), 383.1(79)	chrysoeriol C ² -hex, C ⁻ pen
D28	533.1300	37.10	-0.120	13.5	C ₂₅ H ₂₅ O ₁₃	MS2[533.1]: 515.2(21), 473.2(64), 455.2(8), 443.2(100), 425.3(6), 383.3(20), 353.3(16); MS3[533.1->443.2]: 383.2(31), 353.1(100); MS3[533.1->473.2]: 413.2(28), 383.2(100)	apigenin 6,8-di-C ⁻ pen
D29	533.1300	40.26	-0.120	13.5	C ₂₅ H ₂₅ O ₁₃	MS2[533.1]: 515.3(19), 473.2(59), 455.2(6), 443.3(100), 383.2(8), 353.3(13)	apigenin 6,8-di-C ⁻ pen
D30	563.1406	41.67	-0.051	13.5	C ₂₆ H ₂₇ O ₁₄	MS2[563.1]: 545.3(16), 503.2(49), 485.3(3), 473.2(100), 455.3(4), 443.3(6), 413.2(14), 383.2(22), 353.4(3)	apigenin C ² -hex, C ⁻ pen
D31	577.1563	42.90	0.037	13.5	C ₂₇ H ₂₉ O ₁₄	MS2[577.2]: 559.3(19), 503.2(51), 487.3(19), 473.2(50), 457.2(100), 439.2(9), 413.2(8), 383.3(39), 353.2(33); MS3[577.2->457.2]: 439.2(11), 383.1(53), 353.2(100); MS3[577.2->503.2]: 413.2(14), 383.2(100)	apigenin C ² -hex, C-dhex
D32	533.1300	44.85	-0.120	13.5	C ₂₅ H ₂₅ O ₁₃	MS2[533.1]: 515.2(21), 473.2(61), 443.2(100), 383.2(11), 353.2(14); MS3[533.1->443.2]: 383.3(18), 353.1(100); MS3[533.1->473.2]: 413.2(16), 383.2(100)	apigenin 6,8-di-C ⁻ pen
D33	547.1455	46.62	-0.391	13.5	C ₂₆ H ₂₇ O ₁₃	MS2[547.2]: 529.2(23), 473.3(48), 457.2(23), 443.2(100), 383.2(25), 353.2(26)	apigenin C ⁻ dhex, C ⁻ pen
D34	547.1455	47.87	-0.391	13.5	C ₂₆ H ₂₇ O ₁₃	MS2[547.2]: 529.4(16), 473.2(78), 457.2(37), 443.3(100), 413.3(11), 383.1(37), 353.3(29)	apigenin C ⁻ dhex, C ⁻ pen
T1	725.1935	16.49	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.3(19), 665.4(11), 635.4(100), 605.3(76), 563.3(79), 545.3(17), 515.4(12), 503.3(15), 473.3(52), 443.2(44), 383.3(20), 353.3(23)	apigenin O ⁻ hex, C ⁻ hex, C ⁻ pen
T2	725.1935	19.35	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 635.3(23), 527.2(22), 473.2(80), 443.3(100), 383.2(32), 353.2(35); MS3[725.2->443.3]: 383.2(26), 353.2(100); MS3[725.2->473.2]: 413.2(24), 383.1(100)	apigenin C ⁻ (6''-O ⁻ hex)hex, C ⁻ pen
T3	741.1878	19.67	-0.764	14.5	C ₃₂ H ₃₇ O ₂₀	MS2[741.2]: 723.3(15), 681.3(21), 651.2(100), 543.3(17), 489.2(29), 459.2(40), 453.2(16), 399.3(40), 369.2(43)	luteolin C ⁻ (6''-O ⁻ hex)hex, C ⁻ pen
T4	725.1935	20.97	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.3(21), 665.2(31), 635.3(40), 473.2(25), 443.2(100), 383.2(46), 353.2(46); MS3[725.2->443.2]: 425.2(17), 383.1(49), 353.1(100)	apigenin C ⁻ (6''-O ⁻ hex)hex, C ⁻ pen
T5	725.1935	21.55	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.3(13), 665.4(26), 635.3(43), 605.3(81), 563.2(100), 545.2(24), 515.2(23), 473.3(17); MS3[725.2->563.2]: 545.2(40), 503.2(82), 473.3(100), 443.3(82), 425.3(13), 383.3(50), 353.2(36); MS3[725.2->605.3]: 587.3(19), 545.2(60), 515.3(100)	apigenin O ⁻ hex, C ⁻ hex, C ⁻ pen
T6	725.1935	22.16	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.3(20), 605.3(15), 545.2(100), 527.3(15), 473.3(72), 425.2(19), 353.2(30); MS3[725.2->545.2]: 527.2(100), 515.3(65), 455.3(14), 425.2(91), 407.2(36), 395.3(16); MS3[725.2->473.3]: 383.2(18), 353.2(100); MS4[725.2->545.2->527.2]: 437.2(17), 407.1(100)	apigenin C ⁻ (2''-O ⁻ hex)pen, C ⁻ hex

Peak No.	ion m/z	RT	error (ppm)	RDB	Formula [M-H] ⁻	MS ⁿ data	partial identification ^a
T7	755.2040	22.74	-0.022	14.5	C ₃₃ H ₃₉ O ₂₀	MS2[755.2]: 737.3(9), 695.2(6), 665.4(19), 635.4(4), 575.3(8), 557.3(10), 515.3(5), 503.2(26), 473.2(100), 455.2(8), 443.3(4), 413.3(24), 395.3(6), 383.2(37), 353.2(8)	apigenin C-(6''-O-hex)hex, C-hex
T8	725.1935	22.95	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 635.3(16), 527.2(22), 473.2(48), 443.1(100), 383.2(21), 353.2(56); MS3[725.2->443.1]: 383.2(23), 353.2(100)	apigenin C-(6''-O-hex)hex, C-pen
T9	741.1878	23.69	-0.764	14.5	C ₃₂ H ₃₇ O ₂₀	MS2[741.2]: 723.3(12), 681.4(12), 651.3(100), 561.3(50), 501.3(15), 471.3(23), 459.1(26), 369.2(27)	luteolin 6-C-pen, 8-C-(2''-O-hex)hex
T10	725.1935	23.80	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.4(12), 665.3(23), 635.3(27), 527.2(6), 473.2(24), 443.2(100), 425.4(10), 413.2(8), 383.2(44), 353.2(40); MS3[725.2->443.2]: 425.2(19), 383.1(64), 353.2(100)	apigenin C-(6''-O-hex)hex, C-pen
T11	725.1935	25.49	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.4(22), 665.4(76), 635.3(100), 605.4(43), 575.2(10), 545.3(61), 527.2(16), 473.3(41), 407.1(10), 383.2(11), 353.2(21)	apigenin C-hex, C-pen, O-hex
T12	725.1935	25.86	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.3(37), 665.3(37), 647.3(4), 635.4(100), 617.3(8), 575.3(12), 545.3(40), 527.1(5), 485.4(6), 455.3(20), 395.3(3), 365.2(7)	chrysoeriol C-pen, C-(O-hex) pen
T13	725.1935	26.88	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.3(11), 635.3(8), 545.2(50), 527.2(21), 485.2(37), 443.2(100), 395.3(7), 383.2(7), 353.1(31); MS3[725.2->545.2]: 527.3(58), 485.2(100), 455.1(15), 395.2(4), 365.3(5); MS3[725.2->443.2]: 383.1(24), 353.1(100)	apigenin C-hex, C-pen, O-hex
T14	725.1935	30.47	0.066	14.5	C ₃₂ H ₃₇ O ₁₉	MS2[725.2]: 707.3(9), 635.4(8), 545.3(38), 527.3(21), 485.3(36), 443.2(100), 383.3(9), 353.2(28)	apigenin C-hex, C-pen, O-hex
T15	755.2040	31.06	-0.022	14.5	C ₃₃ H ₃₉ O ₂₀	MS2[755.2]: 737.3(12), 677.3(4), 665.3(19), 575.2(45), 557.3(20), 515.2(30), 485.3(7), 473.2(100), 425.2(13), 383.2(36), 353.2(1)	apigenin C-(3''-O-hex)hex, C-hex
AD1	743.1829	30.17	0.017	18.5	C ₃₅ H ₃₅ O ₁₈	MS2[743.2]: 725.2(2), 545.1(100), 425.1(5); MS3[743.2->545.1]: 425.2(100); MS3[743.2->425.1]: 425.2(11), 365.2(41), 335.2(100), 305.1(20)	apigenin 2''-O-syringoyl, C-hex, C-pen
AD2	713.1722	32.1	-0.172	18.5	C ₃₄ H ₃₃ O ₁₇	MS2[713.2]: 545.1(100), 425.1(6); MS3[713.2->545.1]: 455.2(3), 425.2(100); MS3[713.2->425.1]: 365.2(33), 335.2(100), 305.2(17)	apigenin 2''-O-vanilloyl, C-hex, C-pen
AD3	743.1829	32.46	0.017	18.5	C ₃₅ H ₃₅ O ₁₈	MS2[743.2]: 545.1(100), 425.1(5); MS3[743.2->545.1]: 455.3(4), 425.1(100); MS3[743.2->425.1]: 365.1(47), 335.1(100)	apigenin 2''-O-syringoyl, C-hex, C-pen
AD4	713.1722	35.31	-0.172	18.5	C ₃₄ H ₃₃ O ₁₇	MS2[713.2]: 545.1(100), 425.2(4); MS3[713.2->545.1]: 455.2(3), 425.2(100); MS3[713.2->425.2]: 365.2(59), 335.2(100), 305.1(15)	apigenin 2''-O-vanilloyl, C-hex, C-pen
AD5	709.1775	41.83	0.130	19.5	C ₃₅ H ₃₃ O ₁₆	MS2[709.2]: 563.1(5), 545.1(100), 425.1(5); MS3[709.2->545.1]: 455.2(4), 425.2(100); MS3[709.2->425.1]: 365.2(10), 335.2(100), 305.2(17)	apigenin 2''-O-p-coumaroyl, C-hex, C-pen
AD6	709.1775	43.86	0.130	19.5	C ₃₅ H ₃₃ O ₁₆	MS2[709.2]: 563.1(6), 545.1(100), 425.1(5); MS3[709.2->563.1]: 545.3(33), 503.2(100), 473.2(88), 443.3(90), 425.3(15), 383.3(55), 353.3(39); MS3[709.2->545.1]: 455.3(4), 425.2(100)	apigenin 2''-O-p-coumaroyl, C-hex, C-pen

Peak No.	ion m/z	RT	error (ppm)	RDB	Formula [M-H] ⁻	MS ⁿ data	partial identification ^a
AD7	769.1986	44.56	0.082	19.5	C ₃₇ H ₃₇ O ₁₈	MS2[769.2]: 563.2(3), 545.2(100), 425.3(11); MS3[769.2->545.2]: 455.2(2), 425.2(100); MS3[769.2->425.3]: 365.1(33), 335.1(100), 305.2(17)	apigenin 2''-O-sinapoyl, C-hex, C-pen
AD8	739.1879	44.98	-0.098	19.5	C ₃₆ H ₃₅ O ₁₇	MS2[739.2]: 563.2(2), 545.2(100), 425.3(9); MS3[739.2->545.2]: 455.1(3), 425.2(100); MS3[739.2->425.3]: 365.2(31), 335.2(100), 305.2(17)	apigenin 2''-O-feruloyl, C-hex, C-pen
AD9	769.1986	45.40	0.082	19.5	C ₃₇ H ₃₇ O ₁₈	MS2[769.2]: 563.4(3), 545.3(100), 425.2(10); MS3[769.2->545.3]: 455.2(4), 425.2(100); MS3[769.2->425.2]: 365.1(50), 335.1(100)	apigenin 2''-O-sinapoyl, C-hex, C-pen
AD10	739.1879	46.05	-0.098	19.5	C ₃₆ H ₃₅ O ₁₇	MS2[739.2]: 563.3(3), 545.2(100), 425.2(7); MS3[739.2->545.2]: 455.2(3), 425.2(100); MS3[739.2->425.2]: 365.2(50), 335.2(100), 305.2(15)	apigenin 2''-O-feruloyl, C-hex, C-pen
AD11	739.1879	47.93	-0.098	19.5	C ₃₆ H ₃₅ O ₁₇	MS2[739.2]: 545.2(6), 515.3(100); MS3[739.2->515.3]: 425.1(100); MS4[739.2->515.3->425.1]: 365.2(57), 335.1(100), 305.1(7)	apigenin 2''-O-sinapoyl, C-pen, C-pen
AD12	709.1775	48.10	0.130	19.5	C ₃₅ H ₃₃ O ₁₆	MS2[709.2]: 533.3(2), 515.2(100), 425.2(4); MS3[709.2->515.2]: 425.1(100); MS4[709.2->515.2->425.1]: 407.2(6), 365.1(53), 335.2(100), 307.2(11), 305.2(10), 241.2(10)	apigenin 2''-O-feruloyl, C-pen, C-pen
AD13	709.1775	48.48	0.130	19.5	C ₃₅ H ₃₃ O ₁₆	MS2[709.2]: 533.3(2), 515.2(100), 425.2(3); MS3[709.2->515.2]: 425.2(100); MS4[709.2->515.2->425.2]: 365.1(50), 335.2(100), 305.1(8)	apigenin 2''-O-feruloyl, C-pen, C-pen
AD14	769.1986	49.20	0.082	19.5	C ₃₇ H ₃₇ O ₁₈	MS2[769.2]: 563.1(3), 545.1(100), 425.1(6); MS3[769.2->545.1]: 455.1(3), 425.3(100); MS3[769.2->425.1]: 365.3(41), 335.3(100), 305.1(35)	apigenin 2''-O-sinapoyl, C-hex, C-pen
AT1	901.2408	27.3	0.004	20.5	C ₄₂ H ₄₅ O ₂₂	MS2[901.2]: 739.2(100), 587.1(19), 545.1(92), 425.1(16); MS3[901.2->739.2]: 545.2(100), 425.2(9); MS3[901.2->545.1]: 455.2(3), 425.2(100); MS4[901.2->545.2->425.2]: 365.2(41), 335.1(100), 305.1(12)	apigenin 2''-O-feruloyl, O-hex, C-hex, C-pen
AT2	931.2515	28.25	0.150	20.5	C ₄₃ H ₄₇ O ₂₃	MS2[931.2]: 769.1(39), 587.4(1), 545.1(100), 455.0(2), 425.2(28), 365.1(1), 335.3(2)	apigenin O-sinapoyl, O-hex, C-hex, C-pen
AT3	931.2515	38.9	0.150	20.5	C ₄₃ H ₄₇ O ₂₃	MS2[931.2]: 725.3(2), 707.3(100), 425.3(14); MS3[931.2->707.3]: 617.4(4), 455.2(15), 443.3(2), 425.2(100), 365.3(2), 335.1(3)	apigenin 2''-O-sinapoyl, C-(4'' or 6''-O-hex)hex, C-pen
AT4	931.2515	40.4	0.150	20.5	C ₄₃ H ₄₇ O ₂₃	MS2[931.2]: 725.4(5), 707.3(100), 587.4(22); MS3[931.2->707.3]: 617.1(2), 587.3(100), 335.2(2); MS4[931.2->707.3->587.2]: 425.3(100), 407.2(8), 335.2(31)	apigenin 2''-O-sinapoyl, C-(3'' or 4''-O-hex)pen, C-hex
AT5	901.2408	40.7	0.004	20.5	C ₄₂ H ₄₅ O ₂₂	MS2[901.2]: 707.2(100), 425.2(11); MS3[901.2->707.2]: 587.3(43), 455.2(17), 425.1(100); MS4[901.2->707.2->425.1]: 365.2(36), 335.1(100), 305.3(16)	apigenin 2''-O-feruloyl, C-hex-O-hex, C-pen
AT6	931.2515	43	0.150	20.5	C ₄₃ H ₄₇ O ₂₃	MS2[931.2]: 725.4(2), 707.3(100), 425.2(15); MS3[931.2->707.3]: 617.4(3), 527.2(2), 455.2(16), 443.3(2), 425.2(100), 365.2(2), 335.2(4); MS4[931.2->707.2->425.2]: 365.2(45), 335.3(100)	apigenin 2''-O-sinapoyl, C-(4'' or 6''-O-hex)hex, C-pen

Peak No.	ion <i>m/z</i>	RT	error (ppm)	RDB	Formula [M-H] ⁻	MS ⁿ data	partial identification ^a
AT7	901.2408	44.4	0.004	20.5	C ₄₂ H ₄₅ O ₂₂	MS2[901.2]: 707.3(100), 425.3(15); MS3[901.2->707.3]: 455.1(18), 425.1(100); MS4[901.2->707.3->425.1]: 365.2(47), 335.2(100), 331.1(14), 305.1(13)	apigenin 2'' - <i>O</i> -feruloyl, C-(4'' or 6'' - <i>O</i> -hex)hex, C-pen

^ahex: hexosyl; pen: pentosyl; dhex: deoxyhexosyl