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# Polyphenol Identification Based on Systematic and Robust High-Resolution Accurate Mass Spectrometry Fragmentation

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**High-mass resolution multi-stage mass spectrometry ($MS^n$) fragmentation was tested for differentiation and identification of metabolites, using a series of 121 polyphenolic molecules. The $MS^n$ fragmentation approach is based on the systematic breakdown of compounds, forming a so-called spectral tree. A chip-based nanoelectrospray ionization source was used combined with an ion-trap, providing reproducible fragmentation, and accurate mass read-out in an Orbitrap Fourier transform (FT) MS enabling rapid assignment of elemental formulas to the molecular ions and all fragment ions derived thereof. The used protocol resulted in reproducible $MS^n$ fragmentation trees up to $MS^5$. Obtained results were stable over a 5 month time period, a concentration change of 100-fold, and small changes in normalized collision energy, which is key to metabolite annotation and helpful in structure and substructure elucidation. Differences in the hydroxylation and methoxylation patterns of polyphenolic core structures were found to be reflected by the differential fragmentation of the entire molecule, while variation in a glycosylation site displayed reproducible differences in the relative intensities of fragments originating from the same aglycone fragment ion. Accurate $MS^n$-based spectral tree data are therefore a powerful tool to distinguish metabolites with similar elemental formula, thereby assisting compound identification in complex biological samples such as crude plant extracts.**

One of the bottlenecks in liquid chromatography−mass spectrometry (LC−MS)-based metabolomics is the annotation of the observed molecular ion peaks in an extract. To enable fast elemental formula calculation of detected ions, accurate mass spectrometers, such as time-of-flight (TOF) instruments, Fourier transform ion cyclotron mass spectrometers (FTICR-MS), and Orbitrap Fourier transform mass spectrometry (FTMS) machines are used.[1] Nevertheless, the lack of additional structural information frequently hampers translation of the detected accurate mass into its exact chemical structure.[2] For instance, the elemental formula of the flavonoid rutin, $C_{27}H_{30}O_{16}$, a natural compound commonly present in plants, retrieves 246 different published compound hits in Scifinder (https://scifinder.cas.org/scifinder).

MS fragmentation experiments are used to obtain more structural information of detected molecules. Different types of MS instruments, using either nominal or accurate mass detection, are available to fragment ionized molecules and to detect their charged ion fragments.[3] In the tandem-MS technique, $MS^2$ fragments of the most intense ion or a selected ion are generated. With the use of this approach, several MS/MS databases have been generated in order to facilitate metabolite identification.[4−6] Compared to MS/MS generating $MS^2$ fragments, so-called $MS^n$ approaches, in which ions are specifically selected for sequential fragmentation, result in deeper and more detailed fragmentation pathways, thus enabling more structural information. Up to 5−8 sequential fragmentation spectra can be obtained, depending upon the concentration and ionization efficiency of the compound. With the use of such a multiple-stage mass spectra approach, we generated so-called spectral

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trees[7] (Supplemental Figure 1 in the Supporting Information). So far, most $MS^n$ studies used nominal mass read-out of MS fragments. However, high-resolution accurate mass read-out facilitates rapid assignment of an elemental formula to each MS fragment and thereby to the parent molecule and its fragmentation characteristics.

Mass spectrometry-based metabolomics approaches are important tools in plant sciences.[8−11] A biologically and economically important class of plant metabolites is the flavonoids. Flavonoids are polyphenolic compounds ubiquitously present in plant and involved in numerous physiological processes like protection against damage caused by UV light or microbes and coloring of flowers and fruits.[12] Chemically, the core structure of flavonoids consists of two aromatic and one heteronuclear ring. Flavonoids are divided into several subclasses giving rise to various isomeric aglycones.[13] In plants, flavonoid aglycones normally contain several hydroxyl groups on their aromatic rings. Moreover, one or more sugar moieties can be attached to each of these hydroxyl groups. These and other modifications of the basic flavonoid core have led to a large array of more than 6000 flavonoid species including many different isomers, i.e., structures with similar elemental formulas but different exact chemical configurations. In fact, the large number of different molecules that are possible for a specific elemental formula hampers a fast and unambiguous identification of compounds in biological extracts using MS-based metabolomics.[2] Even in the case of extensive LC separation, in which retention time and absorbance spectra of compounds can be used for identification guidance,[14] the number of isomers having comparable chromatographic behavior and absorbance characteristics prevent an unambiguous annotation based on the elemental formula only.[15]

With the use of tandem-MS technologies, MS/MS spectra of a range of flavonoid structures have been investigated and compared.[14,16] Abad-Garcia et al. recently fragmented a large series of 72 flavonoids with a triple quad MS and derived a number of fragmentation rules based on MS/MS data in the positive ionization mode.[14] Likewise, with the use of ion trap technologies, $MS^n$ spectra of a number of flavonoids have been generated.[17,18] Indeed, specific fragments and (sequential) fragment losses related to specific core structures have been reported using MS/MS and $MS^n$.[12,19−22] The fragments from flavonoids originate from two main fragmentation events.[19,20,22−30] Ring-opening (RO) is characterized by the (subsequentl) loss of small neutral molecules like CO, $CO_2$, and $H_2O$. The other main fragmentation event results in two broken bonds, the so-called cross ring cleavage (CRC) fragmentation, and for polyphenols mostly Retro Diels−Alder (RDA) reactions.

Despite the large number of flavonoid fragmentation studies, data on specific fragmentation patterns that enable discrimination and identification of a range of polyphenolic compounds are scarce.[14,21,31] Either in-depth $MS^n$ fragmentation, with nominal mass read-out, was applied to a small number of flavonoids,[32,33] or in the case of the large study of 72 flavonoids only $MS^2$ was used.[14] In addition, these studies do not provide data on the reproducibility of the obtained fragmentation spectra and thereby on the robustness of the approach used.

In this study we validated and applied an accurate mass $MS^n$ spectral tree approach, and we focused on the possibility to discriminate between positional- and stereoisomeric forms. We used a Nanomate injection system coupled to an ion trap-Orbitrap FTMS system. The NanoMate enabled reproducible and small sample volume infusion, through chip-based nanoelectrospray, into the Ion Trap, which subsequently fragments the molecular ion by normalized collision induced dissociation. The most intense ions in the $MS^2$, $MS^3$, and $MS^4$ fragmentation spectra were further fragmented while the Orbitrap FTMS provided accurate masses of all ion fragments, enabling fast assignment of their elemental formulas. As an example, a large series of flavonoids with increasing number and size of substitutions was chosen for comparison of their fragmentation spectra in both positive and negative ionization mode (Table S1 in the Supporting Information). The method was validated by acquiring $MS^n$ data under different experimental conditions and over different time periods. Following this approach, we aimed to improve the discriminative power of MS by generating reproducible fragment ion spectra and $MS^n$ spectral trees.


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## MATERIALS AND METHODS

**Chemicals.** Phenolic standards were obtained from Apin (Oxon, U.K.), Extrasynthese (Genay, France), Sigma (St. Louis), Fluka (Dorset, U.K.), and Acros (Geel, Belgium). The purity of all compounds was more than 98%. Quercetin-glucosides were produced in-house through bacterial expression of the tomato UDP-glucose flavonoid glycosyl transferase enzyme. The purified protein was incubated with quercetin aglycone and UDP-glucose as substrates for 24 h, allowing the production of a series of mono-, di-, and triglucosides. These quercetin-glucosides were separated by preparative LC and their structures determined using Q-TOF accurate mass MS and 2D-NMR. HPLC grade solvents were obtained from Biosolve (Valkenswaard, The Netherlands) and Merck-Schuchardt (Hohenbrunn, Germany). Ultrapure water was made in purification units present in-house.

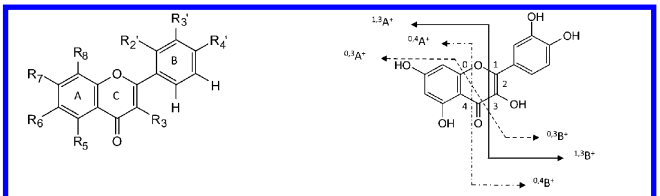
**Preparation of Flavonoid Standard Solutions.** From each polyphenolic molecule, a stock solution of 2.5 $\mu$g/mL in 75% MeOH in $H_2O$ acidified with 0.1% formic acid was prepared and stored at −20 °C. Working solutions were prepared by filling 96 wells plates (Abgene) with 40 $\mu$L of each stock solution, after which the plates were sealed with thermo foil.

**Mass Spectrometry and Data Handling.** A chip-based nanoelectrospray ionization source (Triversa NanoMate, Advion BioSciences) was used for automated direct sample infusion into a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) used in negative and positive ionization mode. For each unique $m/z$ value, a separate Xcalibur method was prepared. The LTQ was programmed to use a window of 10 D to isolate the mass of interest in MS1. The data-dependent fragmentation was set as follows: $MS^2$ fragmentation of most intense ion in MS1; $MS^3$ fragmentation of the 5 most intense fragment ions in $MS^2$; $MS^4$ fragmentation of the 5 most intense fragment ions in each $MS^3$; $MS^5$ fragmentation of the 3 most intense fragment ions in each $MS^4$. The spectrum list with accurate $m/z$ values was used to export the $MS^n$ spectral tree fragmentation data from Xcalibur into Excel where it was processed. Details on instrument settings and data processing can be found in the Supporting Information.

## RESULTS

The chemical structure-spectral tree relationship was studied by individually measuring a series of 121 polyphenolic compounds belonging to different chemical subclasses of flavonoids including various isomeric forms (Table S1 in the Supporting Information), of which a selection is discussed in this article (Figure 1). The resulting spectra were manually checked and processed. Because of the nature of the fragmentation experiments, it was not possible to use a lock mass for automatic recalibration of each scan. Nevertheless, in both ionization modes, the mass deviation was generally less than 1 ppm for $MS^1$ scans and less than 3 ppm for $MS^{2-5}$ scans. In the rare cases that the mass deviation was larger, the mass variation within each scan was always within 2 ppm.

**$MS^n$ Spectral Tree Method Is Highly Reproducible.** *Variations over Time in Fragment Intensities.* During the acquisition time, usually multiple (sometimes up to 20) repetitions of the complete fragmentation tree from the same parent ion were acquired. These repetitive $MS^n$ spectra were averaged and the variation in the intensities of detected fragment ions was calculated, in order




Flavone basic core RDA fragmentation through C-ring, shown on quercetin molecule as example

| ID | Common name | ID | Common name |
|---|---|---|---|
| 1 | 6,4'-dihydroxyflavone | 16 | 3,7-dimethoxyflavonol |
| 2 | 6,2'-dihydroxyflavone | 17 | 5,7-dimethoxyflavone |
| 3 | 6,7-dihydroxyflavone | 18 | 7,8-dimethoxyflavone |
| 4 | 2'-hydroxyflavonol* | 19 | 3,2'-dimethoxyflavonol |
| 5 | 7-hydroxyflavonol | 20 | 3,4'-dimethoxyflavonol |
| 6 | 4'-hydroxyflavonol | 21 | 5,2'-dimethoxyflavone |
| 7 | Morin (5,7,2',4'-tetrahydroxyflavonol) | 22 | quercetin-4'-O-glucoside |
| 8 | Quercetin (5,7,3',4'-tetrahydroxyflavonol) | 23 | quercetin-3-O-glucoside |
| 9 | 3-methoxyflavonol | 24 | quercetin-7-O-glucoside |
| 10 | 5-methoxyflavone | 25 | quercetin-3,7-O-diglucoside |
| 11 | 6-methoxyflavone | 26 | quercetin-3,4'-O-diglucoside |
| 12 | 7-methoxyflavone | 27 | quercetin-3,7,3'-O-triglucoside |
| 13 | 2'-methoxyflavone | 28 | quercetin-3,7,4'-O-triglucoside |
| 14 | 4'-methoxyflavone | 29 | syringetin-3-O-glucoside (5,7,3'-trihydroxy-2',4'-dimethoxyflavonol-3-O-glucoside) |
| 15 | 3,6-dimethoxyflavonol | 30 | syringetin-3-O-galactoside (5,7,3'-trihydroxy-2',4'-dimethoxyflavonol-3-O-galactoside) |

**Figure 1.** The flavone backbone, RDA fragmentation paths, and structures of the discussed polyphenols. The flavone core is displayed at the top left, and retro Diels-Alder (RDA) fragmentations in the flavone backbone are indicated in the top right with their nomenclature for positive ionization mode. *A flavonol refers to a 3-hydroxyflavone.

to determine the reproducibility of fragmentation patterns. Different fragments for negative and positive ionization mode were obtained, but in both modes these patterns were extremely reproducible. Here, we present validation data from negative mode (Figure 2), positive mode spectra can be found in the Supporting Information, Supplemental Figure 2. The relative intensities of the fragments in the $MS^2$ spectra of 6,4′-dihydroxyflavone (**1**) upon combining 6 and 10 spectra are shown in Figure 2A. All 7 selected fragments were detected in all repetitive $MS^2$ scans, and the overall standard deviation of the relative signal intensities of these fragments was less than 1.2%. For example, the fragment C9H5O2 was detected with an average intensity of 22.0% ± 0.83 (mean ±

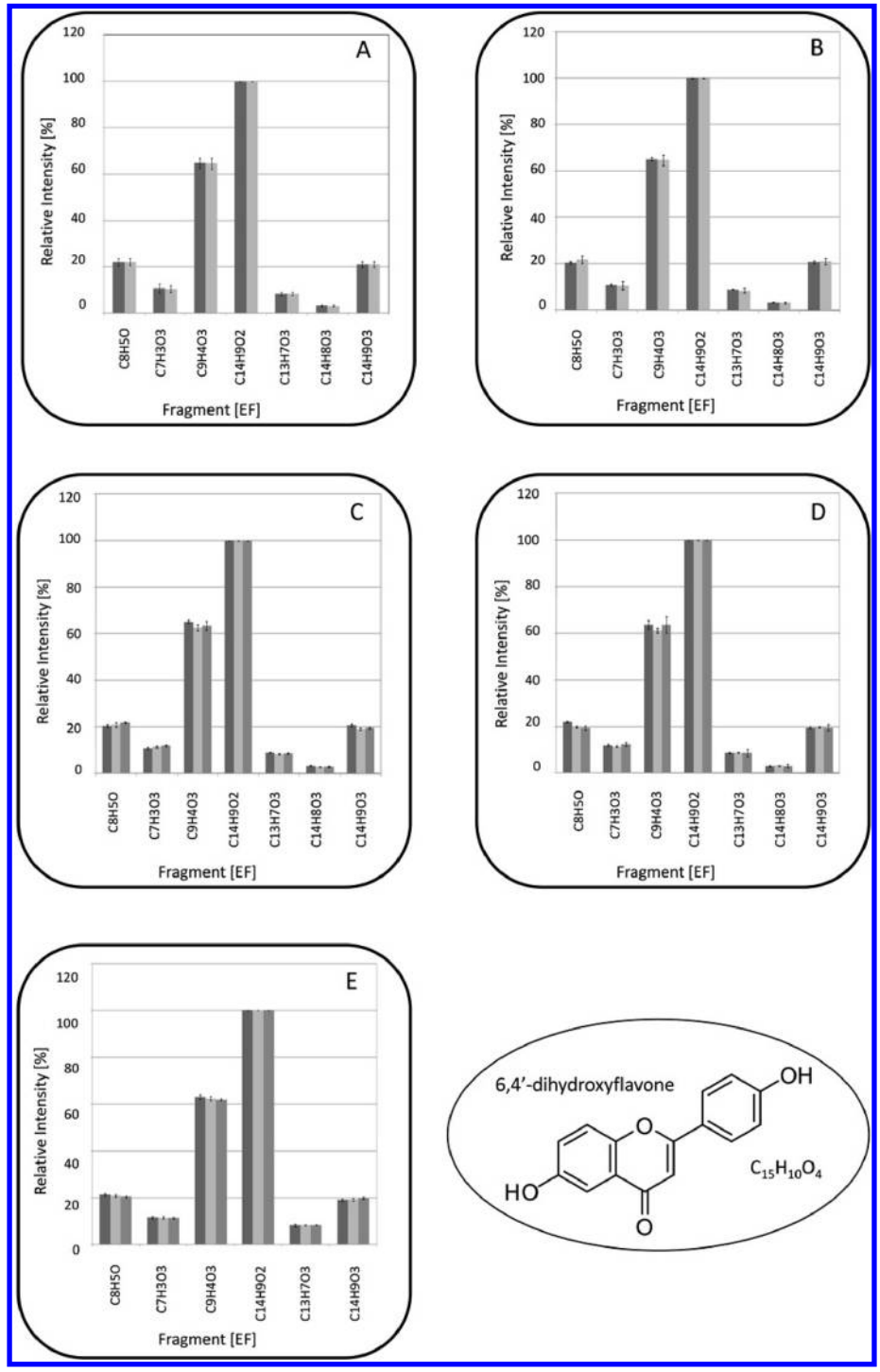



**Figure 2.** Reproducibility of $MS^n$ patterns in negative ionization mode. $MS^2$ fragmentation spectra of 6,4′-dihydroxyflavone (**1**) acquired at different conditions are shown: (A) averaged 6 (dark gray) or 10 times (light gray); (B) two repetitive measurements; (C) at 0 months (dark gray), 3 months (light gray), and 5 months (gray); (D) at 2.5 $\mu$g/mL, 0.5 $\mu$g/mL (light gray), and 50 ng/mL (gray); (E) at a normalized collision energy of 30% (dark gray), 35% (light gray), and 40% (gray). The average of six spectra is shown and the error bars represent a 95% confidence interval.

SD) using 6 repetitive spectra and with an intensity of 22.1% ± 0.79 using 10 repetitive scans. The largest variation observed (<1.2%) was for fragment C9H4O3 for which a relative intensity of 64.7% ± 1.12 over 6 repetitions and 64.5% ± 1.17 over 10 repetitions was found.

We subsequently compared two independent analyses runs of **1**, i.e., two infusions and subsequent spectral tree generation within the same day (Figure 2B). Both repetitive infusions yielded highly comparable spectra with the largest relative intensity difference of 1.5% for the C8H5O fragment, while for the other fragment ions the difference between the analyses was less than 0.6%. Reproducibility of spectral tree generation over time was further determined by analyzing 6,4′-dihydroxyflavone (**1**) and 4′-hydroxyflavonol (**6**), over a 5 month storage (−20 °C) period.

The fragmentation spectra of **1** were highly similar within this time period, e.g., the relative intensity of the most varying $MS^2$ fragment, C9H4O3, differed 2.5% (Figure 2C). For the **6**, three fragments were detected above 3% of the base peak, of which C13H9O2 varied the most (5.2%) (Supplemental Figure 2B in the Supporting Information).

*Variations in Fragmentation Intensities Due to Changes in Compound Concentration and Collision Energy.* To test whether differences in compound concentrations of the parent ion influence the fragmentation patterns, we generated $MS^n$ spectra of 6,4′-dihydroxyflavone (**1**) at decreasing initial concentrations of the molecule. The resulting fragmentation spectra (Figure 2D) indicate that the ion intensity in $MS^1$, i.e., the concentration of the molecule to be fragmented, did not significantly influence the relative intensities and thus the relative order of fragment abundance. The measurement variability slightly increased upon lowering the $MS^1$ ion concentration: from 0.93% at $2.10 \times 10^7$ ions toward 1.8% at $8.20 \times 10^5$ ions for the most varying fragment C9H4O3.

The effect of variations in the collision energy was tested by generation of $MS^n$ spectra at varying normalized collision energies (Figure 2E and Supplemental Figure 2D−F in the Supporting Information). Major changes in neither the relative intensities nor the measurement variation were observed. Only a minor effect was visible in the intensities of all fragments, i.e., on average 1% increase upon increasing the normalized collision energy from 30% to 40%. Thus, small variations in collision energy do not hamper the analysis of the $MS^n$ spectral trees.

**$MS^n$ Fragmentation Generates Unique Flavonoid Spectral Trees.** *Flavonoids with Different Hydroxylation Patterns.* All fragments of the C15H10O4 isomers numbered **1**−**6** (Figure 1) present in the $MS^2$−$MS^5$ spectra in either negative ionization (Table S2 in the Supporting Information) or positive ionization mode (Table S3 in the Supporting Information) were analyzed. Some RO fragments were detected for all isomers, like C14H9O2 and C13H9O2 in negative ionization mode and C13H9 and C12H9 in positive ionization mode. The $MS^2$ spectra showed at least one unique CRC fragment for each isomer, in both ionization modes. In addition, the relative intensities of the three most intense RO fragments in $MS^2$ (i.e., C13H11O2, C14H9O2, and C15H9O3 in positive ionization mode and C14H8O2, C14H9O2, and C14H9O3 in negative ionization mode) differed between all six isomers.

In negative ionization mode, both RO and RDA fragmentation yielded radical fragment ions (Table S2 in the Supporting Information). These radical fragments were sometimes highly abundant in the spectra, for instance the radical C14H8O2 was the most intense fragment ion in the $MS^2$ spectra of (**5**). Despite being potentially reactive, these radicals showed low measurement variation, e.g., standard deviation less than 0.59% for C9H4O3 of **1**.

The two positional isomers of C15H10O7, i.e., morin (**7**) and quercetin (**8**), gave differential fragmentation spectra in both ionization modes (Supplemental Figure 3 in the Supporting Information). The different substitution pattern on the B ring resulted in two intense unique CRC fragments in the negative ionization mode: C6H5O3 for morin and C8H3O5 for quercetin. In the positive ionization mode, the corresponding morin-specific fragment C6H7O3 was detected, though only as a minor fragment, whereas for quercetin the C8H5O5 fragment was not present.

*Flavonoids with Different Methoxylation Patterns.* A series of six isomeric monomethoxylated flavonoids (**9**−**14**, C16H12O3) and 7 dimethoxylated flavonoids (**15**−**21**, C17H15O4) generated fragmentation patterns only in the positive ionization mode and did not ionize well in the negative mode. All fragments collected from the $MS^n$ spectra of the tested monomethoxylated and dimethoxylated compounds are provided in Tables S4 and S5 in the Supporting Information, respectively.

The monomethoxylated flavonoids produced four fragments that were present in all six isomers, of which the radical fragment C15H10O3 was dominant in the $MS^2$ spectra (Table S4 in the Supporting Information). The RO fragments C13H10O and C12H9 and the RDA fragment C6H4O2 were specific for the A ring monosubstituted methoxyflavones, while C11H10 and C7H5O2 were only present in the B ring substituted monomethoxylated flavones. The RO fragment C14H9O2 was specific for the 3-methoxyflavonol. All monomethoxylated isomers showed distinct qualitative and/or quantitative differences in their $MS^n$ fragments. The $MS^2$ spectra of all dimethoxylated structures were dominated by the radical fragment C16H12O4 due to the loss of one $CH_3$ radical. Six of the seven isomers produced one or two unique RDA fragments (Table S5 in the Supporting Information).

*Flavonoids with Different Glycosylation Patterns.* $MS^3$ spectra in the positive mode of all three quercetin-monoglucosides (**22**−**24**) tested showed identical fragmentation behavior, resulting from fragmentation of the C15H11O7 aglycone fragment (Figure 3B). In contrast, in negative ionization mode the differences between their $MS^3$ spectra were striking: the ratios of the four most intense fragments were discriminative for all positional isomers (Figure 3A). The RDA fragment C7H4O3 was the base peak for quercetin-7-*O*-glucoside (**24**), while the RO fragments C14H7O5, and C14H7O6 were much more intense for quercetin-3-*O*-glucoside (**23**). Some minor fragments were also unique for each glucoside, e.g., C13H9O5 for the 7-*O*-glucoside and both C14H6O5 and C14H8O6 for the 3-*O*-glucoside.

Similarly, the di- and triglucosides of quercetin (**25**−**28**) did not show differences in their $MS^n$ spectra in the positive mode, while in the negative mode the intensity patterns of the fragments from the aglycone fragment C15H9O7 (in $MS^4$ and $MS^5$ for the di- and triglucosides, respectively) differed significantly. Quercetin-3,7,3′-*O*-triglucoside (**27**) and its 3,7,4′-*O*-isomer (**28**) could be discriminated solely on the basis of $MS^5$ fragmentation of their quercetin aglycone. In the case of quercetin diglucosides, the patterns of the radical fragments in $MS^2$ and $MS^3$ were markedly different as well.

To test whether the spectral tree approach would be able to differentiate between different hexose sugars attached to the 3-*O* position on the flavonoid aglycone, we compared spectral trees from syringetin-3-*O*-glucose (**29**) and syringetin-3-*O*-galactose (**30**). In line with the quercetin glucosides, the positive ionization mode did not yield any differences in the fragmentation. In the negative mode, the fragmentation behavior was markedly different between both hexose isomers (Figure 3C).

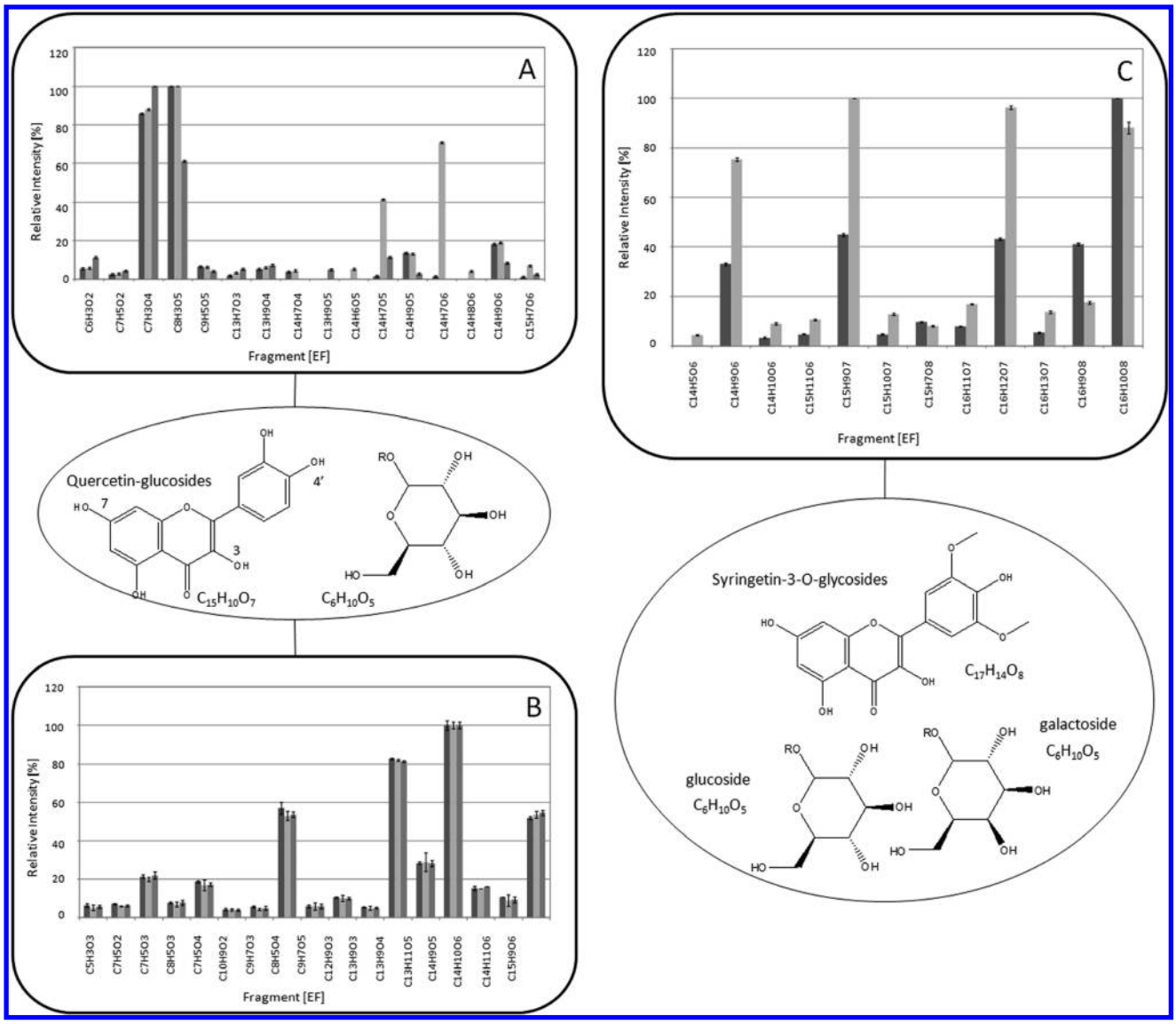



**Figure 3.** $MS^3$ spectra of flavonoid glycoside isomers. The $MS^3$ spectra of the quercetin fragment ions of three quercetin-glucosides (**22**−**24**) are shown for (A) negative and (B) positive ionization mode: 4′-*O*-glucoside (dark gray), 3-*O*-glucoside (light gray), and 7-*O*-glucoside (gray). (C) $MS^3$ fragmentation spectra of the syringetin fragment ions of syringetin-3-*O*-galactoside (**30**) (dark gray) and syringetin-3-*O*-glucoside (**29**) (light gray) in the negative ionization mode. The average of six spectra is shown, and the error bars represent a 95% confidence interval.

## DISCUSSION

MS/MS and $MS^n$ fragmentation are frequently used as tools in metabolite annotation, based on their similarity to reference compounds.[3−7] Here we present an $MS^n$ fragmentation approach using high-resolution Orbitrap FTMS, facilitating elemental formula assignments of all ion fragments and resulting in highly reproducible $MS^n$ fragmentation patterns in both ionization modes. Our method thus enables the detection of even subtle differences in fragmentation patterns. The power of this approach is illustrated by the fact that 119 out the 121 flavonoids tested, including various isomeric forms, could be discriminated based on the presence of either unique fragments (i.e., higher than 3% of the base peak) or marked differences (more than about 30%) in the relative intensity of one or more fragments, resulting in unique spectral trees. Only for the isomeric couple catechin and epicatechin, the $MS^n$ fragmentation approach resulted in identical fragment patterns in both the positive and negative ionization modes, which is in line with previous studies.[19,34,35] The reproducibility and discriminative power of $MS^n$ are important factors in the applicability of spectral trees as a tool in the annotation of known metabolites, based on their unique spectral tree, as well as in the identification of unknown metabolites, based on in-depth fragmentation paths providing structural information of the molecule or its substructures.

The observed characteristics are essential for the generation of an $MS^n$ metabolite database. In existing Web-based MS/MS databases mostly nominal mass data and $MS^2$ data are gathered.[36] Lee and co-workers reported the setup of a fragmentation database for flavonoids using Mass Frontier software.[27] However, the Mass Frontier software has some important limitations like inclusion of ghost peaks and noise signals which interfere with the metabolite fragments. Within

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The Netherlands Metabolomics Centre, new algorithms are under development providing easy processing and analysis of spectra. This will facilitate more global analysis of spectral data, enabling new ways of fragmentation analysis. Therefore, the present study is important in showing that the robust $MS^n$ spectral tree approach can provide the input for a Web-based $MS^n$ fragmentation database, thereby expanding the possibilities of MS in the metabolite identification process. While the described results are based on pure compounds infused by the Nanomate one by one, the practical use and robustness of $MS^n$ fragmentation during LC−MS-based metabolome profiling still needs to be shown. This application of online LC−$MS^n$ in metabolite annotation of crude extracts is currently under investigation. With identical protocols, the comparison of fragmentation data of LTQ-Orbitrap systems at different laboratories, as well as fragmentation data from different ion trap platforms, is facilitated. Currently, a round robin experiment is being conducted in order to examine the intra and interlaboratory variations of the LTQ-Orbitrap platforms.

**Reproducibility and Uniqueness of $MS^n$ Spectral Trees.** Parameters that can potentially influence the $MS^n$ fragmentation patterns, such as normalized collision energy, time interval, and compound concentration, only slightly influenced the spectral tree characteristics, from $MS^2$ up to $MS^5$. For example, the relative intensities of the fragments did not change from an ion concentration range of $\times10^5$ up to $\times10^7$ counts, i.e., a 100-fold difference. However, in case the discrimination is based on specific low abundant fragments present in lower stage $MS^n$ spectra, e.g., $MS^2$ or $MS^3$, or on differences in only higher stage $MS^n$ spectra, e.g., $MS^4$ or $MS^5$, the spectral tree may lose its uniqueness at too low metabolite concentrations. This threshold concentration depends upon both the ionization and fragmentation efficiency of the selected metabolite. In line with Kite and Veitch,[37] we observed that within one measurement series it is very well possible to discriminate between different flavonoid isomers, including the differences in relative intensities of generic fragments. On top of that, we established that the $MS^n$ spectral trees are also highly comparable between different measurement series even over a 5 month period, despite slight variations.

**Accurate Mass $MS^n$.** The studies on flavonoid fragmentation so far were hampered by either limited fragmentation depth in MS/MS[14,27,29,38−42] or limited mass accuracy.[17,18,32,43−45] Here we combined accurate mass with in-depth $MS^n$ fragmentation using an Ion Trap MS coupled to an Orbitrap FTMS. The accurate mass $MS^n$ approach enabled elemental composition calculation of all detected fragments. The assignment of the $MS^2$ fragments from 6,7-dihydroxyflavone (**3**) in positive ionization mode underlined the benefit of high mass resolution and accuracy: the fragments C7H5O4 and C12H9 are close in exact mass, i.e., 51.6 mDa difference. In nominal mass read-out, which has so far been used in all ion trap fragmentation studies on flavonoids,[17,18,32,43−45] the assignment of these two elemental formulas is hampered, thereby preventing their structural elucidation. Compared to accurate mass MS/MS, the present accurate $MS^n$ approach yielded more informative fragmentation spectra, which in some cases was essential to discriminate flavonoid isomers. For instance, differences between flavonoid glycosides were more prominent in $MS^3$ than in $MS^2$, while quercetin-triglucosides showed differential spectra only in $MS^5$.

So far, most studies have used positive ionization mode for polyphenol identification because they conclude that negative mode fragmentation often results in more complex and therefore less informative spectra. Indeed, we observed more complex $MS^n$ fragmentation patterns in negative ionization mode as compared to positive ionization, but nevertheless the fragmentation spectra were stable and reproducible and therefore representing a fingerprint of the fragmented molecule. Moreover, it was only in the negative mode that the various flavonoid glycosides showed differential and specific fragmentations.

The accurate mass $MS^n$ approach led to a collection of fragments occurring in series of hydroxylated and methoxylated flavones (Tables S-2−S-5 in the Supporting Information). Most $MS^n$ fragments we detected from hydroxylated flavonoids have been described in the literature before;[46] however, not all have been described with proposed fragmentation paths. For example, the fragment C12H9 (detected within 2 ppm of its expected mass) was present in the spectra of all dihydroxyflavones in both the positive and negative ionization mode. The generation of this fragment ion is remarkable, as apparently all four oxygen atoms have been lost before generation of the C12H9 ion. We propose a fragmentation pathway from, for instance, the protonated 4′-hydroxyflavonol (**6**) towards C12H9, involving sequential losses of three CO and one $H_2O$ molecules (Supplemental Figure 4 in the Supporting Information). Thus, the accurate mass $MS^n$ spectral tree method can corroborate proposed pathways and develop new ideas about the fragmentation paths present in polyphenolic compounds, thereby facilitating structural elucidation of molecular ions.

**Generation of Radical Fragment Ions.** The production of radicals in the fragmentation of polyphenolic compounds has been observed and described before both in the positive[22,24,46] and in the negative ionization modes.[22,32,37,46−48] The chemistry behind the origin of these radical ions is poorly understood.[47,49,50] We observed similar fragmentation patterns, including patterns upon


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fragmentation of the radical ions, for compounds studied earlier,[21,32] indicating that these radicals are reproducibly generated and are not artifacts of specific MS platforms. Especially the methoxylated polyphenols give rise to many radical fragments. The 3 Da selection window used in the $MS^n$ spectral tree method caused nonradical and radical neighbor ions to be fragmented simultaneously. Nevertheless, these complex and mixed radical and nonradical fragmentation patterns were reproducible and often different for related isomeric compounds.

**$MS^n$ Spectra of Flavonoid Aglycones.** $MS^n$ fragmentation of hydroxylated and methoxylated flavones resulted in specific RDA fragments that were discriminative between the various isomeric forms tested, either in one or both ionization modes, which can be rationalized because the number of hydroxyl groups in each ring influences the mass of the fragments resulting from the RDA reaction. In addition, the relative intensities of a number of RO fragments were influenced by the different substitution patterns. Some of these observations can be explained by fragmentation rules earlier defined for electron impact (EI) fragmentation, e.g., the facilitated loss of water in the case of ortho-positioned hydroxyl groups as in 6,7-dihydroxyflavone (**3**).[30]

By comparison of the fragmentation of dihydroxylated flavonoids with increasingly hydroxylated flavonoids, it appeared that a higher amount of hydroxyl substitutions resulted in a more branched fragmentation tree, i.e., more intense base peak fragments in $MS^2$ and $MS^3$ and a higher number of fragments generating $MS^4$ and $MS^5$ spectra. As a result, more time was needed to generate a single tree. The same fragmentation behavior was found for methoxylated flavonoids, indicating that substitutions on the aromatic rings weaken the bond strengths within the aromatic ring. Likely, the exact positions of the hydroxyl and methoxyl groups in the molecular ions influence the bonding energies and charge (de)locations over the ions, which determine their subsequent fragmentation.

The flavanols catechin and epicatechin were taken as an example in the comparison of the fragmentation patterns of stereoisomeric flavonoid cores, differing only in the stereochemistry of their C3-OH hydroxylation. Apparently the axial or equatorial C3-hydroxylation does not result in differential charge density of the flavonoid core. $MS^2$ and sometimes $MS^3$ fragmentation will discriminate most flavonoid aglycones; however, following our approach up to $MS^5$, more in-depth fragmentation paths will be revealed, facilitating assignment of structures to fragments.

**$MS^n$ Spectra of Flavonoid Glycosides.** For the flavonoid glycosides studied, the $MS^n$ spectra were generally more discriminative in negative ionization mode than in positive ionization mode. In addition, their ionization is generally better in negative mode. This is in contrast to the flavonoid aglycones, for which positive ionization was the favorable mode, especially for those aglycones that did not ionize at all in the negative ionization mode such as (poly)methoxylated flavonoids. $MS^n$ fragmentation was able to differentiate between the three positional monoglucoside isomers of quercetin (**22**−**24**) in the negative ionization mode (Figure 3), in line with recent MS/MS observations by Geng et al.[51] In addition to the monoglucosides, using $MS^n$ up to $MS^5$ we were also able to differentiate between isomers of di- and three-glucosides of quercetin, thus showing the power of $MS^n$. The most likely explanation for the observed differences between fragmentation patterns obtained from the same quercetin fragment ion derived from the various quercetin-glucoside structures is the occurrence of differential charge distributions over the quercetin aglycone fragments. Using the spectral tree approach, we were able to distinguish glucose from a galactose moiety (C4 epimers) located at the 3-*O* position on the rather complex flavonol cores syringetin (Figure 3) as well as quercetin. The difference in fragmentation behavior between, on the one hand, the glycosidic stereo isomers and, on the other hand, the isomeric flavonoid couple of catechin and epicatechin is remarkable, because both couples differ in the orientation of only one hydroxyl group. A possible explanation for the difference in fragmentation behavior may be a lower flexibility of the hydroxyl group attached to the flavonoid core compared to more flexible sugar hydroxyl groups, as well as the larger internal flexibility of the sugar moiety.

## CONCLUSIONS

Accurate mass $MS^n$ fragmentation spectra were reproducibly generated enabling differentiation of isomeric compounds such as polyphenols. For all 121 but 2 compounds tested, the spectral trees showed unique fragments and/or differences in relative intensities of fragment ions. Subtle differences between glucose and galactose conjugations and positions of sugar substitution were observed upon fragmentation of the aglycone fragment, in some cases only apparent in $MS^5$ spectra. The method developed was robust and highly reproducible within and between analyses series. Thus, we conclude that $MS^n$ fragmentation patterns can be used for structural elucidation of polyphenol structures and substructures. Although we cannot yet rule out the influence of different LTQ-Orbitrap instruments, both the reproducibility and robustness of our data and the correspondence with flavonoid $MS^n$ data spectra available in the literature suggest that $MS^n$ databases can be developed to compare and match results from different laboratories.

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## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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