Strategies for differentiation of isobaric flavonoids using liquid chromatography coupled to electrospray ionization mass spectrometry

Short title: Strategies for differentiation of isobaric flavonoids using LC-ESIMS

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Abstract

Flavonoids are a class of secondary plant metabolites existing in great variety. Due to this variety identification can be difficult, especially as overlapping compounds in both chromatographic separations and mass spectrometric detection are common. Methods for distinguishing isobaric flavonoids using MS^2 and MS^3 have been developed. Chromatographic separation of various plant extracts was done with RP-HPLC and detected with positive ESI-MS operated in information dependent acquisition (IDA) mode. Two methods for determination of flavonoid identity and substitution pattern, both featuring IDA criteria, were used together with the HPLC equipment. A third method where the collision energy was ramped utilized direct infusion. With the developed strategies it is possible to differentiate between many isobaric flavonoids. Various classes of flavonoids were found in all of the plant extracts, in the red onion extract 45 components were detected and for 29 of them the aglycone was characterized, while the substituents were tentatively identified for 31 of them. For the strawberry extract those numbers were 66, 30 and 60, and for the cherry extract 99, 56 and 71. The great variety of flavonoids, several of them isobaric, found in each of the extracts highlights the need for reliable methods for flavonoid characterization. Methods capable for differentiating between most of the isobars analyzed have been developed.

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Introduction

Flavonoids are a class of secondary plant metabolites which are commonly found in great diversity, to date over 9000 different flavonoids have been reported.[1] Flavonoids are a type of polyphenolic compounds that can divided into different classes or subgroups such as; Flavones, Isoflavones, Flavonols, Flavanones and Anthocyanins, depending on basic structure of the central aglycone. The composition of different plant extracts is often complex, as the aglycone most often are conjugated with different substituents such as sugars and organic acids[2], and to characterize the sample reliable methods for separation and analysis are required. Analysis of those complex samples could be performed in targeted[3] or non-targeted[4] mode. For successful targeted analysis, accesses to reference standards are often required, however, for a non-targeted approach, reference standards are less critical. The main strategy for analyzing flavonoids is to utilize liquid chromatography (LC) for decreasing the complexity of the sample prior to detection. LC coupled to ultraviolet-visible (UV-vis) diode array detection (DAD) provides good selectivity facilitating differentiation of, for example, the colored (pH dependent) anthocyanins from other flavonoids.[5] DAD can also give valuable information about substituent identity.[6] However, in many cases co-elution of different flavonoids are often encountered in LC making characterizing the flavonoid composition difficult, especially since standards are not always available. Mass spectrometry (MS) with electrospray ionization (ESI) has gained popularity as the technique both provides higher selectivity and information that could be used for identifications. However, selection of ionization mode, positive or negative, will have an influence on the type of information that could be achieved but also on the sensitivity. In the literature a number of different screening or fingerprint methods have been reported,[7-13] where some have used, positive ionization mode,[12, 13] while other have use negative ionization.[9, 10] Information from pos/neg mode provides complementary information and thus some work has

been done utilizing both ionization modes.[8, 11, 14] Generally, in the literature, it is considered that negative ionization provides best sensitivity[9, 14, 15] but it could also be found that positive ionization are more useful for structure elucidation [11, 14, 15]. Co-elution of different flavonoids are often encountered in LC making characterizing the flavonoid composition difficult, especially since standards are not always available. To fully characterizing the flavonoid composition both good selectivity and sensitivity is required. An advantage with MS is that the sensitivity is higher than DAD and by using multiple reaction monitoring (MRM) mode to acquire data the sensitivity can be further improved.[16] Another way to improve selectivity would be metal complexation, in which a metal salt is added to the analyzed solution. Typically, metal complexation will result in a larger ion abundance compared to protonated or deprotonated flavonoids.[17] This would however not work well with anthocyanins, since they already carry a positive charge. Furthermore, anthocyanins are not so well suited for analysis in negative mode, for example Kammerer *et al.* used positive mode for the detection of anthocyanins whereas negative mode was utilized for other phenols.[7] Some work utilizing negative ESI for analyzing anthocyanins could be found in the literature, though.[10]

Due to the systematic classification of flavonoid structures[18] and knowledge of common fragmentation behavior[9, 19] it is possible to calculate theoretically possible MRM transitions. Recently a strategy to identify anthocyanins in plant extract, termed MIAC[16] (multiple reaction monitoring-initiated anthocyanin characterization), were reported which was based on characteristic mass over charge ratios (*m/z*)[19] for the fragmentation of anthocyanins to anthocyanidins. However, a limitation with the MIAC method is that it cannot differentiate between isobaric compounds which are commonly encountered.

In this study, several strategies to overcome this limitation were explored. One question that were of interest to explore was; can the utilization of tandem mass spectrometry such as MS^2

and MS^3, be used to overcome the shortcomings of MIAC? By carefully studying more unique fragments compared to those normally monitored with the MIAC strategy it might be possible to overcome the limitation of the MIAC strategy. Furthermore, can the method be selective enough so that DAD could be omitted. The overall aim is to have a strategy that only requires one LC run, thus the work were focused on LC-ESI-MS operated in positive ionization mode which suites anthocyanins best but also works for the other flavonoids.

Materials and methods

Chemicals. Water, methanol and formic acid (all for LC-MS applications) were obtained from Fisher Scientific GTF AB (Västra Frölunda, Sweden). The following standards were from Polyphenols Laboratories AS (Sandnes, Norway): cyanidin , cyanidin-3-O-glucoside (cy-3-glu), cyanidin-3-O-galactoside (cy-3-gal), cyanidin-3,5-di-O-glucoside (cy-3,5-diglu), cyanidin-3-O-rutinoside (cy-3-rut), delphinidin-3-O-glucoside (del-3-glu), pelargonidin-3-O-glucoside (pel-3-glu) and malvidin-3-O-glucoside (mal-3-glu) as chloride salts (≥98% purity) and quercetin-3-O-glucoside (que-3-glu), quercetin-4´-O-glucoside (que-4´-glu) and quercetin-3,4´-di-O-glucoside (que-3,4´-diglu) as powders (≥98% purity). The following standards were from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany): (+)catechin, apigenin, apigenin-7-O-glucoside (api-7-glu), apigenin-7-O-neohesperidoside (api-7-neo), genistein, hesperetin, hesperetin-7-O-rutinoside (hesp-7-rut), kaempferol, kaempferol-3-O-glucoside (kaempf-3-glu), kaempferol-3-O-rutinoside (kaempf-3-rut), luteolin, luteolin-7-O-glucoside (lut-7-glu), luteolin-8-C-glucoside (lut-8-C-glu), morin and isorhamnetin as powders (analytical quality). Red onion (*Allium cepa L.*) and strawberries (*Fragaria × ananassa*) were purchased at a local supermarket in Uppsala, Sweden, and sour cherries (*Prunus cerasus*) were harvested from a local garden.

Sample Preparation. A standard of each flavonoid was prepared by dissolving it in a 50/50 (v/v) mixture of water and methanol, both acidified with 5% formic acid, and then diluting it 10 times with acidified water giving final concentrations between 20 and 35 μM.

The plant extracts used were prepared as described in more detail previously.[16] The edible part of a red onion was cut in small pieces and extraction was done in a batch minireactor (Autoclave Engineers, Erie, PA) with a water/ethanol/formic acid mixture. The extracts was then filtered into Eppendorf tubes and stored at -18 ^{o}C until used. Both the strawberry and cherry extracts were prepared by cutting the berries to small pieces prior to homogenization and extraction with an ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA).

Instrumentation. An Agilent 1100 series system (Agilent Technologies, Waldbronn, Germany) with a degasser, a binary pump, a thermostated autosampler and a DAD equipped with a Zorbax SB-C18 column (2.1* 100 mm, 3.5 μm, Agilent Technologies, Santa Clara, CA) was used for separation. The mobile phases used were water (A) and methanol (B), both with 5% formic acid. A stepwise gradient was used, starting at 5% B for 2 min, increased to 20% over 2-10 min, held constant at 10-15 min, increased to 30% B over 15-30 min, held at 30% for 30-35 min, increased to 45% B for 35-50 min and finally reequilibration at starting conditions for 50-70 min. The flowrate was 0.200 ml/min. Data were collected using Agilent Chemstation (B.03.01).

The LC system was coupled to a 3200 QTrap triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada) using ESI in positive mode. Analyst (1.4.2) was used to collect MS data. Two different information dependent acquisition (IDA) methods were used schematically shown in Figure 1; method 1, which utilized up-front fragmentation combined with MRM followed by MS^2 detection, and method 2, utilizing MRM followed by MS^2 and MS^3 detection.

The following settings were used in method 1; gas heater temperature (Temp): 600 ^{o}C, curtain gas (Cur, N_2): 15 psi, ion source gas 1 and 2 (GS1 and GS2, N_2): 60 psi, collision energy (CE): 50 eV, ion spray voltage (IS): 5500 V, declustering potential (DP): 90 V and entrance potential (EP): 10 V. 40 MRM transitions were monitored, see Table 1, with an IDA criterion, selecting the most intense signal exceeding 1000 counts per second (cps) for MS^2 in Enhanced Product Ion mode (EPI).

The settings were mostly the same in method 2, however, different CE (30 eV), and DP (50 V) were used. The first experiment consisted of MRM transitions, listed in Table 2, which were followed by an IDA criterion selecting the most intense signal over 1000 cps for, an MS^2 (EPI) experiment. The MS^2 experiment utilized the same settings as the MRM with the exception of CE, which was 40 eV. This was further followed by a second IDA criterion, again selecting the most intense signal over 1000 cps, in the MS^2 spectra, for an MS^3 experiment. The conditions for the MS^3 experiment were the same as the MS^2 experiment, with the addition of a fragmentation voltage (AF2) of 80 V to fragment the selected ion.

A third type of method using direct infusion, method 3, of the standards was also conducted. The settings used were as follows; gas heater temperature (Temp): 200 ^{o}C, Cur: 15, GS1 and GS2: 20 psi, IS: 5500 V, DP: 50 V, EP: 10 V. Injections were done using the mass spectrometer's syringe pump and a 250 µl syringe (Hamilton, Reno, NV) at a flow rate of 10 µl/min. Energy resolved mass spectra (ERMS) were acquired by ramping the CE from 5-100 eV in steps of 1 eV and collecting the data by summation of 10 spectra in full scan EPI mode for each CE.

Results and Discussion

Upfront fragmentation of the standards combined with MS^2 (method 1).

Determination of the identity of aglycones present is a first important step when a sample containing flavonoids should be characterized. One approach found in the literature for achieving this utilizes basic and acidic hydrolysis in such a way that substituents are cleaved off, more or less completely, before chromatographic analysis.[8, 20-22] One limitation with this approach is that information about the number of flavonoids with a specific aglycone could not directly be obtained. Furthermore, C-linked substituents are more resistant against hydrolysis[8], thus identity of those aglycones will not be obtained. As can be seen when comparing lut-7-glu, and lut-8-C-glu (entry **14** and **15** in Table 3), the O-glucoside readily fragments using both methods 1 and 2, whereas no fragments could be seen for the C-glucoside although the loss of the glucoside can be seen with method 2. The main aim with the strategy presented here is to identify which aglycones those are present in the analyzed sample without altering the composition of the sample and at the same time also estimate the number of compounds with a certain aglycone. By increasing the declustering potential when ions are sampled from the ion source into the vacuum system of the mass spectrometer, so-called up-front fragmentation[23] (also referred to pseudo-MS^3[11] or in-source fragmentation[4]), can be promoted. During the sampling step, different flavonoids are mainly converted to the corresponding free aglycone. By acquiring the data in SIM mode it would then be possible to detect the free aglycones at low concentrations, however, the selectivity might not be good enough if the analyzed sample is complex.[16] Instead, by combining up-front CID with ordinary CID the selectivity is increased and at the same time the numbers of possible *m/z* values (parent ions) that have to be monitored to cover a broad range of compounds are reduced, especially compared to the MIAC method.[16] The combination of up-front CID and ordinary full scan CID has been reported previously.[4, 11] However, one limitation with this approach is low sensitivity, and low levels of flavonoids could be difficult to detect. This limitation could be addressed by utilizing MRM, however, isobaric aglycones are rather

common, thus, selection of suitable MRM transitions has to be done carefully. The MRM transitions monitored with method 1 which are listed in Table 1 were chosen to match losses and fragmentations of free aglycones, based on *m/z* values observed upon fragmentation of standards combined with literature values reported by others.[24, 25] MS/MS spectra, with monitored *m/z* values, of the free aglycones and their corresponding structure are shown in Figure S1 and Figure 2 respectively. The flavonoids in Table 1 are grouped based on nominal mass of the positive ion, starting with *m/z* 271 followed by 287, 291, 301, 303, 317 and 331. As can be seen in Table 1 many MRM transitions are not unique for a particular aglycone, for example the transition 271/121 could be generated from cleavage of the C-ring at bound 0 and 2 producing the same $^{0,2}B^+$ fragment of apigenin or $^{0,2}B^+$ fragment of pelargonidin respectively. Further examples are the transition 287/137 which could correspond to cleavage of the C-ring at different locations generating the $^{0,2}B^+$ fragment of cyanidin or luteolin respectively or the $^{0,3}A^+$ fragment of kaempferol. Another example is the 303/153 transition which could be the $^{0,2}B^+$ fragment of delphinidin or the $^{1,3}A^+$ fragment of quercetin according to the nomenclature proposed by Mabry and Markham[26] which was further developed by Ma *et al*.[27] At the end of Table 1 a list of identical MRM transition numbers could be found. Further, detailed data from the standards is shown in Table 1.

One requirement for method 1 to be successful, especially when the number of standards is limited, is that flavonoids with identical aglycones, more or less, should give rise to the same MRM signals with similar signal intensities. In other words, the substituents on the central aglycone should ideally not influence the final MS/MS spectra of the free aglycone which has been selected after up-front CID and subjected to ordinary CID in a collision cell. Upon closer examination of data generated from standards it could be concluded that, for example, for the two first compounds, api-7-glu **1** and api-7-neo **2**, listed in Table 3, the major MRM transitions are

the same with similar relative intensities. However, the third compound in Table 3, the free apigenin **3**, shows slightly different pattern. One reason for this could be that the internal energy of the parent ion (*m/z* 271), selected by Q1, is different due to the large difference in the initial *m/z* values prior to up-front CID. Similar results could be seen for the different cyanidins **6-10**, kaempferols **11-13**, quercetins **21-23** and hesperetins **24, 25**.

A second requirement for method 1 to be successful is that different aglycones should give rise to different MRM signals, however, this is not always that easy to achieve as pointed out above, many MRM transitions are similar. Upon inspecting the MS/MS spectra shown in Figure 3 of the free aglycone generated from the isobaric cy-3-glu **8**, kaempf-3-glu **11** and lut-7-glu **14** it can be concluded that many *m/z* fragments are common. However, there are some differences such as *m/z* 129 being the base peak for **8**, whereas the base peak for **11** and **14** is *m/z* 153. Other differences would be that some major peaks in one spectrum are only minor peaks in the other, such as *m/z* 157 (major peak for **11**), and *m/z* 287 (major peak for **14**). With these differences in the MS spectra alone identification of a compound might be difficult, especially at low signal intensities. A clear distinction between the three isobaric flavonoids can be made by utilizing the chromatographic MRM peak profiles, see supporting info Figure S1. For **8** one of the most intense signals was 287/137 ($^{0,2}A^{+}$), whereas for **11** and **14** the most intense signal was 287/153 ($^{1,3}A^{+}$). To differentiate between **11** and **14** the signal 287/121 ($^{0,2}B^{+}$) can be utilized; **11** shows a signal of approximately 60% of the base peak while **14** shows no such signal at all.

One limitation with method 1 is the problem to differentiate between flavonoids with different substituents but the same aglycone. For example, no unique fragments could be found in the MS^2 spectra of the free aglycone generated from que-3-glu **21**, que-4´-glu **22** and que-3,4´-diglu **23**, respectively. Furthermore, the isobaric cy-3-gal **7** and cy-3-glu **8** which differ only by

the identity of the sugar unit showed no clear unique fragments so the only way to distinguish them is chromatographic retention time.

Even though it has been shown that it is possible to distinguish aglycones with isobaric substituents[28-30] and the same substituent(s) at different positions[29] this is difficult when evaluating the results from compound **7, 8** and **22, 23, 24** with the settings used in method 1 in this study. To address this limitation a CE ramp was used in an attempt to distinguish compound **7, 8** and **22, 23, 24** (see discussion about method 3 below). Information about substitution is, however, not always required and the limitation with method 1 mentioned above will not be a problem if for example, the aim is to estimate the number of quercetin isomers.

MS^2 and MS^3 fragmentation of the standards (method 2).

Instead of characterizing a sample by first promoting the generation of free aglycones, as was done in method 1, method 2 is based on utilizing the knowledge how flavonoids commonly fragment[16] when MRM transitions are selected. As has been observed previously,[24, 31] the loss of the sugar unit(s) is a common fragmentation, although it is dependent on the collision energy. With this in mind, the MRM transitions monitored with method 2 (see Table 2) were chosen to match common flavonoids[32] and the standards. The Q1 values represent *m/z* values of intact flavonoids with common substitutions such as glucosides (+162 u), diglucosides (+324 u) and rutinosides (+308 u) while the Q3 values (*m/z* 271, 287, 301, 303, 317 and 331) represent the free aglycones. Once a chromatographic MRM peak exceeds a set intensity threshold value, MS^2 and MS^3 spectra are acquired and the data is shown in Table 3 for the standards. The collision energy is set generic to 40eV which in most cases resulted in MS/MS spectra dominated by *m/z* values corresponding to the different free aglycones. Thus the free aglycone were then subjected to further fragmentation (MS^3) utilizing the instruments capability to operate the last quadrupole (Q3) as an ion trap. This strategy will then provide increased selectivity compared to method 1.

When comparing the MS^3 spectra, obtained during a LC run, from cy-3-glu **8**, kaempf-3-glu **11** and lut-7-glu **14** (see Figure 4) one can first notice that **11** has more fragments of (comparably) high intensity than the others (**8, 14**). These fragments would be *m/z* 165 ($^{0,2}A^+$), 213 (loss of H_2O and 2CO) and 241 (loss of H_2O and CO). Of somewhat lower intensity are the fragments *m/z* 153 ($^{1,3}A^+$) and 231 (loss of 2CO). The *m/z* 153 fragment is the only one of higher intensity in **14**, while for **8** the *m/z* 213 fragment is of higher intensity and some others, like *m/z* 259 (loss of CO) and 223 (loss of $2H_2O$ and CO), are of lower intensity. From those results it can be concluded that MS^3 spectra, i.e. fragmentation spectra of the intact aglycone, could provide information that facilitates differentiation of isobaric aglycones.

As expected (based on the results from method 1), flavonoids with the same aglycone are much more difficult to identify using the MS^3 spectra alone, for example, when comparing cy-3-gal **7** and cy-3-glu **8** the only thing to tell them apart was the retention times, as they are isobaric and the MS^3 spectra, i.e the fragmentation of the intact aglycone with *m/z* 287, were very similar. An analogous behavior was observed for cy-3,5-diglu **6** and cy-3-rut **9,** where the MS^3 spectra showed no unique fragments for either compound. However, utilizing the additional information from MS^2 spectra that method 2 could provide, identification could be done. The loss of only one saccharide (*m/z* 449) in the MS^2 spectra (see Figure S2) was more intense in the spectra of **6**, whereas the spectra for **9** show a more pronounced loss of both saccharides. Since such behavior has been observed earlier,[33, 34] not only for cyanidin but other flavonoids as well, this would be a possible way to distinguish flavonoids that are disubstituted with monosaccharides from flavonoids monosubstituted with disaccharides. Furthermore, the peak at *m/z* 449 (loss of rhamnose) for cy-3-rut **9** provide information about the carbohydrate sequence and can be used to tell which saccharide is bound to the aglycone.

Plant extracts.

To test the presented strategies a number of different plant extracts, such as red onion, strawberry and cherry, were analyzed. The red onion extract was analyzed with method 1 and method 2 and the total ion chromatograms (TIC) can be seen in Figure 5. In an attempt to provide a discussion that is easy to follow the chromatographic peaks are labeled based on proposed compound identity number listed in Table 4. As a consequence of the complex sample one chromatographic peak could be labeled with several numbers if co-eluting compounds are present. XIC's of the MRM transitions in method 1 can be found in supporting information, Figures S4-S8.

The first major peak detected (entry 8, Table 4) with method 1 at 21.2 min, in Figure 5, was identified as cy-3-glu based on; similar MRM signal profiles as standard analyzed with method 1 (entry **8**, Table 3) and similar MS^2 spectra of the intact flavonoid (*m/z* 449) recorded with method 2. The MS^3 spectra (*m/z* 449→287→xxx, see Figure S9b) for compound **8** shows about the same relative intensity for the most intense fragment (*m/z* 213) compared to the spectra obtained from the standard (5% vs 8%). As cy-3-glu was available as a standard, retention time and UV/Vis spectra confirms the identification.

Four more cyanidin based standards were available (entry **6**, **7**, **9** and **10** in Table 3) and as has been discussed previously, the free aglycone from the conjugated cyanidin standards show similar MRM profiles. Upon evaluating the MRM transitions corresponding to the 287→xxx the results indicates (see Figure S5) that most likely more than 10 cyanidin containing compounds are present in the red onion sample. Peak nr 3, 5, 6, 8, 10, 14, 15, 17, 20, 21, 27, 29, 34, 35, and 39 (see Figure S5), all show MRM patterns that are similar to the cyanidin standards that were analyzed in this study. The peak in Figure S5 with a retention time of 24.3 min (compound 10) has a retention time close to one of the available standards, Cy-3-rut entry **9** in Table 3, however, method 2 indicates that *m/z* 611 is the parent ion and not *m/z* 595. Furthermore, MS/MS of *m/z*

611 only show the loss of 324 Da down to *m/z* 287 indicating that the cyanidin is substituted with a di-hexose, for example Cy-3-laminarbioside which has been reported in red onion previously.[35] Method 2 provides information that at least makes it possible to postulate which substituents that could be present for all those cyanidin containing compounds. The second most abundant compound containing cyanidin compound 27 was, besides the MRM profiles from method 1, identified based on the presence of the 535/287 MRM transition observed with method 2 which indicates a substitution with a hexose and malonic acid. Furthermore, as the MS^2 spectra do not show any signal corresponding to a cy+malonyl ion (*m/z* 373) indicating that the cyanidin was monosubstituted with a malonylhexoside, thus matching Cy-3-(6''-malonoylglucoside) which was observed as one of the major flavonoids in red onion previously.[35] The possible identities of the other cyanidin containing compounds are shown in Table 4. The identity of some of them were further supported by data from the literature,[35-37] data which could also be used to make a more accurate assigning of substituents.

The next major peak (compound 12 in Table 4) at 25.2 min in Figure 5 was identified as que-3,4'-diglu based on; similar MRM signal profiles as standard analyzed with method 1 (entry **21**, Table 3) and similar MS^2 spectra of the intact flavonoid (*m/z* 627) recorded with method 2. In method 2, the presence of the 465/303 and 627/303 MRM transitions gives further strength to the identification that the compound is a quercetin di-substituted with hexoses. In this study 7 flavonoid standards with isobaric aglycones at *m/z* 303 were available. Upon evaluation of the MRM 303→xxx transitions (see Figure S7) it can be concluded that at least 9 quercetin containing compounds (nr: 4, 7, 9, 12, 19, 27, 29, 32, and 44) are present in the extract.

Closer examination of the first major peak eluted at 21.0 min in Figure 5, indicates that some coeluting compounds with lower intensity are present. Method 2 reveals the presence of a 627/303 MRM transition and a slightly weaker (22% of the intensity) 465/303 MRM transition

indicating that likely a delphinidin or quercetin di-substituted with hexoses is present. The co-eluting compound is present at a low concentration, however, the MRM data from method 1 provides good enough signals to state that the co-eluting compound contains quercetin, thus, combining the information from method 1 and method 2 it can be concluded that the identity of compound 9 most likely is a quercetin substituted with two hexoses. It should be pointed out, in this case, that reliable identification could not be done based on the MS/MS spectra of *m/z* 303 obtained with method 1 as the signals were very weak, thus the identification relies on the MRM profiles from method 1 combined with MS/MS data from method 2. As a standard of Que-3,4'-diglu was available this could be excluded from the possible identities as the retention times do not match. Another diglucosides reported in red onion[36, 37] would be Que-7,4'-diglu, however compound **7** also show signals matching this so a clear designation cannot be made.

The third major peak around 34.5 min is a split peak containing at least two compounds. Compound 26 in Table 4 was identified as que-3-glu due to the matching spectra (entry **22**, Table 3) and retention time. The identity of the second compound 27 has already been discussed above.

The fourth major peak at around 38.6 min contains several coeluting compounds. The major compound 31 was identified as quercetin que-4'-glu based on similar MRM signal profiles as standard analyzed with method 1 (entry **23**, Table 3) and similar MS^2 spectra of the intact flavonoid (*m/z* 465) recorded with method 2. The fifth major peak at 50.6 min (entry 43, Table 4) had a fragmentation pattern suggesting a quercetin compound and while no fragments could be seen in method 2 the MRM transitions 465/303 and 551/303, with 465/303 being most intense, suggests disubstitution with hexose and malonic acid. The possible identities of the other quercetin containing compounds are shown in Table 4. Just as for the cyanidin based compounds, the identities of some of the quercetin compounds was supported by previous findings.[35-37]

Compound 32 which elutes on the tail of the fourth major peak, containing compound 30, was identified as kaempf-3-glu based on similar MRM signal profiles (287/153 and 287/121) as standard analyzed with method 1 (entry **11**, Table 3) and similar MS^2 spectra of the intact flavonoid (*m/z* 449) recorded with method 2. The last signal around 38.5 min show only one MRM transition, *m/z* 317/153 (entry 29, Table 4). Three different MRM transitions with *m/z* 317→xxx where monitored with method 1 which according to literature[24, 38] could correspond to isorhamnetin or petunidin. As only one MRM transition was seen for entry 30 identification could not be done. Further evaluation of the MRM 317→xxx transitions (see Figure S8) reveals that at least 5 isorhamnetin or petunidin containing compounds (nr: 18, 36, 37, 42 and 45) are present in the extract. As only one standard with an aglycone of *m/z* 317 was available, different classes of flavonoids cannot be told apart from each other. However, from method 2, there are two compounds containing a hexoside substituent; compounds 18 and 37. Red onion has previously been reported to contain isorhamnetin-4'-glu and isorhamnetin-3,4'-diglu,[36, 37, 39] both which would yield the signal 479/317. The transition corresponding to a dihexoside (642/317) was not monitored, so the compounds cannot be told apart by this. Assuming that the elution order will be the same as for the corresponding quercetin standards, which was observed by Bonaccorsi *et al.*[37], 18 was tentatively identified as isorhamnetin-3,4'-diglu and 37 as isorhamnetin-4'-glu.

Upon evaluation of the MRM 301→xxx transitions (see Figure S6) a few weak peaks are detected which basically show two different types of MRM profiles. Peaks nr: 16, 33 and 1, 22, 38 show similar profiles, respectively, however, none of the profile types resembles the profiles seen from the only available standard (entry **18**, Table 3) thus identification of the aglycon could not be done.

A few very weak peaks are detected upon evaluation of the MRM 271→xxx transitions (see Figure S4), however none of them show similar MRM patterns as the available standards (entry **1-5**, Table 3), thus, identification of the aglycone could not be done.

The methods were also tested on two other types of samples, strawberry and cherry. The main components of the strawberry extract (see supporting information, Figures S10-S14) were identified as cy-3-glu 22, pel-3-glu 27, pelargonidin monosubstituted with hexose and deoxy hexose (peak 31, possibly pel-3-rut reported in strawberry previously[40, 41]), pelargonidin with unknown substituent(s) (peak 53) and another pelargonidin with unknown substituents (peak 56). Peak 39 was not identified. The main components of the cherry extract (see supporting information, Figures S15-S20) were also identified: cyanidin with dihexose (peak 32), cy-3-glu (peak 35), quercetin disubstituted with hexose (peak 40) cy-3-rut (peak 41), quercetin disubstituted with hexose and deoxy hexose (peak 71), kaempferol disubstituted with hexose and deoxy hexose (peak 85) and an unknown flavonoid with hexose (peak 87). Using the combined data from methods 1 and 2 together with previously reported data,[35-37, 40-52] the peaks could be tentatively assigned. The assignments can be found in Table 4 (red onion) and in supporting information, Tables S1 and S2 (strawberry and cherry respectively).

To summarize methods 1 and 2, 45 peaks were detected in the red onion extract. Of these peaks the aglycones were determined for 29 of them. At first this number does not seem very promising, but considering that 11 of the 45 detected peaks were of *m/z* which at most one aglycone (regardless substitution(s)) was analyzed, and thus no comparison between different flavonoid subgroups could be done, the methods show potential. Furthermore from method 2, the substituent identity of 31 of the peaks could be tentatively determined, though the position of the substituent(s) could not be determined based only on the MRM transitions in experiment 2. The proposed methods do well compared to other recent studies where flavonoid analysis has been

the aim (or one of the aims); 16 (eight anthocyanins and eight flavonols)[39], 12 (seven anthocyanins, four flavonols and one unclassified flavonoid)[53] and 16 (eight anthocyanins and eight flavonols)[54] flavonoids respectively has been identified. It would of course be relevant to compare the results also with those obtained previously by MIAC[16] where 33 peaks were reported, some containing several compounds resulting in 47 entries. In a review[55] 54 different flavonoids were reported in onion, among them 25 anthocyanins in red onion, though these flavonoids were distributed over several cultivars and, apart from anthocyanins, not limited to red onions. For the strawberry extract those numbers were 66, 30 and 60, and for the cherry extract 99, 56 and 71. More extensive lists of the signals from the different experiments for each major peak as well as the minor peaks can be found in Table 4 (red onion) and Table S1 supporting information (strawberry extract) and Table S2 (cherry extract).

Energy resolved mass spectrometry (method 3).

Since some isobaric flavonoids could not be distinguished from each other with the settings used in method 1 and 2, an attempt to distinguish them using energy resolved mass spectrometry by ramping the collision energy was made. The ERMS data were recorded by collecting a summation of 10 MS/MS spectra in full scan EPI mode for each CE value. ERMS data, both the peak intensity and the ratio between free aglycone and intact flavonoid, for two monosubstituted quercetin glucosides are shown in Figure 5. By plotting the ratio of the free aglycone over the intact glucosylated flavonoid utilizing logarithmic scale a broader range of values can more easily be visualized. At 5 eV, the glycosylated flavonoid was the base peak for que-4´-glu, whereas the free aglycone was the base peak for que-3-glu. At 30 eV the free aglycone was the base peak for both flavonoids and the ratio of the free aglycone to the glycosylated aglycone 400 for que-3-glu and 9.8 for que-4´-glu. At 40 eV the fragmentation patterns were different; que-4´-glu showed the

most intense fragments above *m/z* 200, whereas que-3-glu had intense signals both above and below *m/z* 200. As can be seen in Figure 5, the substituent at 3-position is more easily lost compared to the substituent at the 4´-position for the quercetins which agrees well with previously reported observations.[15] For the disubstituted quercetin, que-3,4´-diglu, the major signal was *m/z* 465 at 5 eV, but 303 and 627 could also be seen (data not shown), thus the signal at *m/z* 465 would most likely be from the loss of the substituent at the 3 position. At 30 eV the signal at *m/z* 627 had disappeared and the signal from the free aglycone was about 1.5 times as intense as the signal from the monosubstituted flavonoid.

Another example of flavonoids with two sugar substituents are cy-3,5-diglu and cy-3-rut. For cy-3,5-diglu the most intense signal at 5 eV was *m/z* 611 and 449 was more intense than 287. For cy-3-rut the most intense signal was *m/z* 595, but here 287 was more intense than 449. At 30 eV the free aglycone was the most intense signal for both cy-3,5-diglu and cy-3-rut but *m/z* 449 was more intense than 611 for cy-3,5-diglu, whereas 595 was more intense than 449 for cy-3-rut. A third example of a flavonoid with two sugar substituents showing interesting results is hesp-7-rut. When selecting the intact flavonoid (*m/z* 611) in Q1; clear signals corresponding to losses of parts of the substituents. At 5 eV they were more intense between *m/z* 465 and 303, but could be clearly seen between 611 and 449 (see Figure S21). The signals between *m/z* 465 and 303 were still clearly visible at 25 eV, while those between 611 and 465 were not.

Instead of focusing on the intact flavonoids when identification is done analysis of the fragmentation behavior of the free isobaric aglycones could be done by selecting the *m/z* of the free aglycone in Q1. The quercetins all showed the same signals, with the intact aglycone being the base peak up to 44 eV. Fragments corresponding to losses of H_2O's and CO's (mainly *m/z* 285, 257 and 229) were present over the whole range recorded. At lower CE's (up to 25 eV) the ^{0,2}A signal (*m/z* 165) was the highest signal resulting from ring cleavage, while at higher CE's the

^{1,3}A signal (*m/z* 153) were more intense. At 5 eV the intact aglycone (*m/z* 303) was the most intense signal for the hesperetins, with the major fragment being *m/z* 177 followed by 153 and 179. At 25 eV the fragments 177 and 153 were both higher than 303. Morin showed only low intensity signals of fragments (the most intense being *m/z* 285, 257 and 271) at 5 eV with the intact aglycone being the highest signal. At 20 eV the intact aglycone was still the highest signal, but more fragments could be seen (for example *m/z* 153, 229 and 149). At 40 eV the fragments *m/z* 153, 229, 137 and 219 were all higher than the intact aglycone (the most intense about twice the intensity). For del-3-glu, the intact aglycone was the base peak up to 47 eV, when *m/z* 229 reached a similar intensity. Other intense fragments were *m/z* 285, 257, 173 and 139. The 257 signal was actually of greater intensity than the 229 signal up to 32 eV. A figure similar to 5b containing all isobaric aglycones (*m/z* 303) can be seen in the supporting information (Figure S22). From the data it can be seen that the 177/303 ratio optimizes at different collision energies for the different aglycones thus facilitating identification.

An even more challenging analysis for ERMS is the attempt to differentiate between cy-3-glu and cy-3-gal, two very similar flavonoids, with isobaric substituents at the same position. They were difficult to differentiate even in method 3. At 5 eV the substituted flavonoid was the base peak, but the free aglycone could still be seen. At 30 eV the free aglycone was the base peak and the major fragments were the same. Although both flavonoids had some minor fragments that were not seen in the other flavonoid, but these fragments could probably not be used to differentiate the flavonoids as they appeared for the other flavonoid as well at 40 eV. The ratios of the free aglycone over the intact flavonoid were plotted, and as can be seen in supporting information Figure S23a, the trend is that cy-3-glu always has a slightly lower ratio than cy-3-gal. This trend could likely make differentiating between the two cyanidin isobars easier. With the signals from hesp-7-rut in mind (discussed above) the signals between *m/z* 449 and 287 were

studied closer, and at 30 eV a signal at *m/z* 360 could clearly be seen for cy-3-gal but only very weak for cy-3-glu. Likewise at 35 eV cy-3-gal showed more intense signals above *m/z* 400, and after examining some of them at different CE's, the ratio of *m/z* 417 over 449 was examined over a wider CE range (see supporting information Figure S23b) and a clear trend, especially up to a CE of 25eV, can be seen that the ratios differs between the two isomers.

Selectivity in DAD detection.

Differentiation between anthocyanins and other flavonoids[56, 57] could, as has been pointed out in the introduction, be done with DAD detection as anthocyanins have strong (pH dependent[58]) absorption in the visible range. However, the identification could be a great challenge, especially if there are co-eluting compounds in an LC separation.[59] Two flavonoids analyzed in this study with similar structure are for example, del-3-glu and que-3-glu. Del-3-glu has absorption maxima in both the UV range, 277 nm, and the visible range, 524 nm, whereas que-3-glu has maxima only in the UV range, 256 and 354 nm. Figure S24 (in supporting information) shows an example of DAD spectra at a retention time of 21 min (peak **8**, Figure 4) obtained from an LC separation of a red onion extract. Inspection of the front (**8**) and tail (**9**) indicate that the LC peak might contain at least two compounds. DAD spectra from cy-3-gal and que-3-glu standards are also included in Figure S24. This pair is not unique as overlap between anthocyanins and other flavonoids is not uncommon, thus limiting the information the DAD can give to identify one (or more) of some overlapping peaks as anthocyanins. Furthermore, even compounds matching the anthocyanin standards do not always show absorbance in the visible range due to too low concentration, for example cy-3,5-diglu (peak **6**) in the red onion extract shows no DAD signals at all.

Conclusions

To summarize the performance of the methods 1 and 2 it could be concluded that the ion transmission in method 1, in upfront fragmentation mode, is higher than in method 2. This can be seen by the higher ratio of the fragments to the intact compound for method 1 compared to method 2. However, the two methods give higher signal to noise ratio (S/N) in the TIC for different peaks in the red onion extract, for example peak 10 method 2 gives a slightly higher S/N (460 for method 1 and 570 for method 2) whereas for peak 18, method 1 has the higher S/N (1800 for method 1 and 740 for method 2). When looking at only the most intense signals in the peaks (extracted ion chromatogram, XIC), method 1 gives the higher S/N for both peak 10 (5000 for method 1 and 370 for method 2) and peak 18 (1200 for method 1 and 650 for method 2). One advantage with method 1 is that the substitution pattern will not determine if a flavonoid will be detected. This is not the case for method 2, as only flavonoids whose transitions are listed in the MRM experiment could be detected. Finally, the strategy employed in method 1 has another advantage as it provides improved identification possibilities as the chromatographic MRM profiles are monitored. While not all flavonoids could be differentiated using method 1 and 2, the methods can differentiate between most isobaric aglycones. A majority of the compounds in each plant extract could be partly identified; that is the aglycone could be determined with methods 1 and 2, and the general structure of the substituent(s) could be identified although the position(s) could not be determined with the information obtained in method 2.

Method 3 has the advantage over MRM acquisition that prior knowledge of generated fragment ions is not required which allows more possibilities when the data is evaluated. Furthermore, by plotting and evaluating the intensity ratio of suitable ions over a broader collision energy range the identification will become more reliable. As signal intensity can vary

greatly depending on the collision energy, the use of a logarithmic scale for signal intensity can be used to easier visualize data.

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Tables

Table 1. The MRM transitions used in method 1 and the proposed corresponding loss.

Elemental Comp. + ion	Exact mass	MRM Trans. nr:	Q1 mass	Q3 mass	Compound*	Loss or fragment
$C_{15}H_{11}O_5$	271.2449	1	271.1	119.1	Apigenin	$^{1,3}B^+$
		2	271.1	121.1		$^{0,2}B^+$
		3	271.1	141.1		$-H_2O-4CO$
		4	271.1	145.1		$^{0,4}B^+ -H_2O$
		5	271.1	153.1		$^{1,3}A^+$
		6	271.1	169.1		$-H_2O-3CO$
		7	271.1	197.1		$-H_2O-2CO$
		8	271.1	243.1		$-CO$
$C_{15}H_{11}O_5$	271.2449	9	271.1	121.1	Pelargonidin	$^{0,2}B^+$
		10	271.1	141.1		$-H_2O-4CO$
		11	271.1	145.1		$^{0,4}B^+ -H_2O$
		12	271.1	169.1		$-H_2O-3CO$
		13	271.1	197.1		$-H_2O-2CO$
		14	271.1	215.1		$-2CO$
$C_{15}H_{11}O_5$	271.2449	15	271.1	141.1	Genistein	$-H_2O-4CO$
		16	271.1	153.1		$^{1,3}A^+$
		17	271.1	169.1		$-H_2O-3CO$
		18	271.1	197.1		$-H_2O-2CO$
		19	271.1	215.1		$-2CO$
$C_{15}H_{11}O_6$	287.0550	20	287.1	115.1	Cyanidin	$-4CO-C_2H_2O-H_2O$
		21	287.1	121.1		$^{0,3}A^+$
		22	287.1	137.1		$^{0,2}B^+$
		23	287.1	157.1		$-H_2O-4CO$
		24	287.1	165.1		$^{0,3}B^+$
		25	287.1	185.1		$-H_2O-3CO$
		26	287.1	213.1		$-H_2O-2CO$
		27	287.1	241.1		$-H_2O-CO$
$C_{15}H_{11}O_6$	287.0550	28	287.1	121.1	Kaempferol	$^{0,2}B^+$
		29	287.1	137.1		$^{0,3}A^+$
		30	287.1	153.1		$^{1,3}A^+$
		31	287.1	157.1		$-H_2O-4CO$
		32	287.1	165.1		$^{0,2}A^+$
		33	287.1	185.1		$-H_2O-3CO$
		34	287.1	241.1		$-H_2O-CO$
$C_{15}H_{11}O_6$	287.0550	35	287.1	115.1	Luteolin	$-4CO-C_2H_2O-H_2O$
		36	287.1	135.1		$^{1,3}B^+$
		37	287.1	137.1		$^{0,2}B^+$
		38	287.1	153.1		$^{1,3}A^+$
		39	287.1	165.1		$^{0,2}A^+$
		40	287.1	213.1		$-H_2O-2CO$
		41	287.1	241.1		$-H_2O-CO$
$C_{15}H_{15}O_6$	291.0863	42	291.1	123.1	(+)Catechin	$^{0,3}A^+$
		43	291.1	139.1		$^{1,3}A^+$
$C_{16}H_{13}O_6$	301.0707	44	301.1	153.1	Diosmetin	$^{1,3}A^+$
		45	301.1	229.1		$-CH_3-COH-CO$
		46	301.1	257.1		$-CH_3-COH$
		47	301.1	287.1		$-CH_2$
$C_{15}H_{11}O_7$	303.0499	48	303.1	150.1	Delphinidin	$^{0,2}A^+$
		49	303.1	153.1		$^{0,2}B^+$

		50	303.1	165.1		-3H_2O-3CO
		51	303.1	173.1		-H_2O-4CO
		52	303.1	201.1		-H_2O-3CO
		53	303.1	229.1		-H_2O-2CO
$C_{15}H_{11}O_7$	303.0499	54	303.1	137.1	Morin	$^{0,2}B^+$
		55	303.1	149.1		$^{1,3}B^+$
		56	303.1	153.1		$^{1,3}A^+$
		57	303.1	177.1		$^{0,4}B^+$ -H2O
$C_{15}H_{11}O_7$	303.0499	58	303.1	117.1	Quercetin	$^{1,3}A^+$ - 2H_2O
		59	303.1	150.1		$^{1,3}B^+$
		60	303.1	153.1		$^{1,3}A^+$
		61	303.1	165.1		$^{0,2}A^+$
		62	303.1	173.1		-H_2O-2CO
		63	303.1	177.1		$^{0,4}B^+$ -H2O
		64	303.1	201.1		-H_2O-3CO
		65	303.1	229.1		-H_2O-2CO
$C_{16}H_{15}O_6$	303.0863	66	303.1	117.1	Hesperetin	$^{1,3}A^+$ - 2H_2O
		67	303.1	153.1		$^{1,3}A^+$
		68	303.1	177.1		$^{0,4}B^+$ -H2O
$C_{16}H_{13}O_7$	317.2697	69	317.1	153.1	Isorhamnetin	$^{1,3}A^+$
		70	317.1	229.1		-CH_3-OH-2CO
		71	317.1	285.1		-CH_3-H_2O
$C_{16}H_{13}O_7$	317.2697	72	317.1	153.1	Petunidin	$^{0,2}B^+$-CH_2
		73	317.1	229.1		-CH_3O-COH-CO
		74	317.1	285.1		-CH_3-H_2O
$C_{17}H_{15}O_7$	331.0812	75	331.1	201.1	Malvidin	-H_2O-4CO
		76	331.1	213.1		-CH_3-COH-H_2O-2CO
		77	331.1	241.1		-CH_3-COH-H_2O-CO
		78	331.1	287.1		-CH_3-COH

The following MRM transitions have the same *m/z* values: 2,9; 3,10,15; 4,11; 5,16; 6,12,17; 7,13,18; 14,19; 20,34; 21,28; 22,29,36; 23,31; 24,32,37; 25,33; 26,38; 27,39; 48,55; 47,58; 48,55,59,66; 49,60; 50,61; 51,63; 52,64; 56,62,67; 69,72; 70,73; 71,74

Table 2. The MRM transitions used in method 2 with proposed substitution and aglygone identity.

Q1 mass	Q3 mass	Neutral loss	Substituent(s)	Aglycone*
433.1	271.1	162	Hexose	Pelargonidin
533.1	271.1	262	Hexose+Succinic acid	Pelargonidin
549.1	271.1	278	Hexose+Malonic acid	Pelargonidin
579.1	271.1	308	Hexose+Deoxy hexose	Pelargonidin
639.1	271.1	368	Hexose+Sinapic acid	Pelargonidin
697.1	271.1	426	Aldopentose+Aldopentose+Hexose	Pelargonidin
449.1	287.1	162	Hexose	Cyanidin
491.1	287.1	204	Hexose+Acetic acid	Cyanidin
535.1	287.1	248	Hexose+Malonic acid	Cyanidin
595.1	287.1	308	Hexose+Deoxy hexose	Cyanidin
611.1	287.1	324	Hexose+Hexose	Cyanidin
625.1	287.1	338	Hexose+Ferulic acid	Cyanidin
697.1	287.1	410	Hexose+Hexose+Malonic acid	Cyanidin
463.1	301.1	162	Hexose	Peonidin
549.1	301.1	248	Hexose+Malonic acid	Peonidin
465.1	303.1	162	Hexose	Delphinidin
477.1	303.1	174	Deoxy hexose+Formic acid	Delphinidin
551.1	303.1	248	Hexose+Malonic acid	Delphinidin
611.1	303.1	308	Hexose+Deoxy hexose	Delphinidin
627.1	303.1	324	Hexose+Hexose	Delphinidin
655.1	303.1	352	Hexose+Hexose+Formic acid	Delphinidin
479.1	317.1	162	Hexose	Petunidin
579.1	317.1	262	Hexose+Succinic acid	Petunidin
493.1	331.1	162	Hexose	Malvidin

*Examples given are anthocyanins, the transitions would work for any isobaric flavonoid

Table 3. The retention times (t_r) and signals of the standards. Maximum of five signals are listed.

Comp. nr:	Compound identity	*m/z* intact ion $[M]^{+\cdot}$ or $[M+H]^+$	Retention time (min)	DAD maxima (nm)	Method 1, MRM signals	Method 1, EPI fragments (Parent ion in bold)	Method 2, MRM signals	Method 2, EPI fragments (Parent ion in bold)
1	Api-7-glu	433.1	39.2	338 (100) 266 (87)	271.1/153.1 (100) 271.1/119.1 (43) 271.1/145.1 (30) 271.1/121.1 (23)	**271** 153 (100) 145 (65) 119 (35) 121 (24) 115 (20)	433.1/271.1 (100)	**433**→271 (100) 153 (18) 145 (3) 229 (3) 225 (3)
2	Api-7-neo	579.2	42.5	340 (100) 266 (92)	271.1/153.1 (100) 271.1/119.1 (44) 271.1/145.1 (36) 271.1/121.1 (28)	**271** 153 (100) 121 (33) 119 (29) 169 (29) 145 (24)	579.1/271.1 (100) 433.1/271.1 (2)	**579**→271 (100) 153 (15) 229 (7) 243 (5) 213 (4)
3	Apigenin	271.1	47.9	n.d.	271.1/153.1 (100) 271.1/215.1 (30) 271.1/197.1 (27) 271.1/121.1 (19) 271.1/145.1 (16)	**271** 153 (100) 115 (93) 141 (62) 168 (29) 127 (29)	n.d.	n.d.
4	Pel-3-glu	433.1	24.7	500 (100) 278 (64) 428 (45) 330 (14)	271.1/121.1 (100) 271.1/141.1 (23) 271.1/169.1 (12) 271.1/197.1 (11) 271.1/145.1 (10)	**271** 121 (100) 115 (76) 141 (51) 127 (39) 197 (24)	433.1/271.1 (100)	**433**→271 (100) 253 (9) 197 (9) 145 (9)
5	Genistein	271.1	53.8	260 (100)	271.1/153.1 (100) 271.1/141.1 (41) 271.1/169.1 (31) 271.1/197.1 (29) 271.1/215.1 (28)	**271** 115 (100) 153 (83) 141 (65) 127 (35) 169 (33)	n.d.	n.d.

6	Cy-3,5-diglu	611.2	16.5	514 (100) 278 (85)	287.1/137.1 (100) 287.1/213.1 (53) 287.1/157.1 (33) 287.1/121.1 (31) 287.1/115.1 (30)	**287** 111 (100) 121 (100) 129 (78) 143 (78) 196 (59)	611.1/287.1 (100) 449.1/287.1 (18)	**611**→287 (100) 259 (5) 231 (5) 213 (5) 189 (5)
7	Cy-3-gal	449.1	19.1	516 (100) 280 (66)	287.1/137.1 (100) 287.1/213.1 (49) 287.1/121.1 (31) 287.1/115.1 (28) 287.1/157.1 (27)	**287** 115 (100) 128 (100) 137 (96) 139 (92) 121 (60)	449.1/287.1 (100)	**449**→287 (100) 231 (6) 213 (5) 137 (4) 175 (3)
8	Cy-3-glu	449.1	21.0	516 (100) 280 (67)	287.1/137.1 (100) 287.1/213.1 (55) 287.1/121.1 (31) 287.1/115.1 (30) 287.1/157.1 (26)	**287** 115 (100) 128 (85) 137 (85) 139 (63) 157 (63)	449.1/287.1 (100)	**449**→287 (100) 213 (8) 171 (3) 149 (3)
9	Cy-3-rut	595.2	23.7	520 (100) 280 (67)	287.1/137.1 (100) 287.1/213.1 (55) 287.1/121.1 (44) 287.1/115.1 (33) 287.1/157.1 (32)	**287** 115 (100) 157 (67) 128 (57) 139 (57) 213 (57)	595.1/287.1 (100) 449.1/287.1 (6)	**595**→287 (100) 213 (8) 231 (6) 241 (4) 137 (4)
10	Cyanidin	287.1	48.9	518 (100) 280 (67)	287.1/137.1 (100) 287.1/213.1 (54) 287.1/121.1 (43) 287.1/157.1 (31) 287.1/115.1 (30)	**287** 128 (100) 139 (76) 115 (71) 137 (71) 121 (67)	n.d.	n.d.
11	Kaempf-3-glu	449.1	40.8	266 (100) 348 (78) 298 (59)	287.1/153.1 (100) 287.1/121.1 (51) 287.1/213.1 (26) 287.1/157.1 (21) 287.1/165.1 (17)	**287** 153 (100) 121 (74) 128 (47) 127 (36) 157 (31)	449.1/287.1 (100)	**449**→287 (100) 213 (14) 241 (13) 165 (8) 231 (4)
12	Kaempf-3-rut	595.2	42.0	266 (100) 348 (80) 298 (60)	287.1/153.1 (100) 287.1/121.1 (60) 287.1/157.1 (20)	**287** 153 (100) 121 (55)	595.1/287.1 (100) 449.1/287.1 (91)	**595**→287 (100) 241 (27) 165 (18)

					287.1/213.1 (20) 287.1/137.1 (16)	128 (38) 157 (35) 139 (26)		121 (11) 213 (11)
								449→287 (100) 213 (23) 241 (23) 145 (15) 157 (12)
13	Kaempferol	287.1	43.9	n.d.	287.1/153.1 (100) 287.1/121.1 (59) 287.1/157.1 (22) 287.1/213.1 (21) 287.1/165.1 (20)	**287** 153 (100) 128 (42) 139 (42) 111 (28) 141 (28)	n.d.	n.d.
14	Lut-7-glu	449.1	32.7	350 (100) 254 (86)	287.1/153.1 (100) 287.1/135.1 (30) 287.1/137.1 (15) 287.1/241.1 (12)	**287** 153 (100) 161 (50) 139 (38) 115 (31) 185 (31)	449.1/287.1 (100)	**449**→287 (100) 153 (8) 245 (3) 241 (2) 219 (2)
15	Lut-8-C-glu	449.1	25.3	350 (100) 256 (86)	n.d.	n.d.	449.1/287.1 (100)	n.d.
16	Luteolin	287.1	53.8	350 (100) 254 (81)	287.1/153.1 (100) 287.1/135.1 (32) 287.1/137.1 (15) 287.1/241.1 (12)	**287** 153 (100) 139 (45) 161 (44) 135 (29) 287 (27)	n.d.	n.d.
17	(+)Catehcin	291.1	4.8	n.d.	291.1/123.1 (100) 291.1/139.1 (41)	**291** 123 (100) 119 (29) 115 (23) 147 (19) 139 (17)	n.d.	n.d.
18	Diosmetin	301.1	*	n.d.	301.1/229.1 (100) 301.1/153.1 (77) 301.1/257.1 (48) 301.1/287.1 (11)	**301** 258 (100) 229 (68) 153 (50) 203 (18)	n.d.	n.d.

						286 (11)		
19	Del-3-glu	465.1	17.6	524 (100) 278 (58)	303.1/229.1 (100) 303.1/173.1 (52) 303.1/153.1 (48) 303.1/201.1 (23) 303.1/165.1 (10)	**303** 229 (100) 127 (84) 115 (68) 121 (42) 131 (42)	465.1/303.1 (100)	**465**→303 (100) 257 (20) 229 (20)
20	Morin	303.1	48.4	n.d.	303.1/153.1 (100) 303.1/149.1 (14)	**303** 153 (100) 134 (24) 149 (16) 117 (13)	n.d.	n.d.
21	Que-3,4'-diglu	627.1	25.0	264 (100) 344 (75)	303.1/153.1 (100) 303.1/229.1 (51) 303.1/201.1 (28) 303.1/173.1 (22) 303.1/165.1 (15)	**303** 153 (100) 155 (37) 127 (33) 173 (30) 115 (30)	465.1/303.1 (100) 627.1/303.1 (30)	**465**→303 (100) 257 (11) 229 (10) 285 (8) 165 (5)
22	Que-3-glu	465.1	33.4	256 (100), 354 (81)	303.1/153.1 (100) 303.1/229.1 (50) 303.1/201.1 (31) 303.1/173.1 (21) 303.1/165.1 (16)	**303** 153 (100) 127 (85) 155 (52) 121 (39) 137 (30)	465.1/303.1 (100)	**465**→303 (100) 229 (59)
23	Que-4'-glu	465.1	39.3	252 (100), 364 (95)	303.1/153.1 (100) 303.1/229.1 (64) 303.1/201.1 (37) 303.1/173.1 (18) 303.1/165.1 (15)	**303** 153 (100) 155 (75) 127 (75) 229 (34) 201(30)	465.1/303.1 (100)	**465**→303 (100) 257 (52) 285 (29) 229 (27) 165 (19)
24	Hesp-7-rut	611.1	39.0	284 (100)	303.1/153.1 (100) 303.1/117.1 (18)	**303** 153 (100) 117 (28) 134 (22) 128 (17)	611.1/303.1 (100) 465.1/303.1 (51)	**611**→177 (100) 179 (100) 303 (70)
25	Hesperetin	303.1	55.5	288 (100)	303.1/153.1 (100) 303.1/117.1 (31)	**303** 153 (100) 134 (27) 117 (21) 149 (15)	n.d.	n.d.

						137 (10)		
26	Isorhamnetin	317.1	*		317.1/153.1 (100) 317.1/229.1 (36) 317.1/285.1 (16)	**317** 153 (100) 217 (65) 245 (49) 203 (35) 229 (35)	n.d.	n.d.
27	Mal-3-glu	493.1	31.1	528 (100) 278 (61)	331.1/287.1 (100) 331.1/213.1 (36) 331.1/241.1 (36) 331.1/201.1 (29)	**331** 315 (100) 287 (89) 241 (53) 242 (53) 229 (44)	493.1/331.1 (100)	**493**→331 (100) 299 (68) 287 (51) 315 (38) 270 (12)

*Not eluted during the analysis time

Table 4. The retention times, signals and tentative identification of the peaks in the red onion extract. MS data are listed as percentages of the base peak.

Comp. Nr:	Retention Time (min)	Method 1, MRM signals	Method 1, EPI fragments Parent ion in bold	Method 2, MRM signals	Method 2, MS^3 fragments Parent ion in bold	Identity
1	5.0	301.1/153.1 (100)	**301** 153 (100) 107 (49)	n.d.	n.d.	?
2	12.5	271.1/169.1 (100) 271.1/141.1 (90) 271.1/197.1 (50) 271.1/145.1 (37)	n.d.	n.d.	n.d.	Pel+?
3	14.3	287.1/137.1 (100)	n.d.	535.1/287.1 (100) 449.1/287.1 (17) 697.1/287.1 (10)	**535**→287 (100)	Cy+Hex+(Hex+Malonic acid) **Cy-3(malonoyl-glucoside)-5-glu**
4	14.6	303.1/153.1 (100) 303.1/229.1 (37) 303.1/173.1 (23) 303.1/201.1 (16) 303.1/165.1 (13)	**303** 153 (100) 135 (75) 137 (75)	627.1/303.1 (100) 465.1/303.1 (24)	**627**→303 (100) 229 (80) 257 (80) 165 (65) **465**→303 (100) 257 (85) 229 (85) 165 (67)	Que+Hex+Hex **Que-3,7,4'-triglu**
5	15.4	287.1/137.1 (100)	n.d.	611.1/287.1 (100) 449.1/287.1 (33)	**449**→287 (100) 213 (22)	Cy+Hex+Hex
6	16.6	287.1/137.1 (100)	n.d.	611.1/287.1 (100) 449.1/287.1 (33)	**611**→287 (100)	Cy-3,5-diglu
7	17.0	303.1/153.1 (100) 303.1/229.1 (33) 303.1/201.1 (33) 303.1/117.1 (20) 303.1/165.1 (18)	**303** 115 (100) 129 (100)	627.1/303.1 (100) 465.1/303.1 (81)	**627**→303 (100) 229 (19) 247 (19) 257 (19) 285 (19) **465**→303 (100) 229 (20) 165 (20) 257 (15) 285 (15)	Que+Hex+Hex **Que-7,4'-diglu (possibly Que-3,7-diglu)**

8	21.2	287.1/137.1 (100) 287.1/213.1 (47) 287.1/121.1 (31) 287.1/115.1 (30) 287.1/157.1 (24)	**287** 137 (100) 139 (97) 115 (90) 128 (90) 121 (66)	449.1/287.1 (100)	**449→**287 (100) 213 (5) 241 (4) 259 (4) 185 (3)	Cy-3-glu
9	21.5	303.1/153.1 (100) 303.1/229.1 (51) 303.1/201.1 (26) 303.1/173.1 (23) 303.1/165.1 (22)	n.d.	627.1/303.1 (100) 465.1/303.1 (22)	**627→**303 (100) 257 (12) 229 (9) 165 (7) 285 (5)	Que+Hex+Hex **Que-7,4'-diglu (possibly Que-3,7-diglu)**
10	24.3	287.1/137.1 (100) 287.1/213.1 (50) 287.1/121.1 (34) 287.1/115.1 (32) 287.1/157.1 (32)	**287** 128 (100) 121 (100) 139 (75) 137 (75) 157 (75)	611.1/287.1 (100)	**611→**287 (100) 287 (100) 231 (8) 213 (6) 141 (5)	Cy+(Hex+Hex) **Cy-3-laminarbioside**
11	24.8	271.1/121.1 (100)	n.d.	433.1/271.1 (360)	n.d.	Pel-3-glu
12	25.2	303.1/153.1 (100) 303.1/229.1 (60) 303.1/201.1 (33) 303.1/173.1 (18) 303.1/165.1 (14)	**303** 153 (100) 155 (61) 137 (51) 127 (49) 229 (39)	465.1/303.1 (100) 627.1/303.1 (42)	**465→**303 (100) 257 (74) 229 (60) 165 (47) 285 (29)	Que-3,4'-diglu
13	25.2	317.1/153.1 (100)	n.d.	n.d.	n.d.	?
14	25.9	287.1/137.1 (100)	n.d.	697.1/287.1 (100)	n.d.	Cy+(Hex+Hex+Malonic acid) **Cy-3(6''-malonoyl-laminarbioside)**
15	26.9	287.1/137.1 (100) 287.1/213.1 (42) 287.1/121.1 (27) 287.1/115.1 (23) 287.1/157.1 (20)	**287** 139 (100) 157 (100) 171 (100) 128 (78) 213 (78)	535.1/287.1 (100) 449.1/287.1 (1)	**535→**287 (100) 259 (3) 175 (3) 165 (2) 213 (2)	Cy+(Hex+Malonic acid) **possibly Cy-3(3''-malonoylglucoside)**
16	28.0	301.1/229.1 (100) 301.1/257.1 (33)	**301** 201 (100)	463.1/301.1 (100)	**463→**301 (64) 287 (100)	?+Hex

		301.1/287.1 (6)	229 (78) 187 (59) 155 (37) 150 (29)			
17	28.6	287.1/137.1(100) 287.1/213.1(63) 287.1/121.1(41)	n.d.	535.1/287.1 (100) 449.1/287.1 (3)	**535→**287 (100) 216 (13)	Cy+(Hex+Malonic acid) **possibly Cy-3(3''-malonoylglucoside)**
18	29.1	317.1/153.1 (100) 317.1/229.1 (35) 317.1/285.1 (16)	**317** 153 (100) 217 (93) 245 (80) 203 (67) 121 (31)	479.1/317.1 (100)	**479→**317 (100)	?+Hex **possibly Iso-3,4'-diglu**
19	29.4	303.1/153.1 (100) 303.1/229.1 (55) 303.1/201.1 (32) 303.1/173.1 (23) 303.1/165.1 (15)	**303** 153 (100) 155 (49) 137 (42) 127 (29) 145 (26)	655.1/303.1 (100) 465.1/303.1 (81)	**655→** 303 (100) 257 (16) 229 (14) 165 (10) 285 (5) **465→**303 (100) 257 (11) 165 (9) 229 (6) 153 (4)	Que+Hex+(Hex+Formic acid)
20	29.6	287.1/137.1 (100) 287.1/213.1 (53)	n.d.	491.1/287.1 (100) 449.1/287.1 (10)	**491→**287 (100) 199 (12)	Cy+(Hex+Acetic acid) or Cy+(Aldopentose+Oxalic acid)
21	31.0	287.1/137.1 (100) 287/213.1 (50)	n.d.	449.1/287.1 (100) 491.1/287.1 (57)	**491→**287 (100)	Cy+Hex+Acetic acid or Cy+Aldopentose+Oxalic acid
22	31.4	301.1/153.1 (100) 301.1/229.1 (45)	n.d.	n.d.	n.d.	?
23	32.6	n.d.	n.d.	465.1/303.1 (100) 655.1/303.1 (63)	**655→**303 (100) 257 (40) 285 (20) 247 (20)	Que+Hex+190
24	33.0	287.1/121.1 (100) 287.1/137.1 (19)	**287** 121 (100)	n.d.	n.d.	Cy+?
25	33.8	317.1/153.1 (100)	n.d.	n.d.	n.d.	?
26	33.9	303.1/153.1 (100)	**303**	465.1/303.1 (100)	**465→**303 (100)	Que-3-glu

		303.1/229.1 (50) 303.1/201.1 (30) 303.1/173.1 (20) 303.1/165.1 (16)	153 (100) 155 (63) 137 (48) 127 (48) 229 (38)		257 (8) 165 (6) 285 (6)	
27	34.6	287.1/137.1 (100) 287.1/213.1 (53) 87.1/121.1 (31) 287.1/115.1 (29) 287.1/157.1 (27)	**287 (29)** 137 (100) 115 (90) 128 (75) 139 (70)	535.1/287.1 (100) 449.1/287.1 (1)	**535→**287 (100) 213 (17) 231 (9) 241 (7)	Cy+(Hex+Malonic acid) **Cy-3(6''-malonylglucoside)**
28	35.6	303.1/153.1 (100) 303.1/229.1 (53) 303.1/201.1 (31) 303.1/173.1 (21) 303.1/117.1 (11)	**303** 153 (100) 127 (83) 155 (55) 145 (38) 137 (33)	655.1/303.1 (100) 465.1/303.1 (11)	**665→**303 (100) 165 (13) 257 (11)	Que+Hex+(Hex+Formic acid)
29	37.5	287.1/137.1 (100) 287.1/213.1 (47) 287.1/121.1 (42) 287.1/157.1 (34) 287.1/115.1 (26)	**303** 128 (100) 139 (83) 137 (64) 109 (64) 121 (56)	697.1/287.1 (100)	**697→**287 (100) 213 (5) 175 (2) 231 (2) 185 (2)	Cy+(Hex+Hex+Malonic acid) **Cy-3(6''-malonoyl-lamin arbioside)**
30	38.5	317.1/153.10 (100)	**317** 145 (100) 181 (59)	n.d.	n.d.	?
31	38.6	303.1/153.1 (100) 303.1/229.1 (55) 303.1/201.1 (20) 303.1/173.1 (15) 303.1/165.1 (13)	**303** 153 (100) 127 (53) 155 (53) 137 (46) 229 (44)	465.1/303.1 (100)	**465→**303 (100) 257 (69) 229 (67) 165 (52) 285 (31)	Que-4'-glu
32	39.5	287.1/153.1 (100) 287.1/121.1 (90)	n.d.	449.1/287.1 (100)	**449→**287 (100) 258 (30) 243 (20) 213 (20)	Kaempf-3-glu
33	41.5	301.1/229.1 (100) 301.1/257.1 (40)	n.d.	n.d.	n.d.	?
34	42.8	287.1/137.1 (100) 287.1/213.1 (43) 287.1/121.1 (28)	**287** 109 (100) 127 (78)	491.1/287.1 (100)	**491→**287 (100) 231 (8) 259 (6)	Cy+(Hex+Acetic acid) or Cy+(Aldopentose+Oxalic acid)

		287.1/115.1 (27)	128 (78)			
		287.1/157.1 (22)	141 (78)			
			139 (59)			
35	43.7	287.1/137.1 (100)	n.d.	449.1/287.1 (100)	n.d.	Cy+Hex
		287.1/213.1 (48)				
36	44.0	317.1/153.1 (100)	n.d.	n.d.	n.d.	?
		317.1/229.1 (35)				
		317.1/285.1 (14)				
37	45.6	317.1/153.1 (100)	**317**	479.1/317.1 (100)	**479→**317 (100)	?+Hex
		317.1/229.1 (34)	153 (100)		285 (43)	**Iso-4'-glu**
		317.1/285.1 (14)	217 (75)		302 (34)	
			245 (47)		139 (19)	
			203 (47)		261 (14)	
			229 (27)			
38	45.6	301.1/153.1 (100)	n.d.	n.d.	n.d.	?
		301.1/229.1 (35)				
39	47.8	271.1/153.1 (100)	n.d.	n.d.	n.d.	Apigenin
40	49.2	287.1/137.1 (100)	n.d.	595.1/287.1 (100)	n.d.	Cy+(Hex+Deoxy hexose)
		287.1/213.1 (64)				
41	50.2	271.1/169.1 (100)	n.d.	n.d.	n.d.	?
42	50.4	317.1/153.1 (100)	**317**	n.d.	n.d.	?
			127 (100)			
			153 (100)			
			245 (100)			
43	50.6	303.1/153.1 (100)	**303**	465.1/303.1 (100)	**465→**303 (100)	Que+Hex+Malonic acid
		303.1/229.1 (63)	153 (100)	551.1/303.1 (22)	257 (22)	
		303.1/201.1 (34)	155 (74)		165 (22)	
		303.1/165.1 (18)	127 (63)			
			137 (50)			
			229 (47)			
44	53.4	271.1/119.1 (100)	n.d.	579.1/271.1 (100)	n.d.	?+(Hex+Deoxy hexose)
45	55.4	317.1/153.1 (100)	n.d.	n.d.	n.d.	?
		317.1/229.1 (30)				

n.d. not detected. A question mark (?) means no reliable identification can be made. A or B means identity not ascertained, peak is either A or B. A parenthesis around two or more substituents indicate that those substituents are on the same position, for example Cy+(Hex+Hex) would mean cyanidin monosubstituted with a dihexose whereas Cy+Hex+Hex would mean cyanidin disubstituted with monohexoses. Boldface denotes compounds tentatively identified with the help of previously published data.[35-37]

Figures

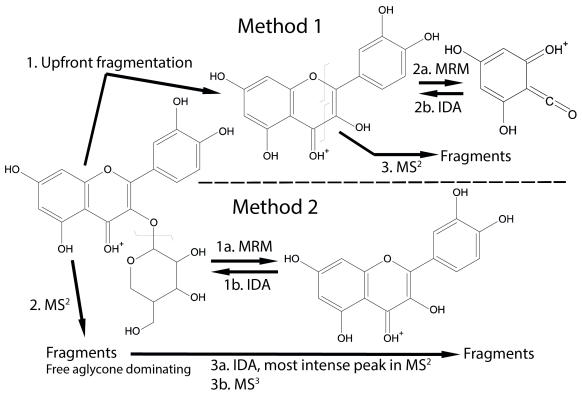

Figure 1. A schematic illustration of the MS experiments, showing what information is obtained from each experiment. While method 1 fragments the aglycone in the first experiment, method 2 gives information about substitution(s) in the first experiment.

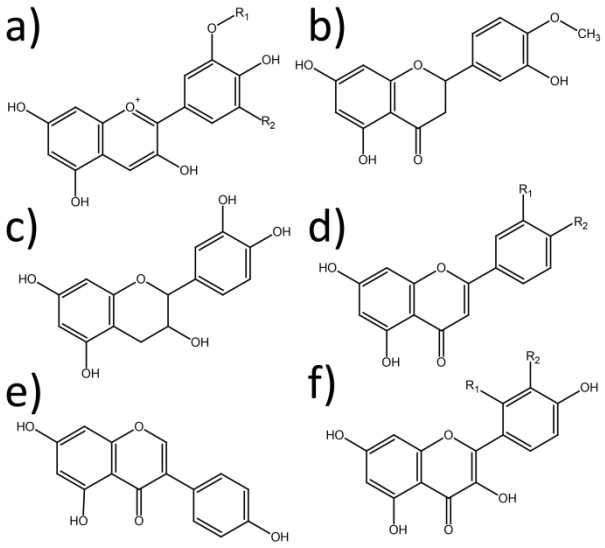

Figure 2. Structures of the flavonoid standards; a) anthocyanidins: cyaniding (R_1 = OH, R_2 = H), delphinidin (R_1 = R_2 = OH), malvidin (R_1 = R_2 = OCH_3) and pelargonidin (R_1 = R_2 = H), b) hesperetin (the only flavanone standard), c) (+)catechin (the only flavan-3-ol standard), d) flavonones: apigenin (R_1 = H, R_2 = OH), diosmetin (R_1 = OH, R_2 = OCH_3) and luteolin (R_1 = R_2 = OH), e) genistein (the only isoflavone standard), flavonols: isorhamnetin (R_1 = H, R_2 = OCH_3), kaempferol (R_1 = R_2 = H), morin (R_1 = OH, R_2 = H) and quercetin (R_1 = H, R_2 = OH).

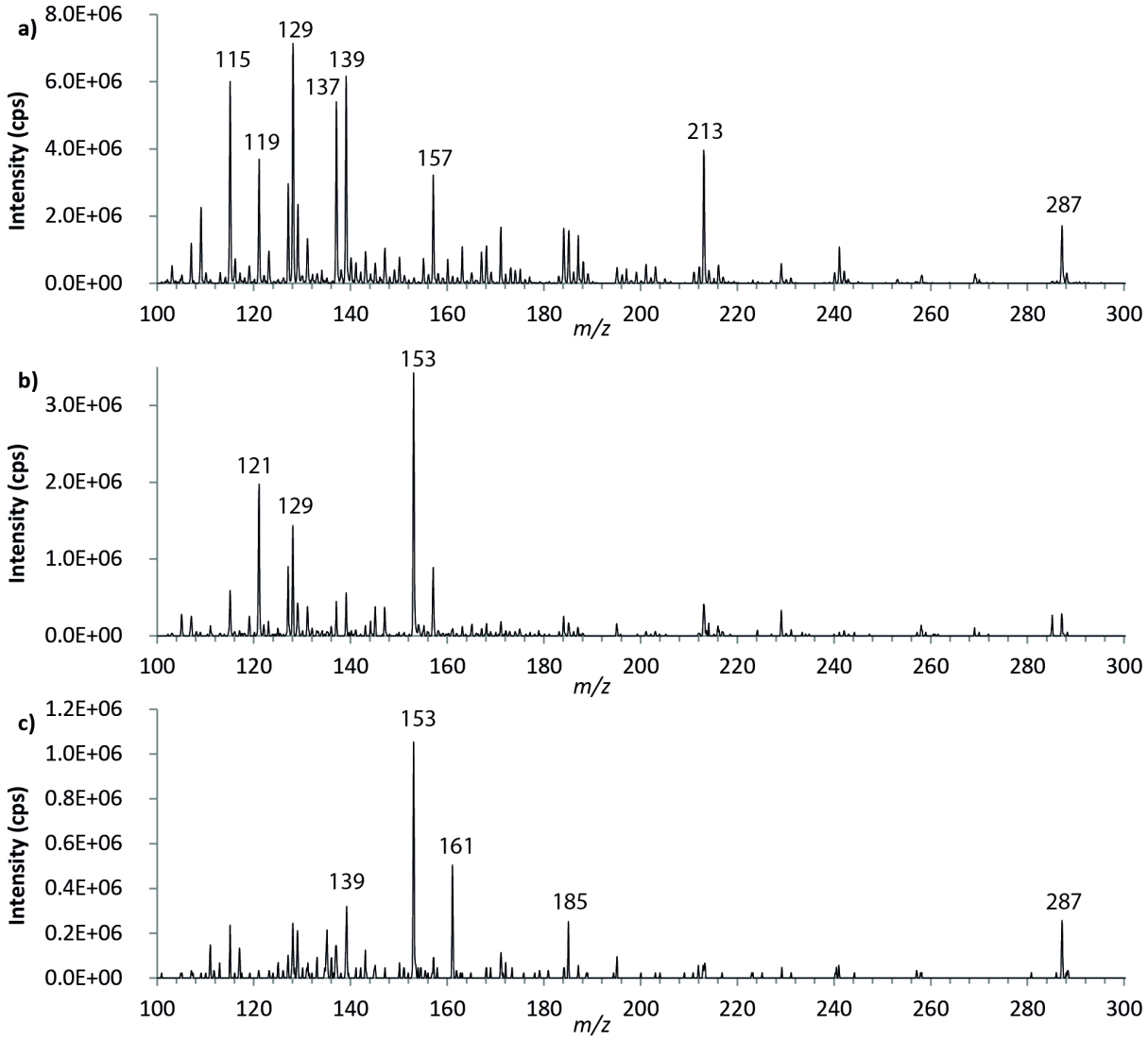

Figure 3. MS^2 spectra of a) cy-3-glu, b) kaempf-3-glu and c) lut-7-glu from method 1. In the upfront step the free aglycone (*m/z* 287) is formed which is selected for MS^2. For cy-3-glu the signal at *m/z* 129 (loss of 5 CO, H_2O) is the most intense whereas for kaempf-3-glu and lut-7-glu the most intense peak is *m/*z 153 ($^{1,3}A^+$).

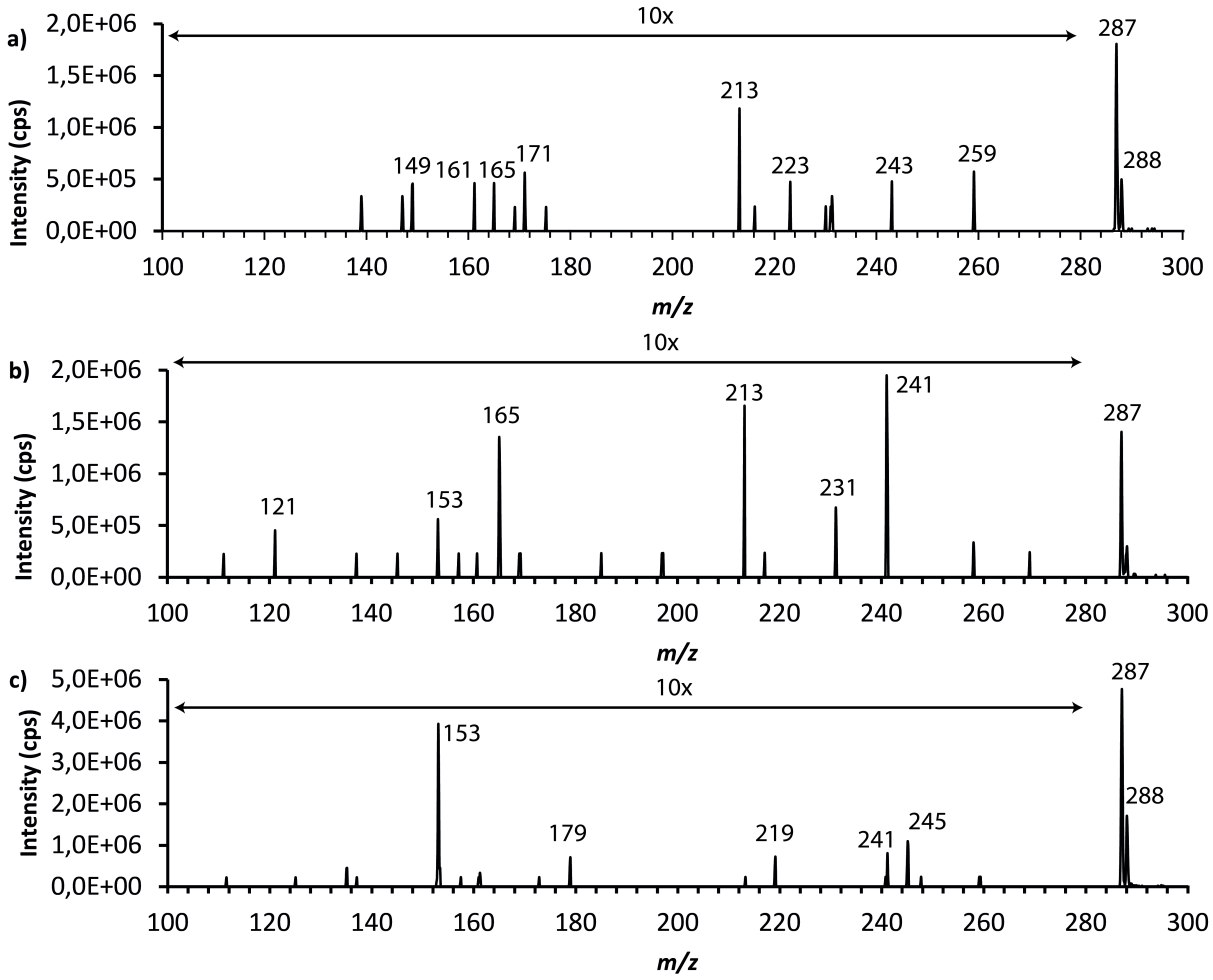

Figure 4. MS^3 spectra of a) cy-3-glu, b) kaempf-3-glu and c) lut-7-glu from method 2. The intact molecule is selected by Q1 and fragmeted in the collision cell to generate the free aglycone which is trapped in Q3 and further excited and fragmented in Q3. The most intense fragment of cy-3-glu and the second most intense for kaempf-3-glu is *m/z* 213 (loss of H_2O and 2CO), the most intense fragment for kaempf-3-glu is *m/z* 241 (loss of H_2O and CO). For lut-7-glu the most intense fragment is *m/z* 153 ($^{1,3}A^+$). The range *m/z* 100-280 has been amplified 10 times in each spectrum (indicated by arrows).

Figure 5. TICs of red onion extract from a) method 1 and b) method 2. For peak identity, see Table 4.

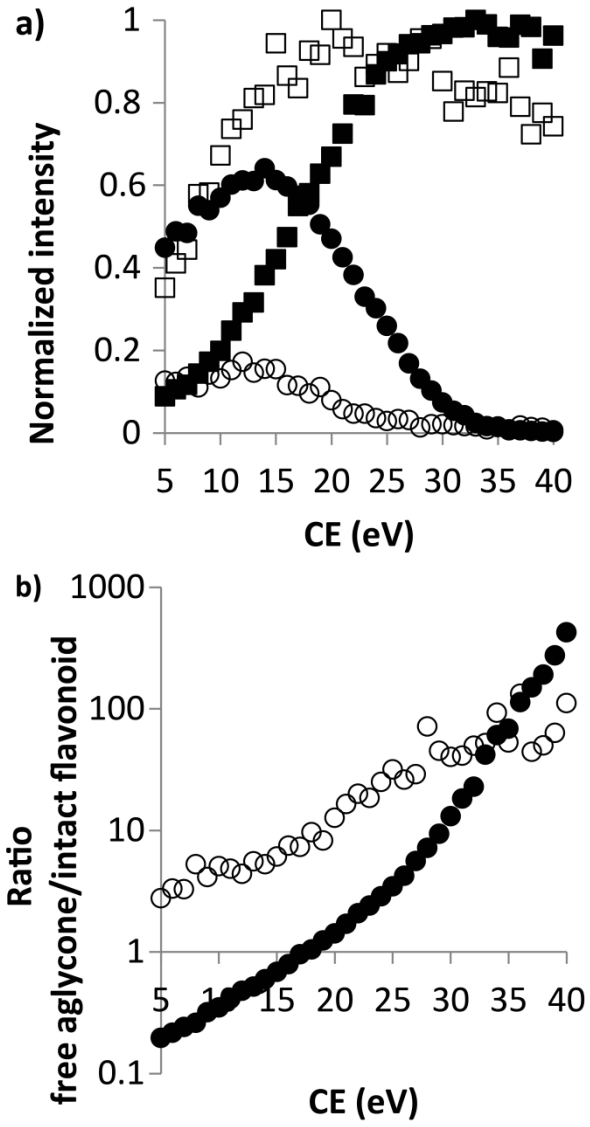

Figure 6. a) Normalized signal intensities at different collision energies que-3-glu (white circles), free quercetin from que-3-glu (white squares), que-4´-glu (black circles) and free quercetin from que-4´-glu (black squares). b) Ratio of the free aglycone over the intact flavonoid for que-3-glu (white circles) and que-4´-glu (black circles).