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Three-step HPLC–ESI-MS/MS procedure for screening and identifying non-target flavonoid derivatives

Gábor Rak, Péter Fodor, László Abrankó*

Corvinus University of Budapest, Faculty of Food Science, Department of Applied Chemistry, 29-33 Villányi, Budapest, H-1118, Hungary

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ABSTRACT

A three-step HPLC–ESI-MS/MS procedure is designed for screening and identification of non-target flavonoid derivatives of selected flavonoid aglycones. In this method the five commonly appearing aglycones (apigenin, luteolin, myricetin, naringenin and quercetin) were selected. The method consists of three individual mass spectrometric experiments of which the first two were implemented within a single chromatographic acquisition. The third step was carried out during a replicate chromatographic run using the same RP-HPLC conditions. The first step, a multiple reaction monitoring (MRM) scan of the aglycones was performed to define the number of derivatives relating to the selected aglycones. For this purpose the characteristic aglycone parts of the unknowns were used as specific tags of the molecules, which were generated as in-source fragments. Secondly, a full scan MS experiment is performed to identify the masses of the potential derivatives of the selected aglycones. Finally, the third step had the capability to confirm the supposed derivatives. The developed method was applied to a commercially available black currant juice to demonstrate its capability to detect and identify various flavonoid glycosides without any preliminary information about their presence in the sample. As a result 13 compounds were detected and identified in total. Namely, 3 different myricetin glycosides and the myricetin aglycone 2 luteolin glycosides plus the aglycone and 3 quercetin glycosides plus the aglycone could be identified from the tested black currant sample. In the case of apigenin and naringenin only the aglycones could be detected.

1. Introduction

Flavonoids are secondary plant metabolites that belong to the large group of phenolic compounds. This diverse group of phytochemicals is involved in many physiological activities in plants. They are also synthesized in normal development such as plant pigmentators and in some cases are involved in primary metabolism. However, their main biological importance is relating to their protective functions. Flavonoids are synthesized by plants in higher quantities in response to various stresses such as UV irradiation, wounding, and fungal infection [1–3]. The precise physiology of their involvement in disease resistance mechanisms is still to be cleared. In addition to the inhibitory effects of flavonoids on microorganisms that plays important role in disease resistance of plants they also exhibit several physiological activities in animals and humans. They are potent antioxidants, free radical scavengers, they inhibit lipid peroxidation and among others have anti-inflammatory, anti-hypertensive, anti-allergic, anti-thrombic and anti-carcinogenic properties [1,2,4,5]. It should be noted that their occurrence in animal tissues and nonplant materials is generally due to the ingestion of plant foods [6]. In food products they also contribute to bitterness, astringency, color, flavor, odor and oxidative stability of products [4]. The above outlined properties of flavonoids explain the intense interest towards plant phenolics research, thus the need for appropriate analytical methods that are suitable to serve various researches in the fields of plant breeding, food processing and medical sciences.

From the analytical point of view even the qualitative analysis of flavonoids can be a complex task since the estimated number of flavonoids, which are only a sub-group of plant phenolics according to different literatures, varies between 4000 and 8000 [5,7]. On the contrary to the extreme diversity all flavonoids are structurally related molecules having either a flavone or a flavonone skeleton (Fig. 1) or in the case of isoflavons the phenyl substituent is in the C_3 position [6,8].

Flavonoids are generally hydroxylated in 3, 5, 7, 3′, 4′ and/or 5′ positions of the skeleton, moreover quite often the hydroxyl groups in positions 3 and/or 7 have various mono- or diglycosides substituents. These latter derivatives of the aglycone molecules are called the O-glycosides (Fig. 1). Besides the above-described frequent derivatives several others are also known, for instance the C-glycosides that have the sugar groups directly bound to a carbon

* Corresponding author. Tel.: +36 1 482 6163; fax: +36 1 466 4272.
E-mail address: laszlo.abranko@uni-corvinus.hu (L. Abrankó).

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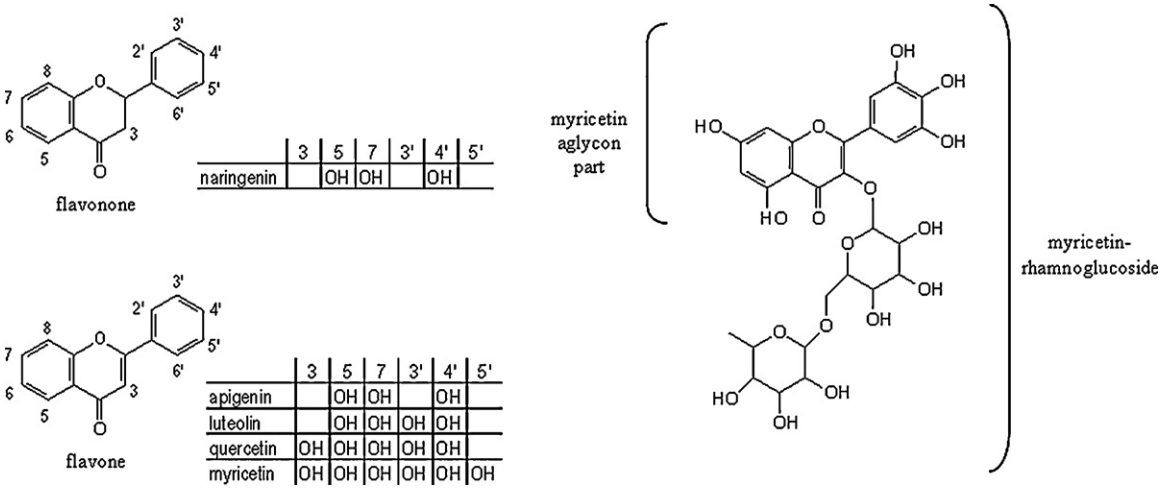

Fig. 1. The flavone and flavonone skeletons of flavonoids and indication of the actual structures of the selected aglycone molecules (left). As an example for flavonoid glycosides the structure of myricetin-3-O-rhamnoglucoside is given (right). The myricetin aglycone (3′, 4′, 5, 5′ 7 pentahydroxy-flavon-3-ol) is a characteristic part of the glycoside molecule.

of the aglycone or the more complex group of O and C-glycosides with acylated saccharide parts [9,10,29].

Among the numerous developed methods used for flavonoid analysis two distinct groups can be recognized. According to the aim of the study there are a group of methods focusing on predefined flavonoid compounds where identification of the detected analytes is made by comparison with standard substances [11–14]. Within this group there are also applications where only the determination of the aglycone molecules is targeted, hence a chemical hydrolysis using hydrochloric [2–5,15,16] or formic acid or alternatively enzymatic hydrolysis of the glycosides is performed before the chromatographic analysis [2]. As a result, the extract of a sample originally containing several derivatives of a given aglycone produces only the chromatographic peak of the free aglycone after hydrolysis. By this method the interpretation of complex flavonoid profiles can dramatically be simplified, which is useful in cases where appropriate standards of the suspected glycosides are not available. The major disadvantage of such methods is the reduction in information content of the original flavonoid profile of the sample [17].

Apart from the above outlined methods that are focusing only on target analytes recently methods with the concept of involving non-target analytes also seem to be of great importance [13,18–21]. In various research fields such as metabolomics [22] or plant physiology where metabolic pathways and regulation mechanisms are investigated [13,21] or in chemotaxonomic studies where specific compounds should be found [23–25], beside the analysis of the predefined target analytes (various flavonoid aglyca and/or their derivatives) the interest expanded to the detection and identification of possibly all flavonoid compounds (including non-target analytes) in a single extract in order to provide a comprehensive inventory of the flavonoid fingerprint of a sample. The major challenge in these so-called profiling methods is to cover as much compounds as possible including unknown flavonoid derivatives. Concerning the applied detection techniques today tandem mass spectrometry (MS/MS or MS^n) usually with electrospray ionization (ESI) often combined with a diode array detector (DAD) appeared to be the most important technique for profiling methods [2,5,10]. Several MS/MS methods with a variety of identification approaches for unknown flavonoid derivatives have been published and are thoroughly reviewed among others by Harnly et al. [5] and de Rijke et al. [2]. In the majority of the published methods the identification concept is common and is based on the determination of the aglycone part of the flavonoid conjugates completed with mass difference calculations between the intact parent ions and the corresponding characteristic fragments that have lost their sugar moieties. It should be noted however, that most of these methods using the fragmentation information of compound subunits provide only provisional identification of unknown derivatives and NMR often turns out to be an indispensable tool to arrive at an unambiguous structural characterization of unknown compounds [2,21].

For the separation of flavonoids and their derivatives high performance liquid chromatography (HPLC) is perhaps the most popular and reliable system among all chromatographic separation techniques. The chromatographic conditions of the HPLC methods include the use of, almost exclusively; a reversed-phase C18 column and a binary solvent system containing acidified water and a polar organic solvent [26–28].

In such methods, which are targeting a wide variety of different flavonoid derivatives in addition to the use of appropriate separation and detection techniques, the extraction method should also be carefully chosen. Since the solubility of the analytes among others depends on the type of compound (e.g., glycosides are more polar than aglycones) there is no uniform or completely satisfactory procedure that is suitable for the extraction of all phenolics or even a specific class of phenolic substances in plant materials [4]. As a practical solution in the case of qualitative methods the goal is the preparation of a sample extract that is as much as possible uniformly enriched in all components of interest [3]. A variety of aqueous solvents (methanol, ethanol, acetone, acetonitrile and dimethyl sulfoxide), water/solvent ratios, and techniques for physical solvent–sample interaction (sonication, microwave-assisted extraction, high pressure temperature extraction, stirring and shaking) have been described in the literature [13]. Most frequently aqueous methanol between 50 and 80% has been used for extracting flavonoids. Higher water composition in the solvent can aid the extraction of glycosides, while especially for aglycones non-aqueous solvents can also be used [26]. In order to be able to identify all potential derivatives of every aglycone, thus providing a complete flavonoid fingerprint of the sample extract hydrolysis should of course be prevented and as a rule of thumb harsh extraction conditions and heating should be avoided [2]. In the case of liquid samples sample preparation often consists of only a simple filtration step. However, reduced sample preparation procedures and the lack of further sample clean-up manipulations require enhanced selectivity in the subsequent analysis [3].

In this study a three-step HPLC–ESI-MS/MS procedure is presented designed for screening and identification non-target flavonoid derivatives of selected flavonoid aglycone molecules. The developed method is applied for a commercially available black currant juice to demonstrate its capability to detect and identify various flavonoid glycosides without any preliminary information about their presence in the sample.

2. Materials and methods

Crystalline flavonoid aglyca (apigenin, luteolin, myricetin, naringenin and quercetin) along with three derivatives apigenin-7-glucoside, naringin and rutin were obtained from Sigma (Sigma–Aldrich, Budapest, Hungary). HPLC grade acetonitrile, methanol and formic acid were purchased from Scharlau (Barcelona, Spain). Water used in all experiments was high purity water (18 MΩ cm^{-1}) provided by a Milli-Q system (Billerica, MA, USA). Black currant juice used as a test sample was purchased in a local supermarket.

Experiments were performed with an Agilent (Agilent Technologies, Waldbronn, Germany) 1200 HPLC system coupled to an Applied Biosystems (Foster City, CA, USA) 3200 Q-Trap hybrid triple quadrupole/linear ion trap MS/MS instrument equipped with a Turbo-V ESI ion source that was used in the negative ionization mode.

For chromatographic analysis a 2.1 mm × 50 mm Agilent Zorbax Rapid Resolution C18 column with 1.8-μm particle size was used. The column was protected with an inline filter having 0.45 μm pore size. For the elution 0.1% (v/v) formic acid in high purity water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) were used as solvents at a flow rate of 300 μl/min. The gradient program started at 3% B and after 1.5 min of isocratic run the following program was performed. From 1.5 to 35 min solvent B was increasing linearly from 3 to 30%, then from 35.1 to 40 min to 99% and finally from 40.1 solvent B was set back to 3% and was running until 45 min for the equilibration of the initial solvent composition. Additionally to the use of high resolving HPLC column and MS detection – in accordance with the literature [5,18] – a relatively long chromatographic run was performed, since the separation of several unknown compounds over the same range of polarities was targeted.

A commercially available black currant juice was applied in this study to demonstrate the suitability of the developed method. A simple filtration was carried out on the sample using a 0.45 μm PTFE membrane filter before injecting 3 μl to the HPLC.

3. Results and discussion

The basic idea of the method rests on the utilization of the fact that every flavonoid derivative contains a characteristic aglycone part that can be considered as a specific tag of every analyte molecule. For instance myricetin is the characteristic molecular tag in the compound shown in Fig. 1 and also in every other derivative of myricetin. Therefore, the developed analytical method in the first step is focusing only on the detection of the aglycone part of flavonoid derivatives using it as a label that can indicate the presence of various derivatives of a certain aglycone that might be present in a sample. By this means the number of screened analytes can easily be multiplied significantly and it becomes feasible to monitor possibly all derivatives of a given aglycone. However, the number of aglycone molecules is much more limited than the relevant derivatives, nevertheless it is not possible and neither rational to incorporate all the existing aglyca in one method. Therefore, it should be predetermined which aglycone molecules will be involved for screening. In our experiments the five commonly appearing aglyca namely, apigenin, luteolin, myricetin, naringenin and quercetin were selected (see Fig. 1).

3.1. Step I: the MRM experiment

The multiple reaction monitoring (MRM) scan mode was used for the detection of the aglycone tags. Two characteristic fragments were chosen for qualitative identification. Negative ion mode was used in this and all further experiments of the entire study, since according to literature [10,18] and the authors own experience negative ion mode is more sensitive in the case of flavonoids and their derivatives. The parameters of the MRM method were optimized using standard substances. The results of the optimization along with the retention times of the investigated compounds obtained from a standard MRM chromatogram are presented in Table 1.

In order to produce 'input' to the MRM scan the aglycone tag of flavonoid derivatives should be provided as an individual subunit having the appropriate mass that was set in the MRM method as precursor mass. For that purpose the aglycone tags were intended to be provided as in-source fragments of the original derivatives. To encourage the formation of in-source aglycone fragments an unusually high declustering potential (DP) value – that source parameter is originally used to minimize solvent cluster ions – was applied. Since various unknown derivatives of the selected aglycone molecules are expected during the chromatographic acquisition in-source fragmentation conditions should be generalized. In order to find the consensus DP value in-source fragmentation characteristics was tested by introducing standard solutions of various different flavonoid derivatives. The selected derivatives were apigenin-7-O-glucoside, naringenin 7-O-rutinoside (naringin) and quercetin 3-O-rutinoside (rutin) representing a variety of aglyca, glycan parts and glycosylation positions. Flavonoid derivatives were infused individually to the MS while only m/z values of the relevant aglycone subunits $[M-H]^-$ were monitored in a single MS experiment (Fig. 2).

Table 1
Retention times of the investigated compounds and their optimized parameters in negative ionization mode. RT: retention time (min); DP: declustering potential (V); EP entrance potential (V); CEP: cell entrance potential (V); CE 1 and 2 collision energies for selected transitions (eV); CXP 1 and 2: cell exit potentials for selected transitions (V).

Target analytes	Parent ion $[M-H]^-$	Selected transitions and annotation of fragments[a]		RT	DP	EP	CEP	CE1	CE2	CXP1	CXP2
Apigenin	269	117 ($^{1,3}B^-$)	149 ($^{1,4}B^-$+2H)	30.7	−65	−4	−14	−42	−32	0	0
Naringenin	271	151 ($^{1,3}A^-$)	119 ($^{1,3}B^-$)	29.4	−45	−3	−14	−24	−32	−2	0
Luteolin	285	133 ($^{1,3}B^-$)	151 ($^{1,3}A^-$)	27.0	−65	−5.5	−22	−44	−34	0	0
Quercetin	301	151 ($^{1,3}A^-$)	179 ($^{1,2}A^-$)	26.5	−50	−4.5	−14	−28	−26	0	0
Myricetin	317	151 ($^{1,3}A^-$)	137 ($^{1,2}B^-$)	21.3	−65	−5.5	−36	−34	−34	−2	0
Apigenin-7-glucoside	431	268 ($A-2H^{\bullet -}$)	117 ($^{1,3}B^-$)	21.7	−80	−8.5	−20	−42	−84	0	0
Naringin	579	271 ($M-H^-$)	151 ($^{1,3}A^-$)	21.5	−80	−10	−30	−40	−50	0	0
Rutin	609	300 ($A-2H^{\bullet -}$)	301 ($A-H^{\bullet -}$)	18.6	−90	−9	−20	−48	−40	0	−2

Ion source capillary voltage: −4500 V; ion source temperature: 350 °C; exhaust and nebulizer gases: 50 psi.

[a] Annotation of fragments is following the nomenclature proposed by Ma et al. [30].

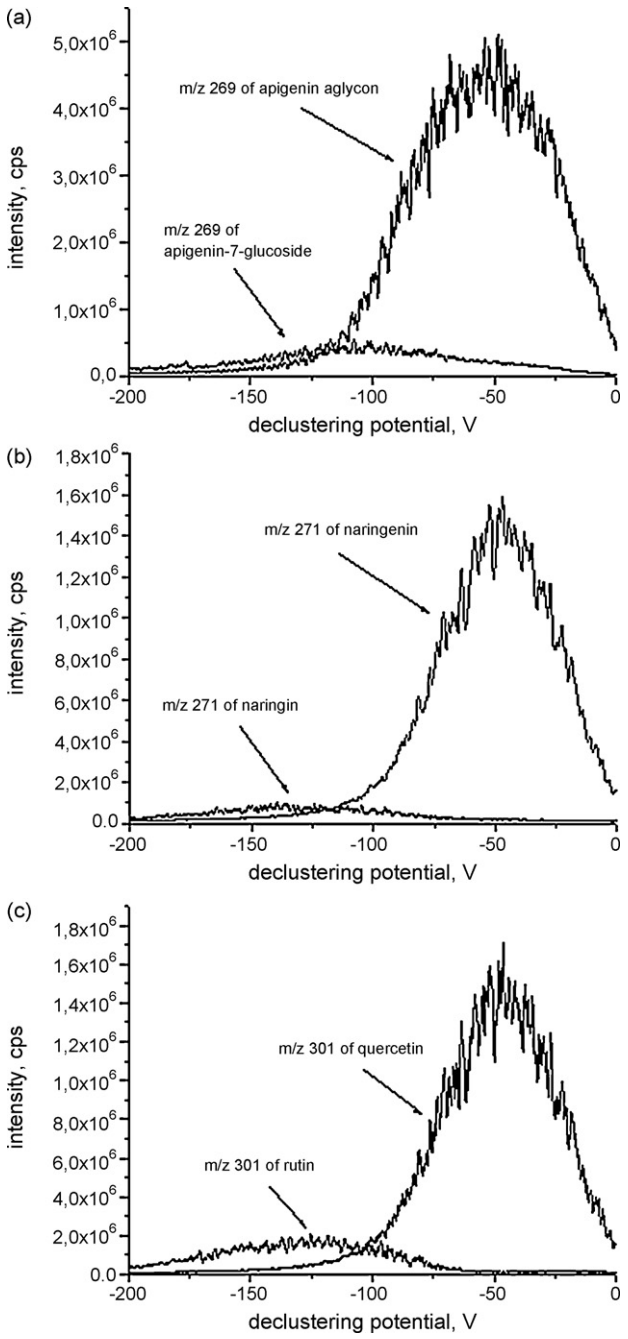

Fig. 2. Formation of in-source aglycone fragments from apigenin-7-glucoside (a) naringin (b) and rutin (c) as a function of declustering potential (DP). Each flavonoid derivative and authentic aglycone standard solution was infused individually at 10 μg ml^{-1} concentrations from a syringe pump dissolved in 1:1 dilution of the mobile phases applied for chromatographic separation.

As can be seen in Fig. 2 signal intensities of in-source aglycone fragments coming from derivatives reached their maxima at lower DPs compared to the ones of the authentic aglycone standards. Moreover, at optimum DP values signals for those aglycone subunits formed in-source were approximately one order of magnitude less intense than that of their authentic counterparts. On the other hand, optimum values for declustering potential were reasonably close to each other in the case of the tested compounds showing that a consensus DP value can be applied independently from the type of aglycone, sugar moiety and glycosylation position. A DP value of −120 V was chosen as a consensus in-source fragmentation condition and set in the MRM method. In order to test the method with a real sample a commercially available black currant juice was measured. The obtained MRM chromatograms of the screened five aglycones are shown in Fig. 3.

In Fig. 3 it can be seen that except for apigenin compounds relating to the remaining 4 aglycones (luteolin, myricetin, naringenin and quercetin) produced peaks at multiple retention times. In the case of apigenin only traces of the aglycone could be detected at 30.7 min and the compound eluting at 11.9 min was not an apigenin derivative. The aglycone is generally eluting as the last compound since being the less polar one compared to the various derivatives due to the lack of attached polar moieties such as saccharides. Beside the aglycone the other peaks providing the same MRM transitions with same transition ratios are quite probable to be various derivatives (e.g., glycosides) of the aglycone. Fig. 4 shows only the extracted chromatograms of both myricetin aglycone transitions.

As can be seen in the chromatogram in Fig. 4 there are only 3 peaks at retention times of 15.7, 16.0 and 21.3 min, respectively where both transitions give signal. According to the generally accepted mass spectrometric qualifying protocol that is based on the simultaneous detection of two characteristic fragments of a given compound it means that each compound can be considered to somehow relate to myricetin. Based on the retention times the last peak at 21.3 was confirmed to be the myricetin aglycone. The remaining two peaks are unknowns, most possibly derivatives of myricetin and the in-source formed aglycone fragment ions served as precursors in the MRM experiment. At this point nothing more exact can be claimed about these two derivatives except that they supposed to be more polar than the aglycone, since being eluted earlier under reversed-phase chromatographic conditions. After the above-discussed MRM experiment a second MS step was introduced in order to provide more information on the found compounds. In the forthcoming discussion the remaining steps of method development will be detailed based on the example of the above myricetin results.

3.2. Step II: full scan experiment

In the first MS experiment the number of compounds corresponding to any of the selected aglyca can be defined. However, the MRM experiment does not provide any further qualitative information on the intact derivative of which the in-source aglycone fragment originates. That is the reason why a single-stage full scan MS experiment in the mass range 250–900 m/z was performed right after the MRM experiment in the same chromatographic run. In Fig. 5 the full scan spectra of the two unknown peaks are given.

Beside m/z 479 the mass peaks of m/z 316 and 317 are also common in the two chromatographic peaks. Both 316 and 317 correspond to myricetin aglycone that are formed in-source as a result of the −120 DP value. The mass 317 is the deprotonated in-source fragment of the aglycone $[M-H]^-$ and m/z 316 corresponds to the radical $[M-2H]^-$ in-source aglycone fragment of myricetin formed by a homolytic cleavage. According to our previous experiments the intensity of the radical aglycone was experienced to be varying as a function of DP. At high negative values (i.e., below −80 V) the radical aglycone fragment becomes more abundant in the cases of the investigated derivatives than the even-electron counterparts (data not shown). Finally, it is possible that at a DP value where intensity of the even-electron in-source aglycone fragment reaches its maximum the radical aglycone will still be more abundant. Nevertheless, according to the authors' opinion in the MRM experiment it is appropriate to use the even-electron in-source aglycone fragment as a precursor for the MRM scan, since only transitions relating to this precursor mass can be confirmed by standard substances. In the particular case of myricetin the significant dominancy of m/z 316 over 317 can be further justified by the observations that in negative ESI-MS/MS the abundance ratio of the radical aglycone to the

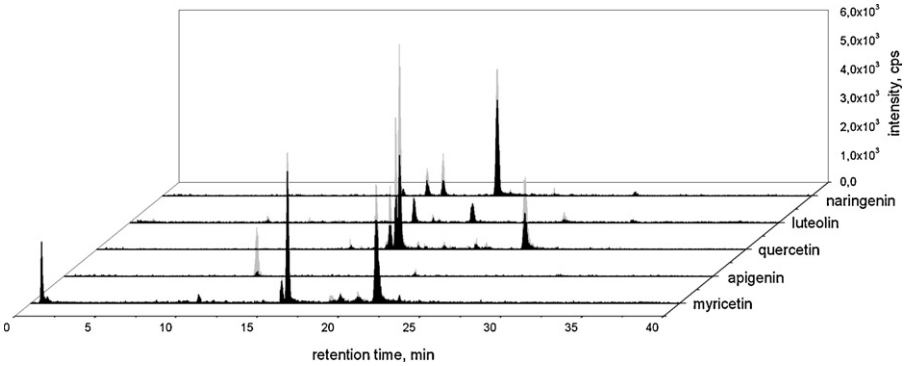

Fig. 3. Extracted MRM chromatograms of the measured black currant juice sample. Dark and light signals showing the two selected transitions of the investigated five aglycone molecules.

regular (i.e., even-electron) aglycone originating from the cleavage at the 3-O-glycosidic bond with a concomitant H-rearrangement increases with increasing OH substitution on the B-ring [10].

Apart from the mass peaks relating to the myricetin aglycone there are other dominant masses present in the full scan mass spectra of the two unknowns. These masses (m/z 479 in the peak at 15.7 min and m/z 479 and 625 in the peak eluting at 16.0) are considered to correspond to the original masses of the intact unknown flavonoid derivatives. In the case of the myricetin derivative eluting at 15.7 min the picture is simple. The only dominant mass peak above the aglycone is m/z 479. The difference between the two m/z of 479 and 317 equals 162 amu. Therefore, it is quite probable that the loss of 162 corresponds to the cleavage of a hexosyl moiety, which was originally conjugated via a glycosidic bond to the aglycone. In the case of the unknown compound eluting at 16.0 min apart from the aglycone masses there are two more dominant masses in the full scan spectrum that makes the understanding somewhat complicated. One of the possible explanations can be that there is only one compound in the given chromatographic peak. In this case the highest mass (m/z 625) is addressed to be the deprotonated ion of the original compound and m/z 479 is another in-source fragment of the original compound similarly to the aglycone peaks at m/z 316 and 317. The mass difference of 146 amu between m/z 625 and 479 indicates a loss of a deoxyhexosyl group. Following the previous theory the original flavonoid derivative may be a myricetin-disaccharide, presumably a myricetin-hexosyl-deoxyhexoside. It should be noted that this provisional identification method is fundamentally based on the hypothesis that the detected individual mass peaks that are simultaneously present in a single chromatographic peak are related to each other, i.e., the lower masses are in-source fragments of the higher one(s). However, another possibility can be that they are independent from each other and relating to two or more individual compounds that are partly or fully co-eluting as a result of poor chromatographic separation. In order to clear up the above-described uncertainty a third MS experiment was applied to support the identification procedure.

3.3. Step III: generating selected product ion spectra

Target masses of the supposed derivatives were obtained as a result of the second (full scan) MS experiment applying the previously described methodology. Based on this data a third MS step

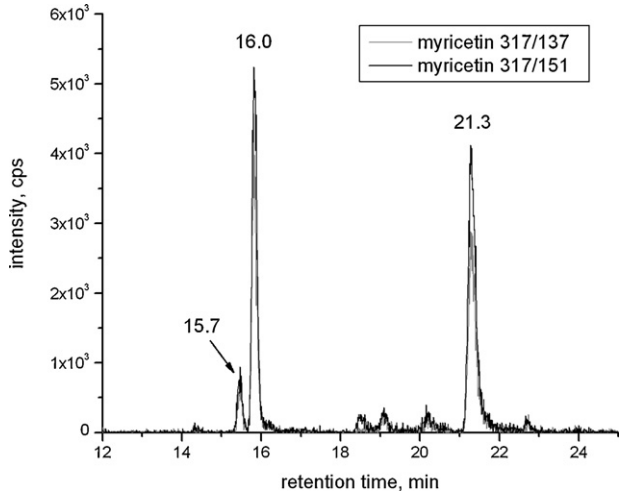

Fig. 4. Extracted chromatograms of the two characteristic myricetin transitions of 317/151 and 317/137 obtained from a black currant juice sample.

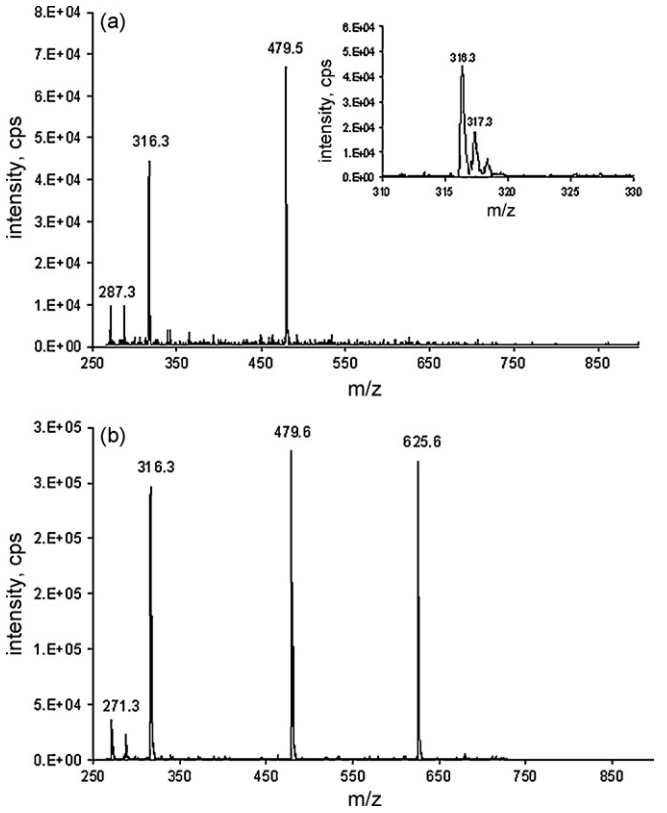

Fig. 5. Full scan spectra of peaks at 15.7 min (a) and 16.0 min (b) of the measured black currant juice sample.

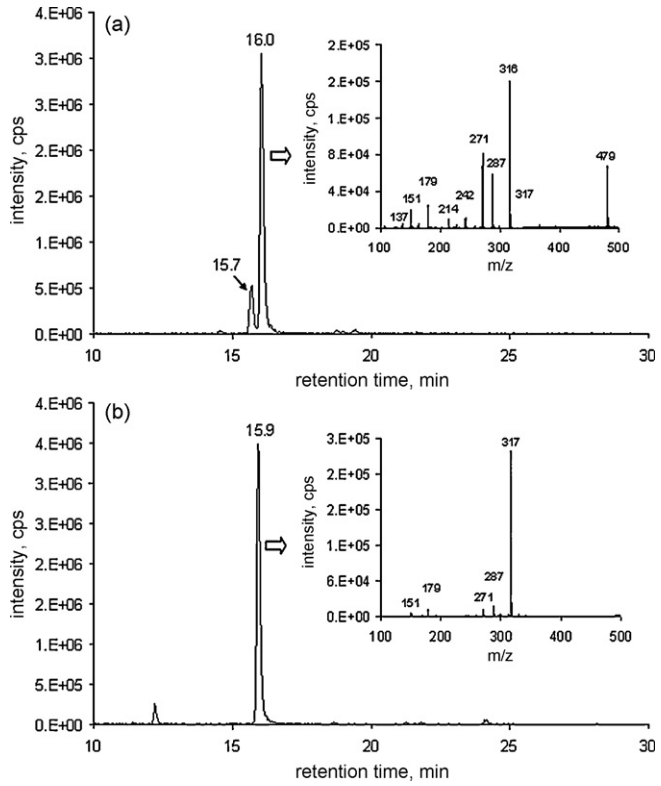

Fig. 6. Total ion chromatograms of the enhanced product ion (EPI) scans of *m*/*z* 479 (a) and *m*/*z* 625 (b) of the investigated black currant juice sample. The insets are showing enhanced product ion (EPI) spectra of *m*/*z* 479 of the peak eluting at 16.0 min (a) and the one of *m*/*z* 625 of the peak at 15.9 min (b).

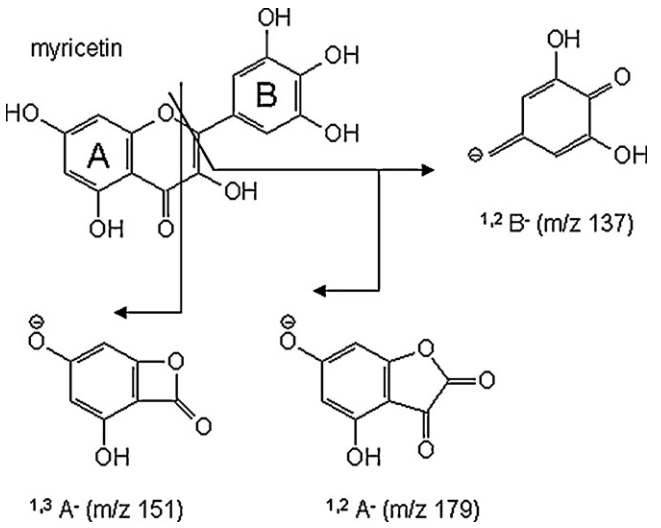

Fig. 7. Putative fragmentation pathway of myricetin aglycone. Annotation of fragments is following the nomenclature proposed by Ma et al. [30].

was performed to generate the product ion (PI) scans of the targeted masses of the unknown compounds. The third MS experiment could not be incorporated into the same MS method implementing the first and the second MS experiments, since the target precursor masses of the planned PI scans are not preliminary known. That means another individual chromatographic run was required of the same sample to complete the third MS experiment. Product ion spectra were acquired using the enhanced product ion (EPI) scan mode of the instrument that provides higher sensitivity, since in this scan mode the third quadrupole of the MS instrument functions as a linear ion trap. When generating EPI spectra at an intermediate scanning speed of 1000 amu s^{-1}, it was experienced that the number of simultaneously acquired product ion scans should ideally not exceed 4, otherwise the total cycle time of the MS scans will become too long to obtain acceptable peak shapes and moreover, it deteriorated sensitivity.

In the case of the previous example of myricetin derivatives precursor masses of 479 and 625 were subjected to EPI scans. The resulting TIC chromatograms along with the product ion spectra of the chromatographic peaks at 15.9 and 16.0 min are shown in Fig. 6.

The two peaks in Fig. 6a are relating to isomers of the myricetin-deoxyhexosides. Due to the beneficial chromatographic characteristics of the applied 1.8-μm particle size stationary phase in the RP-HPLC column satisfactory separation of such structural isomers could be carried out. On the contrary, the supposed myricetin-hexosyl-deoxyhexoside (*m*/*z* 625) eluting at 15.9 min (see Fig. 6b) could not appropriately be separated from the myricetin-deoxyhexoside isomer at 16.0 min. This example clearly shows that in the case when unknown derivatives are screened even high resolution chromatographic separation cannot be considered as an ultimate tool to avoid co-elutions that might result in misidentification of compounds. However, problems relating to co-elutions can be well handled by using the product ion scan as a third confirmatory MS experiment in the developed methodology. Product ion scanning in the third step of the procedure provides further advantageous information to tentatively confirm the identity of the precursor molecules. As it can be seen in the inset of Fig. 6b showing the product ion spectrum of the peak eluting at 15.9 min *m*/*z* 479 is not appearing among the product ions of the supposed myricetin-hexosyl-deoxyhexoside (*m*/*z* 625). This further justifies the fact of co-elution.

Beside this fact some characteristic product ions do appear in both spectra. For instance, the mass peak of *m*/*z* 317 corresponds to the regular (even-electron) myricetin aglycone fragment. It is worth noting that *m*/*z* 317 (myricetin aglycone fragment) is more intensive if dissociation of the intact molecules was induced collisionally with N_2 using collision energy of 30 eV as a general parameter instead of generating them as in-source fragments (see spectrum in the inset of Fig. 6b). Moreover, the product ions with *m*/*z* values lower than the one of the aglycone (i.e., *m*/*z* <317) like *m*/*z* 179 ($^{1,2}A^-$), 151 ($^{1,3}A^-$) and 137 ($^{1,2}B^-$) are typical to the retro Diels-Alder (RDA) fragmentation of flavon-3-ols having a dihydroxylated A ring and *m*/*z* 137 is a typical fragment of the trihydroxylated B-ring [30]. That means these three fragments together with *m*/*z* 317 of the aglycone are quite characteristic to myricetin. The putative fragmentation pathway of myricetin aglycone is given in Fig. 7. Following the above-described logic 12 further flavonoid derivatives could be detected and identified in the test sample.

3.3.1. *Identification of compounds other than myricetin derivatives*

Applying the above-described three-step procedure in total 13 compounds were detected and identified. Beside the 3 different myricetin glycosides and the myricetin aglycone 2 luteolin glycosides plus the aglycone and 3 individual quercetin glycosides plus the aglycone could be identified from the tested black currant sample. In the case of apigenin and naringenin only the free aglycone molecules could be detected.

The MRM screening for luteolin indicated three compounds of which the latest eluting one was the aglycone and the ones at 17.8 and 21.3 min were both luteolin-hexoside isomers providing dominant mass peaks at *m*/*z* 447 in the acquired full scans. Both of them were confirmed to be luteolin derivatives in the third MS step.

In case of quercetin the MRM scan provided four peaks at 18.4, 18.7, 19.0 and 26.7 min. The compound eluting at 26.7 min was confirmed to be the quercetin aglycone. The other found compounds at 18.4 and 19.0 were quercetin-hexoside isomers with m/z 463. The compound eluting at 18.7 was found to be a quercetin-hexosyl-deoxyhexoside with an m/z value of 609. Beside the three-step identification procedure this compound could be confirmed by comparing the retention times of the found compound and that of obtained from the measurement of a rutin standard solution. The comparison resulted in retention time matching proofing that the found compound was the quercetin-3-O-rhamnoglucoside conventionally called rutin.

4. Conclusions

A three-step analytical procedure was presented applying three mass spectrometric experiments of which the first two were implemented within a single chromatographic acquisition. The third step was carried out during a replicate chromatographic run using the same HPLC conditions. The first step, an MRM experiment was designed to define the number of derivatives relating to the selected aglycone subunits along with their chromatographic retention times. By this means it was demonstrated that a complete profile of the derivatives of the selected aglycone molecules could be recognized if they were present at least in concentrations, which are detectable during the MRM scan. The second full scan MS experiment was performed to identify the masses of the potential compounds that can be derivatives of the selected aglyca. Again, this step allowed the recognition of any derivatives of the given aglycone subunits if their original masses are within the applied mass range and their concentrations are at a level that is detectable in the single-stage MS scan. Finally, the third step had the capability to confirm that the supposed mass of the derivative was really a precursor of the selected aglycone. By this means it became possible to distinguish between co-eluting compounds that have different glycan moieties but are derivatives of the same aglycone. The applied methodology was demonstrated to be suitable for a wide-scope qualitative flavonoid profiling in a real sample without any preliminary knowledge on the presence of the derivatives of the selected aglycones. However, a limitation of the method is evidently its unsuitability to identify structural isomers having for instance hexoside groups such as glucose or galactose attached to the same aglycone.

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