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High-performance liquid chromatography with electrospray ionization mass spectrometry and diode array ultraviolet detection in the identification of flavonol aglycones and glycosides in berries

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Abstract

High-performance liquid chromatography–electrospray ionization-mass spectrometry (HPLC–ESI-MS) was used to study flavonol aglycones and glycosides in berries. For the identification of aglycones, photodiode-array detection (DAD) was also used. The HPLC–ESI-MS technique is highly valuable in the identification of flavonol aglycones and glycosides from berry extracts. This ionization technique provides information on the structure of the aglycones and glycosides without time-consuming pre-purification or derivatization steps. Quercetin aglycone was identified with both ESI-MS and DAD in all of the berries studied. Myricetin aglycone was identified with both techniques in three berries. Hexose, deoxyhexose–hexose and pentose derivatives of quercetin were the most abundant flavonol glycosides identified. Two glycosides of myricetin and one glycoside of kaempferol were identified in blackcurrant. To confirm the data obtained using the HPLC–ESI-MS procedure, fractions of the glycosides from four berries were separated, hydrolyzed, silylated and the sugars were analyzed using gas chromatography–mass spectrometry.

Keywords: Food analysis; Flavonols; Aglycones; Glycosides; Quercetin; Myricetin; Kaempferol; Flavonoids

1. Introduction

Flavonoids are polyphenolic compounds that contain a C_{15} flavone skeleton and represent a large group of secondary plant metabolites. Flavonols (e.g. quercetin, myricetin and kaempferol) are a group of flavonoids that occur in foods as O- and C-glycosides (Fig. 1). These natural products are of interest because of their apparent health-promoting effects as antioxidants [1,2] and anticarcinogens [3]. Recent studies indicate that flavonols (e.g. quercetin) can be absorbed from the diet as glycosides by humans [4,5]. There is thus an interest in analyzing flavonol glycosides from various dietary sources.

Our main interest is in the flavonoid and phenolic acid contents of Finnish berries, their bioavailability and effects on carcinogen metabolism. We have recently analyzed aglycones of flavonoids and phenolic acids from several berries by high-performance liquid chromatography (HPLC) methods [6,7]. The

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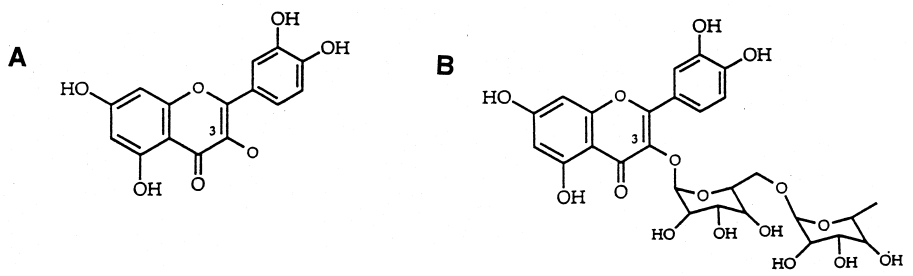

Fig. 1. Structures of (A) quercetin aglycone and (B) quercetin-3-O-β-rutinoside (rutin).

methods previously used for the analysis of flavonoid glycosides in berries are thin-layer chromatography (TLC) and gas chromatography (GC) in blackcurrant [8] and TLC and UV spectrophotometric techniques in strawberries, raspberries [9] and blueberries [10].

Catechins [11] and various groups of polyphenols, including flavonol O-glycosides [12], have been studied from tea using thermospray liquid chromatography–mass spectrometry (LC–MS). Positive ion fast atom bombardment MS and tandem mass spectrometry have been used to study the glycosidic linkages in diglycosyl flavonoids [13]. HPLC–electrospray ionization (ESI)-MS offers advantages in terms of sensitivity and capacity for the analysis of large, thermally labile and highly polar compounds [14]. Catechins [15] and glycosides of apigenin [16] have been studied from tea using HPLC–ESI-MS.

Positive ion ESI mass spectra usually show protonated molecules as base peaks and only low amounts of fragment ions. However, fragmentation can be easily induced in the high pressure regions of the ion passageway from the source into the mass analyzer. This method is often called source-induced dissociation (SID) or high orifice potential fragmentation [17]. An elegant application of alternating skimmer region voltage was used to produce phosphate fragment ions to identify phosphorylated peptides in tryptic digests [18]. In the present study, a similar approach was used to screen quercetin, myricetin and kaempferol glycosides in the berry samples.

The purpose of this study was to identify flavonoid (flavonol) aglycones and major flavonol glycosides in berries using HPLC–ESI-MS or with UV–Vis photodiode-array detection (DAD). To confirm the capability of HPLC–ESI-MS to identify flavonol glycosides, some sugar moieties of flavonol glycosides in berries were further characterized by GC–MS after fractionation with HPLC–ESI-MS, hydroxylation and silylation.

2. Experimental

2.1. Standards and solvents

Kaempferol, quercetin and rutin were purchased from Sigma (St. Louis, MO, USA). Myricetin was obtained from Fluka (Buchs, Switzerland). The standards were dissolved in methanol. Methanol (Lab-Scan, Dublin, Ireland) and acetonitrile (Rathburn, Walkerburn, UK) were of HPLC grade. Formic acid (Merck, Darmstadt, Germany) and hydrochloric acid (Riedel-de Haën, Seelze, Germany) were of analytical grade. D-(−)-Arabinose, D-(+)-galactose and L-rhamnose were obtained from Sigma and D-(+)-glucose was from BDH (AnalaR grade; Poole, UK). *N,O*-Bismethylsilyltrifluoroacetamide (BSTFA) was from Sigma and pyridine was from Riedel-de Haën.

2.2. Berries

The berries studied were of Finnish origin and harvested or picked in 1997. Cultivated blackcurrants (*Ribes nigrum* 'Öjebyn'), strawberries (*Fragaria x ananassa* 'Senga Sengana') and red raspberries (*Rubus idaeus* 'Ottawa'), harvested in eastern Finland, as well as wild lingonberries (*Vaccinium vitis-idaea*) and bilberries (*Vaccinium myrtillus*), were obtained from Pakkasmarja, Suonenjoki. Rowanberries (*Sorbus aucuparia*), chokeberries (*Aronia mitchurinii* 'Viking') and sea buckthorn berries

(*Hippóphaë rhamnoides*) were harvested from the botanical garden of the University of Kuopio. Wild crowberries (*Empetrum hermaphroditum*) were picked from Lapland, bog whortleberries (*Vaccinium uliginosum*) from Muuruvesi, eastern Finland, and cranberries (*Vaccinium oxycoccos*) were from Kurikka, western Finland. The berries were stored for four–six months at −18°C until used for analysis.

2.3. Sample preparation for HPLC–ESI-MS and HPLC–DAD

The frozen berries (100 g) were thawed in a microwave oven and crushed in a food processor. Flavonoid glycosides were extracted and hydrolyzed to corresponding aglycones with a method modified from that of Hertog et al. [19]. The berry sample (5 g) was diluted with purified water to 15 ml, and 25 ml of methanol, which contained 2 g/l *tert.*-butylhydroxyquinone (TBHQ), were added. Moreover, 10 ml of 6 M HCl were added (final HCl concentration, 1.2 M). The mixture (in a 100-ml round-bottomed bottle) was refluxed for 2 h at 85°C. The extract was allowed to cool and was then filtered. A 20-ml portion of the filtrate was evaporated to dryness using a rotary evaporator and a 35°C water bath. The residue was dissolved in 1.5 ml of methanol and filtered through a 0.45-μm filter that was compatible with organic solvents (cellulose acetate, Lida, USA) prior to injection into the HPLC–ESI-MS or HPLC–DAD system.

For the extraction of flavonoid glycosides, the thawed and homogenized berries (5 g) were diluted with purified water to 25 ml, and 25 ml of methanol were added. The remaining air in the 100-ml bottle was replaced by nitrogen gas. The mixture was shaken at room temperature (+21°C) for 2 h. The extract was centrifuged (3000 *g*, 10 min, +4°C) and 20 ml of the supernatant were evaporated to dryness. The sample was dissolved in 1.5 ml of methanol and filtered as described above.

2.4. Chromatographic systems for the HPLC–ESI-MS and HPLC–DAD

In both HPLC–MS and HPLC–DAD analyses, a LiChroCART column (125×3 mm I.D., Purospher RP-18e, 5 μm; generously provided by S. Fuchs, Merck, Finland) protected with a LiChroCART guard column (4×4 mm I.D., Purospher RP-18e, 5 μm) (Merck, Darmstadt, Germany) was used. Solvent A was 1% formic acid and solvent B was acetonitrile. The elution system was: 0–10 min, 10–13% of B in A; 10–25 min, 13–70% of B in A; 25–29 min, 70% of B in A; 29–30 min, 70–10% of B in A; 30–35 min, 10% of B in A. The flow-rate was 0.5 ml/min, and the injection volume was 20 μl for HPLC–MS and 5 μl for HPLC–DAD.

2.5. HPLC–ESI-MS and HPLC–DAD analyses

The system used for HPLC–MS analysis was a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with a Rheos 400 HPLC pump (Danderyd, Sweden). The ionization parameters were optimized using constant infusion of rutin to the ion source. The heated capillary and voltage were maintained at 225°C and 4.5 kV, respectively. The full scan mass spectra of the flavonols from m/z 200 to 1000 were measured using 500 ms for collection of the ions in the trap; two micro scans were summed. Tandem mass spectrometry was performed using helium as the collision gas, and the collision energy was set at 30%. All mass spectrometry data were acquired in the positive ionization mode. Total ion chromatograms (TICs) were measured and, in addition, the instrument was set to alternatively measure four events: (1) full scan source induced dissociation (SID) was used to screen the samples for kaempferol- (fragment ion at m/z 287), quercetin- (m/z 303) or myricetin (m/z 319)-containing glycosides in the berry samples. (2) MS (full scan) was used to measure the $[M+H]^+$ ions, revealing the molecular masses of the components, (3) MS–MS was used to break down the most abundant $[M+H]^+$ ion from MS with dependent collision-induced dissociation (CID) and (4) MS^3 was used to break down the most abundant fragment ion from MS–MS with CID.

The HPLC system for UV–Vis analyses (HPLC–DAD) was a Hewlett-Packard (Waldbronn Analytical Division, Germany) instrument with a quaternary pump (HP 1050), an autosampler (HP 1050) and a photo-diode array detector (HP 1040M). Retention times and UV–Vis spectra of the flavonoids in berry samples were compared with those of aglycone

standards. Spectra were recorded up-slope, apex and downslope (220–450, 2 nm steps). The spectra of each peak were superimposed after subtraction of the corresponding base spectrum. Peaks were considered to be pure when there was a correspondence of >900 among the spectra.

2.6. Sample preparation for GC–MS

Flavonol glycosides from chokeberry, rowanberry, lingonberry and sea buckthorn berry were fractionated. The berry samples were extracted as described in Section 2.3 and 20 μl of the extract were injected into the HPLC–ESI-MS system. From the 500 μl/min flow, 90 μl were directed into the mass spectrometer and 410 μl were collected when the desired ion was detected (m/z 300–800). Fractions from two runs were combined and the eluent was evaporated to dryness in an 80°C water bath under nitrogen. Methanol (50 μl), deionized water (90 μl) and 6 M HCl (10 μl) were added to the residue (final concentrations, 0.4 M HCl in 33% methanol) and the mixture was hydrolyzed for 30 min in an 80°C heat block. The hydrolyzed solution was evaporated to dryness in an 80°C heat block under nitrogen and the residue was kept in the evaporator overnight. Derivatization of the sugars in the residue was performed with 30 μl of BSTFA and 30 μl of freshly distilled pyridine by incubating for 2 h in an 80°C heat block.

2.7. Identification of the derivatized sugars in GC–MS

The derivatized sugars were identified in a GC–MS system using an HP 5890 gas chromatograph (Hewlett-Packard, Germany) equipped with an HP 7673 autosampler and a HP-5-column (0.11 μm, 25 m×0.20 mm I.D., 5% phenylmethylsilicone). A VG Masslab TRIO-2 mass spectrometer (Manchester, UK) was used. Operating conditions were as follows: injection port, 260°C; column, 0.5 min at 100°C, then 8.0°C/min to 280°C where the temperature was kept for 5 min. The total run time was 30 min. Helium was used as the carrier gas. The volume of injected sample was 1 μl. The derivatized sugars were ionized using electron ionization (ionization energy, 18 eV; temperature of ion source, 200°C). The positive ions formed were analyzed using a quadrupole mass analyzer (scan range, 50–800 m/z; scan time, 900 ms). The chromatograms were compared with those of sugar standards. Identification was based on retention times in GC–MS and on comparison with the mass spectra of monosaccharide standards.

3. Results and discussion

3.1. Identification of the flavonoid aglycones

In Fig. 2A and B, an HPLC–DAD chromatogram and an extracted ion chromatogram for the ion m/z 303 (quercetin) in sea buckthorn berry sample are shown. Quercetin eluted at 19.9 min in the HPLC–DAD system (Fig. 2A) and at 18.3 min in the HPLC–MS system (Fig. 2B). An MS spectrum (Fig. 2C) of m/z 303.3 ion resulted in a fragmentation spectrum MS–MS (Fig. 2D) in which the main ions matched with the fragmentation spectrum of quercetin. Using these techniques, it was possible to identify flavonol aglycones in 11 berries (Table 1). Quercetin aglycone was identified with both ESI-MS and DAD in all of the berries studied. Myricetin was identified with both techniques in cranberry, crowberry and blackcurrant. These identification techniques have also been used more recently in the analysis of the flavonol content in Finnish berries [20].

The signal-to-noise ratio was six when the quercetin content in methanol was 40 ng/injection (2 μg/ml) and 30 when the injected quercetin content was 200 ng. In hydrolyzed red raspberry samples, the injected quercetin content was approx. 150 ng; the quercetin ion could be fragmented and compared to the MS–MS results for the quercetin standard. For strawberry, in which the quercetin content is about the same as in red raspberry, the analysis of fragmented quercetin ion was not successful because of a stronger background noise.

3.2. Identification of flavonol glycosides from berries

TIC and SID chromatograms, as well as MS, MS–MS and MS^3 spectra, were used to identify a diglycoside in the unhydrolyzed chokeberry sample

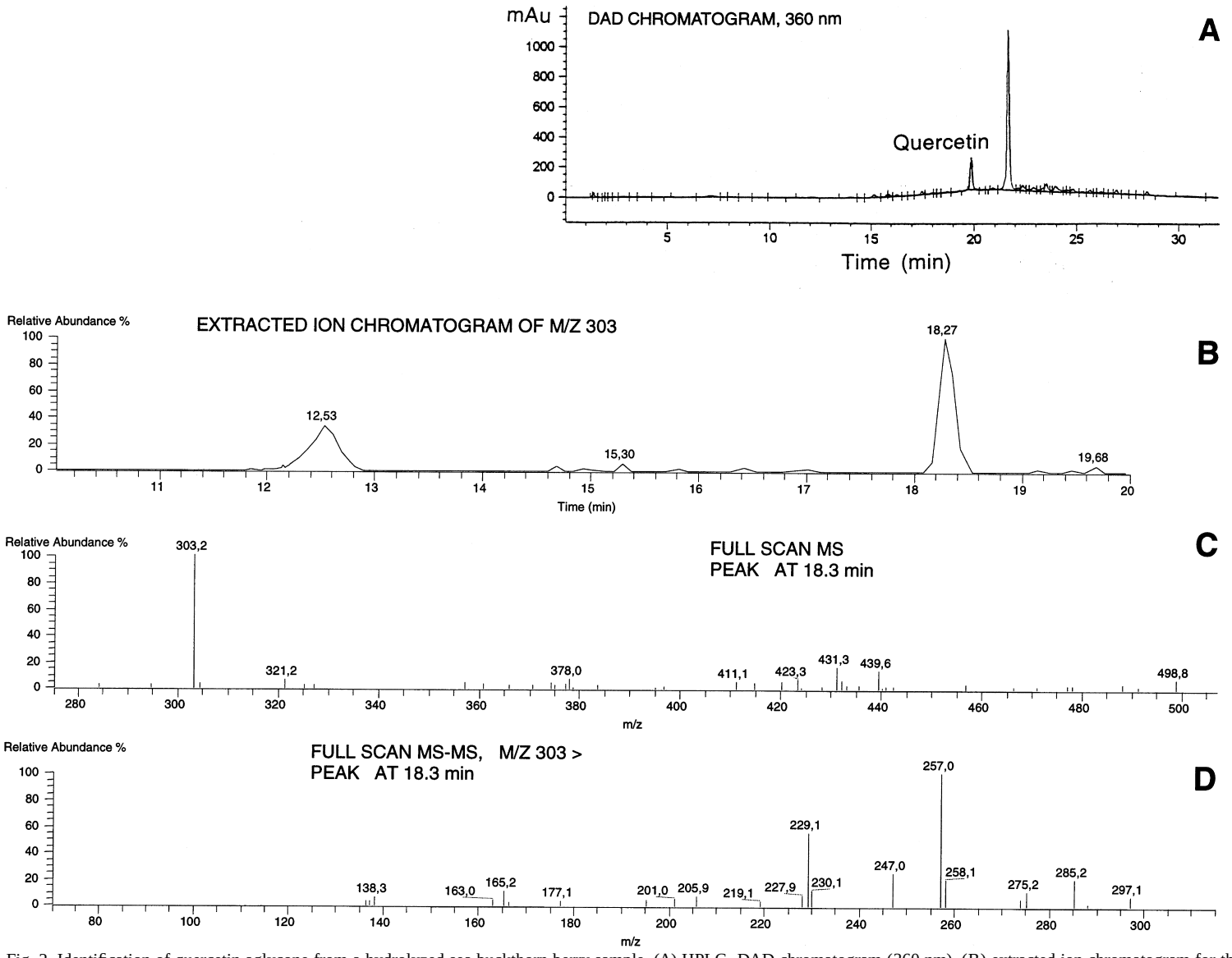

Fig. 2. Identification of quercetin aglycone from a hydrolyzed sea buckthorn berry sample. (A) HPLC–DAD chromatogram (360 nm), (B) extracted ion chromatogram for the quercetin ion (m/z 303), (C) full scan MS spectrum of the peak at 18.3 min, (D) fragmentation spectrum (MS–MS) of the m/z 303.2 ion.

Table 1
Flavonol aglycones identified in hydrolyzed berry extracts based on m/z values of $[M+H]^+$, MS–MS match with the aglycone standard MS–MS and peak purity match in DAD

Berry	Retention time (min)	$[M+H]^+$ in MS	MS–MS match with the aglycone standard MS–MS	Flavonol aglycone	DAD, peak purity factor >900
Bog whortleberry	16.9	319	x	M	
	17.9	303	x	Q	x
	19.4	287		K	
Chokeberry	15.9	319		M	
	17.8	303	x	Q	x
Rowanberry	17.7	303	x	Q	x
	19.7	287		K	
Cranberry	16.1	319	x	M	x
	17.7	303	x	Q	x
Strawberry	18.3	303		Q	x
	19.9	287		K	x
Crowberry	16.6	319	x	M	x
	18.3	303	x	Q	x
Lingonberry	18.3	303	x	Q	x
Bilberry	16.5	319	x	M	
	18.3	303	x	Q	x
Blackcurrant	16.7	319	x	M	x
	18.4	303	x	Q	x
Sea buckthorn berry	18.3	303	x	Q	x
	19.9	287	x	K	
Red raspberry	18.3	303	x	Q	x

M, myricetin; Q, quercetin; K, kaempferol.

(Fig. 3). SID-generated ion chromatograms of aglycone ions were used for screening flavonols (e.g. quercetin, m/z 303.3) in the HPLC–MS chromatogram (Fig. 3B). From the MS results (Fig. 3C), it appears that the compound having a retention time of 14.1 min has an $[M+H]^+$ ion at 611. The MS–MS spectrum of the $[M+H]^+$ ion of the diglycoside shows an ion at 465 (Fig. 3D), which indicates a loss of a 146 mass unit (u) ion and also exhibits an ion of m/z 303.3. The loss of 146 ion is often indicative of a deoxyhexose sugar. A difference between the two masses of 465 and 303 (i.e. 162 u) suggests a further loss of a hexose sugar. The m/z 303.3 ion is indicative of the base component, quercetin. The MS^3 spectrum of the m/z 303.3 ion (Fig. 3E) resulted in a fragmentation spectrum in which the main ions matched with the fragmentation spectrum of quercetin. Using these techniques, we identified several glycosides in berries (Table 2) without time-consuming pre-purification steps or optimization of chromatographic procedures. Hexoses, deoxyhexose–hexoses and pentoses of quercetin (Q) were the most abundant glycosides identified. Hexose and deoxyhexose–hexose of myricetin (M) and deoxyhexose–hexose of kaempferol (K) were identified in black-currant. To confirm the data of the HPLC–ESI-MS procedure, fractions of the glycosides from four berries (lingonberry, rowanberry, sea buckthorn berry, chokeberry) were separated, hydrolyzed, silylated and analyzed with GC–MS in order to identify the sugar moieties of the flavonol glycosides. Although the mass spectra of different sugars were very similar, the sugar peaks (two peaks per sugar) could be identified based on their retention times when compared to those of standard sugars. In Table 3, the retention times of the standard sugars and the fractionated berry flavonol sugars are presented.

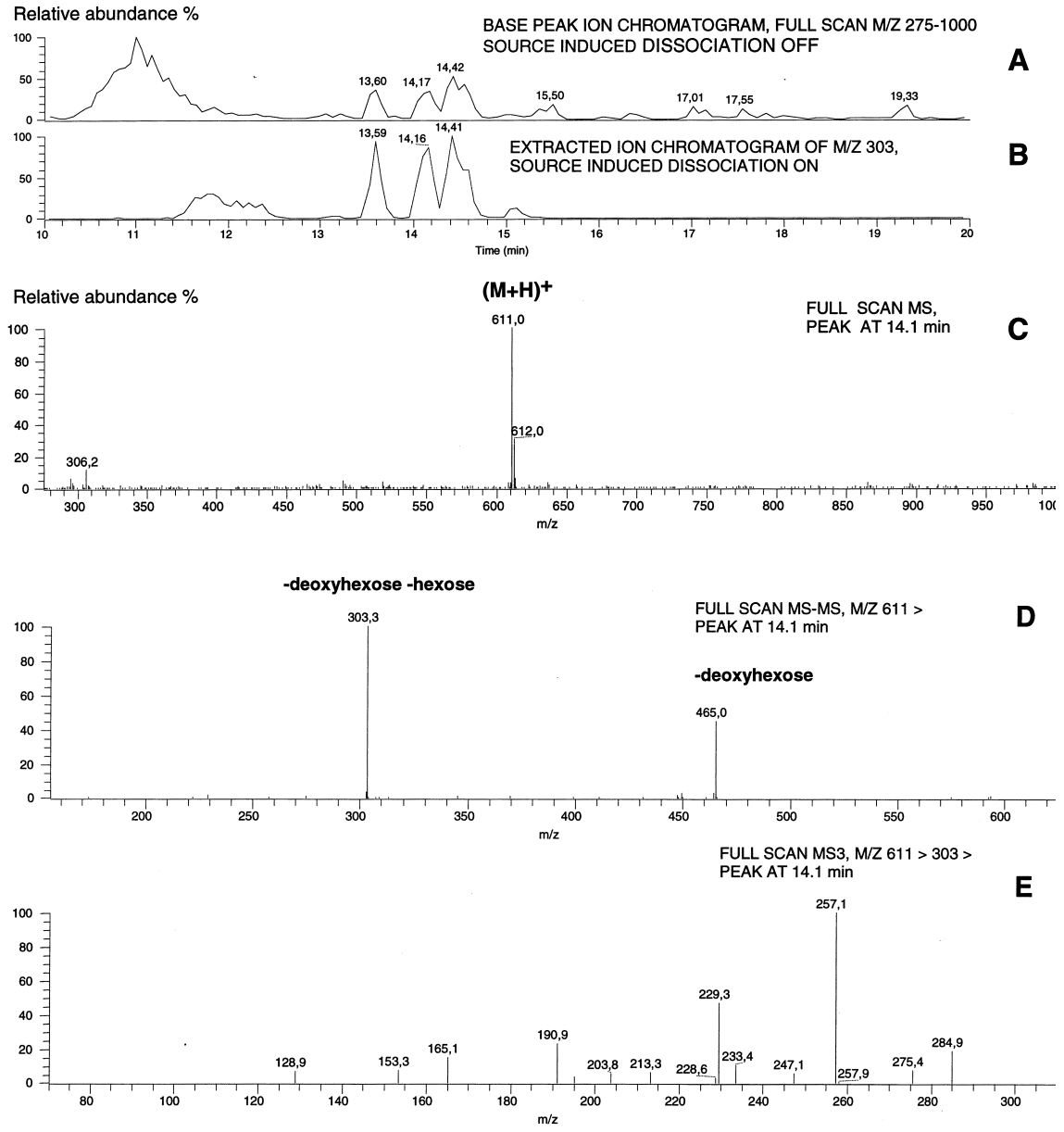

Fig. 3. Identification of a flavonoid diglycoside from unhydrolyzed chokeberry sample. (A) Positive total ion chromatogram, (B) ion source collision-induced dissociation chromatogram for the ion m/z 303 (quercetin), (C) full scan MS spectrum of the peak at 14.1 min, (D) full scan MS–MS spectrum of the $[M+H]^+$ ion, (E) fragmentation spectrum (MS^3) of the m/z 303.3 ion.

From lingonberry, we identified Q-hexose, Q-pentose and Q-deoxyhexose with ESI-MS; according to Kühnau [21], the sugars are galactose, arabinose and rhamnose, respectively. In our GC–MS analysis of sugars, we identified both Q-glucoside and Q-galactoside from lingonberry extract (Table 3). M-3-Rutinoside, M-3-glucoside, Q-3-rutinoside and K-3-rutinoside have been identified in blackcurrant [22]; this is in agreement with our studies on flavonol glycosides in blackcurrant (M-deoxyhexose–hexose,

Table 2
m/z values of $[M+H]^+$ ions (MS) of flavonol glycosides, major fragment ions (MS–MS), the match of the MS^3 with the aglycone standard MS–MS and tentative identification of the flavonol glycosides using ESI-MS of the unhydrolyzed berry extracts

Berry	Retention time (min)	$[M+H]^+$ in MS	Y_1^+ in MS–MS	Y_0^+ in MS–MS	MS^3 match with aglycone standard MS–MS	Tentative structure
Bog whortleberry	5.2	435	303		x	Q-pentose
	14.4	465	303		x	Q-hexose
	15.0	435	303		x	Q-pentose
	17.9	303			x	Q
Chokeberry	14.1	611	465	303	x	Q-deoxyhexose+hexose
	14.4	465	303		x	Q-hexose
	15.5	597	465	303	x	Q-hexose+pentose
Rowanberry	10.5	627	465	303	x	Q-hexose+hexose
	14.1	611	465	303	x	Q-deoxyhexose+hexose
	14.4	465	303		x	Q-hexose
	14.9	595	449	287	x	K-hexose+pentose
Cranberry	14.3	465	303		x	Q-hexose
	15.0	435	303		x	Q-pentose
	15.4	449	303		x	Q-deoxyhexose
	17.8	569	303		x	Q-pentose+pentose
Crowberry	14.9	465	303		x	Q-hexose
	16.6	319			x	M
Lingonberry	14.7	611	465	303	x	Q-hexose+deoxyhexose
	14.5	597				Q-hexose+pentose
	14.9	465	303		x	Q-hexose
	15.4	435	303		x	Q-pentose
	15.8	449	303		x	Q-deoxyhexose
Blackcurrant	13.3	627	465	319	x	M-deoxyhexose+hexose
	13.8	481	319		x	M-hexose
	14.5–15.0	611	465	303	x	Q-deoxyhexose+hexose[a]
	15.2	611	303		x	Q-deoxyhexose+hexose[a]
	15.7	595	287		x	K-deoxyhexose+hexose
Sea buckthornberry	12.3	611	449	303	x	Q-deoxyhexose+hexose[b]
	14.3	611	464	303	x	Q-hexose+deoxyhexose[b]
Bilberry	15.0	479	303		x	Q-hexose+CH_3

Q, quercetin; K, kaempferol; M, myricetin.
[a] There are two diglycosides with m/z 611 in blackcurrant; they have different retention times in HPLC and different MS–MS spectra. In the faster-eluting diglycoside, the sugars are fragmented separately. In the more slowly eluting glycoside, the fragmented sugars are linked together.
[b] There are two diglycosides with m/z 611 in sea buckthorn berry; they have different retention times in HPLC and different MS–MS spectra. In the faster-eluting diglycoside, 449 is the strongest ion in MS–MS, indicating the initial loss of a hexose sugar (162). In the more slowly eluting diglycoside, 464 is the strongest ion in the MS–MS, indicating the initial loss of deoxyhexose sugar (146).

M-hexose, Q-deoxyhexose–hexose and K-deoxyhexose–hexose, respectively). Q-Hexose and Q-deoxyhexose–hexose were identified from rowanberry by HPLC–ESI-MS; this is in agreement with an earlier study where Q-3-glucoside and rutinoside have been identified from rowanberry [21]. The structure of Q-rutinoside was confirmed by our GC–MS method (Table 3). We also identified, from rowanberry, Q-dihexose in which both sugar moieties were glucoses, acccording to GC–MS. On the other hand, we did not identify Q-3-rhamnoside [21] from rowanberry. Two deoxyhexose–hexoses (m/z 611) of quercetin were identified from sea buckthorn berry using HPLC–ESI-MS (retention times, 12.3 and 14.3

Table 3
Retention times of the sugar standards and sugars of fractionated flavonol glycosides from berries, as analyzed with GC–MS

	Retention time, peak 1 (min)	Retention time, peak 2 (min)	Sugar structure of the sample ion
Sugar standards			
Arabinose	9.97	10.43	
Rhamnose	10.17	11.08	
Glucose	13.80	14.97	
Galactose	13.38	13.95	
Flavonol glycoside fractions from berries			
m/z 465 (15.5 min) in lingonberry	13.37	13.93	Galactose
	13.78	14.93	Glucose
m/z 611 (13.5 min) in sea buckthorn berry	10.17	11.08	Rhamnose
	13.80	14.95	Glucose
m/z 611 (14.8 min) in sea buckthorn berry	10.17	11.08	Rhamnose
	13.80	14.95	Glucose
m/z 611 (14.6 min) in rowanberry	10.17	11.08	Rhamnose
	13.80	14.95	Glucose
	13.38	13.95	Galactose
m/z 627 (12.9 min) in rowanberry	13.80	14.95	Glucose
m/z 597 (14.2 min) in chokeberry	9.78	10.43	Arabinose
	13.80	14.97	Glucose
m/z 611 (14.8 min) in chokeberry	10.17	11.09	Rhamnose
	13.38	13.95	Galactose
	13.80	14.95	Glucose

min). In the GC–MS analysis of both fractions of m/z 611, glucose and rhamnose were identified. In agreement with our studies, an aglycone of quercetin in addition to Q-3-rutinoside has been identified from sea buckthorn berry [21]. We found Q-hexose, Q-pentose, Q-deoxyhexose and Q-dipentose in our ESI-MS study on cranberry *Vaccinium oxycoccos*. Kühnau [21] identified Q-3-galactoside, Q-3-arabinoside and Q-3-rhamnoside in cranberry *Vaccinium macrocarpum*. To our knowledge, no results of the flavonol glycosides of chokeberry, crowberry and bog whortleberry have been published previously. In chokeberry, we identified Q-deoxyhexose–hexose (m/z 611), Q-hexose–pentose (m/z 597) and Q-hexose (m/z 465) using HPLC–ESI-MS. With GC–MS, we identified rhamnose and glucose in fraction m/z 611 and glucose and arabinose in fraction m/z 597 (Table 3). These are in good accordance with our HPLC–ESI-MS results.

4. Conclusions

HPLC–ESI-MS is of high value in the identification of flavonol aglycones and glycosides from berries. This ionization technique provides information on molecular ions and their fragmentation spectra when compared to corresponding standards. Valuable information can also be obtained about the structure of the glycosides. Moreover, photodiode-array detection can be performed under the same conditions, thus providing UV–Vis spectra with confirmatory data.

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