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Detection of Isorhamnetin Glycosides in Extracts of Apples (*Malus domestica* cv. "Brettacher") by HPLC-PDA and HPLC-APCI-MS/MS

Andreas Schieber,[1]* Petra Keller,[1] Petra Streker,[1] Iris Klaiber[2] and Reinhold Carle[1]

[1]Hohenheim University, Institute of Food Technology, Section Plant Foodstuff Technology, Garbenstrasse 25, D-70599 Stuttgart, Germany
[2]Hohenheim University, Institute of Chemistry, Section Bioorganic Chemistry, Garbenstrasse 30, D-70599 Stuttgart, Germany

Extracts of apple fruits (*Malus domestica* cv. "Brettacher") were analysed by HPLC with photodiode array detection. An unknown peak was monitored displaying the same retention time as isorhamnetin 3-*O*-glucoside. Preliminary identification of the isorhamnetin aglycone was performed by comparison of UV spectral data of the unknown compound with a reference substance. Using atmospheric pressure chemical ionisation mass spectrometry in the negative ion mode, the presence of an isorhamnetin glycoside was supported by loss of 162 amu from the pseudomolecular ion (*m/z* 477). MS^2 product ion analysis of the parent ion *m/z* 477 provided a fragmentation pattern identical to the reference. Collision-induced dissociation of the aglycone (*m/z* 315) in the MS^3 product ion analysis allowed the differentiation of rhamnetin and isorhamnetin, and unambiguous assignment by comparison with standard compounds. A second isorhamnetin glycoside eluting prior to the glucoside was tentatively identified as isorhamnetin 3-*O*-galactoside. To the best of our knowledge, this is the first report of isorhamnetin glycosides in apple fruit extracts. Results are discussed with respect to chemotaxonomic relevance within the genera *Malus* and *Pyrus*, and especially in consideration of the control of the authenticity of apple products. **Copyright © 2002 John Wiley & Sons, Ltd.**

Keywords: HPLC-MS; authenticity control; flavonoids; isorhamnetin; *Malus domestica* cv. 'Brettacher'; *Pyrus communis.*

INTRODUCTION

Phenolic compounds have been widely recognised as indicators of adulteration of fruit juices (Fernández de Simón *et al.*, 1992; Wald and Galensa, 1989), jellies (Silva *et al.*, 2000), jams (García-Viguera *et al.*, 1993, 1997), and puree (Andrade *et al.*, 1998). It is generally accepted that the dihydrochalcone derivatives phloridzin (phloretin 2′-*O*-*β*-D-glucoside) and phloretin 2′-*O*-(6″-*β*-D-xylosyl)-*β*-D-glucoside are characteristic constituents of apples (*Malus domestica*; Spanos *et al.*, 1990; Spanos and Wrolstad, 1992; Tomás-Barberán *et al.*, 1993). In the case of pear (*Pyrus communis*), however, there is still disagreement as to which phenolics should be taken as markers. Whilst isorhamnetin 3-*O*-glucoside has been described to be indicative of pear (Wald and Galensa, 1989; Fernández de Simón *et al.*, 1992), this flavonol glycoside has not been detected by Andrade *et al.* (1998). Hofsommer and Koswig (1999) suggested that arbutin (hydrochinone *O*-*β*-D-glucoside) might be used as a marker to detect adulterations of apple juice with pear juice, however, without considering isorhamnetin 3-*O*-glucoside.

Recently, a method for the determination of the phenolic compounds of apples and pears has been developed using a stationary phase with hydrophilic endcapping (Schieber *et al.*, 2001). Since excellent resolution, especially of flavonol glycosides and dihydrochalcones, has been obtained, this analytical system has also been applied to the control of the authenticity of apple juices and pear juices. Investigations of the phenolic composition of a number of pear cultivars revealed that isorhamnetin 3-*O*-glucoside was omnipresent, whereas arbutin could not be detected in all cultivars examined.

Whole apple extracts have been shown to inhibit the growth of colon- and liver-cancer cells *in vitro* in a dose-dependent manner. It has been suggested that this strong inhibition of tumour-cell proliferation could be due to phenolic acids and flavonoids (Eberhardt *et al.*, 2000). Therefore, a screening of phenolic compounds of 19 apple cultivars grown in Southern Germany has been carried out using a new HPLC method (Keller *et al.*, 2001; Schieber *et al.*, 2001). In this paper, the presence of isorhamnetin glycosides in extracts of apples cv. 'Brettacher' is reported for the first time.

EXPERIMENTAL

Standards. Standards used for identification purposes with HPLC and MS were purchased from Roth (Karlsruhe, Germany; quercetin 3-*O*-rutinoside, querce-

* Correspondence to: A. Schieber, Hohenheim University, Institute of Food Technology, Section Plant Foodstuff Technology, Garbenstrasse 25, D-70599 Stuttgart, Germany.
Email: schieber@uni-hohenheim.de

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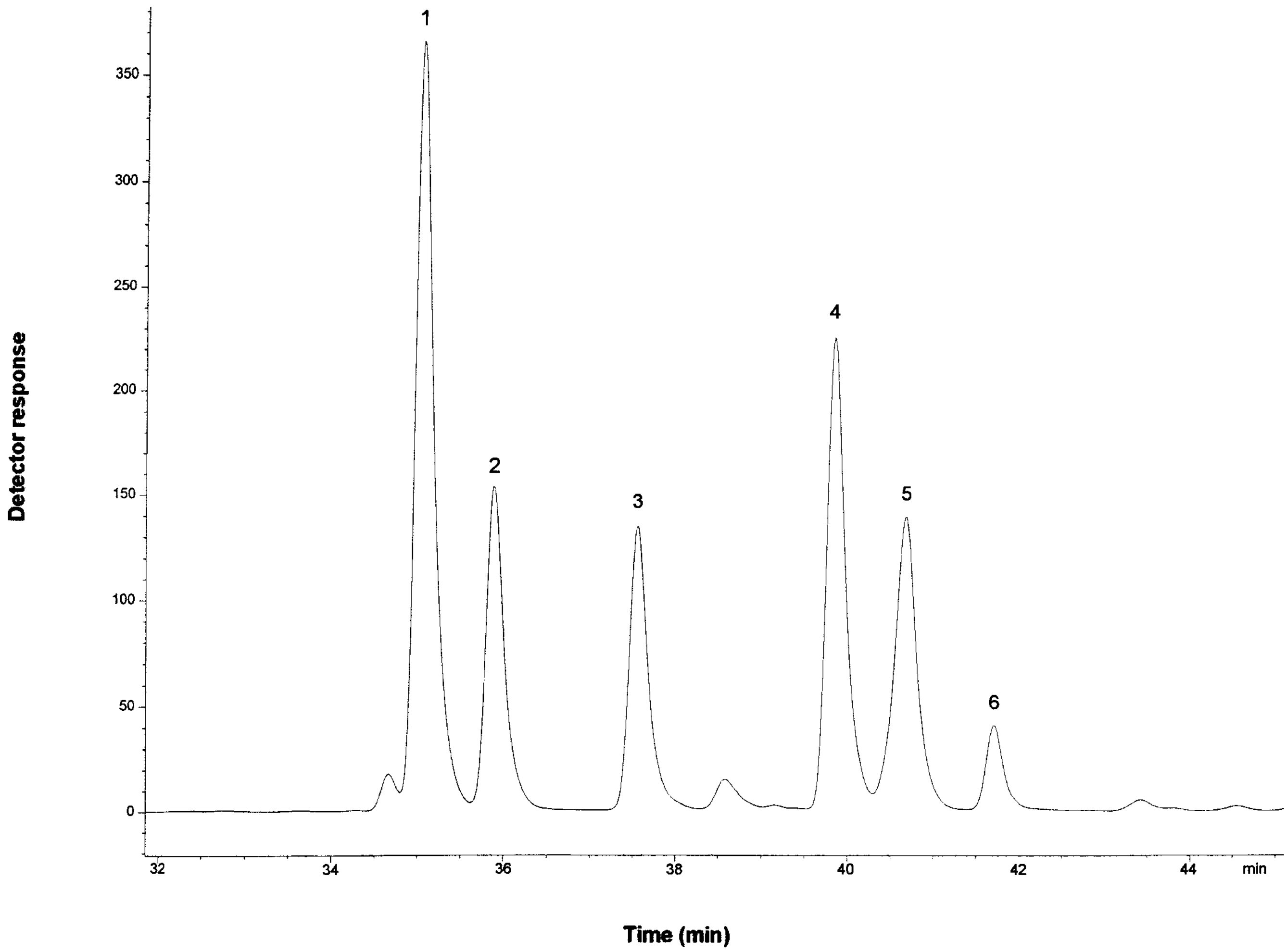

Figure 1. Separation of flavonol glycosides extracted from fruits of the apple cv. "Brettacher". Key to peak identity: (**1**) quercetin 3-*O*-galactoside, (**2**) quercetin 3-*O*-glucoside, (**3**) quercetin 3-*O*-xyloside, (**4**) quercetin 3-*O*-arabinoside, (**5**) quercetin 3-*O*-rhamnoside and isorhamnetin 3-*O*-galactoside, (**6**) isorhamnetin 3-*O*-glucoside (detection at 370 nm; for chromatographic protocol see Experimental section).

tin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside, isorhamnetin 3-*O*-glucoside), from Plantech (Reading, UK; quercetin 3-*O*-xyloside) and from Extrasynthese (Lyon, France; rhamnetin).

Sample preparation. Samples were prepared from "Brettacher" apples, harvested in 2000 and obtained from local producers, by the method described by Schieber *et al.* (2001). Mature apples of four lots were washed and minced. After addition of ascorbic acid (1 g) and sodium chloride (50 mg) to prevent enzymatic browning, amounts of 50 g were homogenised using an Ultraturrax model T25 (Janke and Kunkel, Staufen, Germany) and extracted by stirring with acetone for 1 h at ambient temperature. After centrifugation, acetone was removed *in vacuo* (280 mbar, 30°C). The aqueous solution was adjusted to pH 7.0 and pH 1.5, respectively, and extracted three times with ethyl acetate (50 mL each). After evaporation to dryness, the residues were dissolved in methanol, membrane filtered (0.2 μm), and used for HPLC. In a second set of experiments, peels, flesh and core from five apples were pooled and separately extracted as described above.

HPLC analysis. The separation of phenolic compounds was performed, according to the method of Schieber *et al.* (2001), using a Hewlett Packard (Waldbronn, Germany) HPLC series 1100 chromatograph equipped with ChemStation software, a degasser model G1322A, a binary gradient pump model G1312A, a thermo-autosampler model G1329/1330A, a column oven model G1316A, and a photodiode array detector (PDA) model G1315A. The column used was a Phenomenex (Torrance, CA, USA) C_{18} Aqua (250 × 4.6 mm i.d.; 5 μm particle size), with a C_{18} ODS guard column (4.0 × 3.0 mm i.d.), operated at a temperature of 25°C. The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and of 0.5% acetic acid in water and acetonitrile (50:50, v/v; eluent B). The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min) at a flow rate of 1 mL/min. The injection volume for all samples was 10 μL. Simultaneous monitoring was performed at 280 nm (dihydrochalcones, catechins, proanthocyanidins, benzoic acids), 320 nm (hydroxycinnamic acids) and 370 nm (flavonols). Spectra were recorded from 200 to 600 nm (peak width 0.2 min; data rate 1.25/s).

HPLC-MS analyses. Analyses were performed with the same stationary phase and eluents as described above. The system consisted of an HPLC Pro Star pump (Varian,

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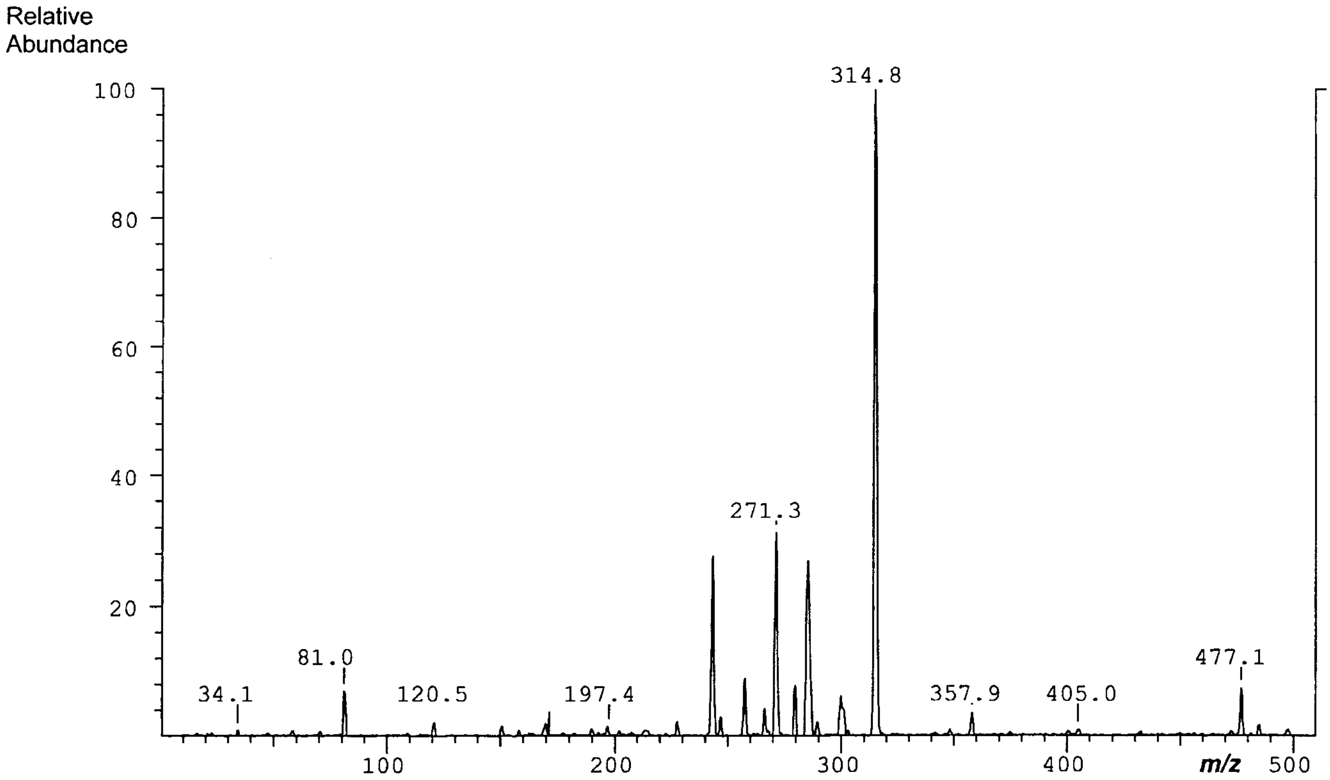

Figure 2. APCI-MS/MS (negative ion mode) of isorhamnetin 3-*O*-glucoside (peak 6 in Fig. 1).

Darmstadt, Germany) and an Applied Biosystems (Weiterstadt, Germany) UV detector model ABI 785 connected in series with a Finnigan (Bremen, Germany) model TSQ 700 mass spectrometer fitted with an APCI source. Negative ion MS (in the range *m/z* 10–800) of the column eluate were recorded. The temperature of the capillary was set to 200°C and that of the vaporiser was maintained at 450°C. Nitrogen was used as the sheath gas at a pressure of 4.76 atm. Collision-induced dissociation spectra were obtained at 20 eV using xenon as the collision gas (1.2×10^{-6} atm). Isorhamnetin 3-*O*-glucoside was used for the optimisation of ionisation parameters.

RESULTS AND DISCUSSION

The separation of flavonol glycosides in a "Brettacher" apple extract is presented in Fig. 1. As can be seen, the analytical method allowed the determination of five quercetin glycosides, four of which could be readily identified as quercetin 3-galactoside (peak **1**), quercetin 3-glucoside (peak **2**), quercetin 3-xyloside (peak **3**), and quercetin 3-rhamnoside (peak **5**) by comparison with standards. The fifth glycoside (peak **4**) showed a spectral maximum of 355 nm and an *m/z* of 433 and was therefore assigned to quercetin 3-arabinoside (see below). An unknown compound (peak **6**) eluting after quercetin 3-rhamnoside displayed the characteristic UV spectrum of an isorhamnetin glycoside. Co-injection of isorhamnetin 3-*O*-glucoside revealed identical retention times, therefore, the presence of this glycoside in the apple extracts was assumed.

In order to confirm this assumption, the extract was analysed by HPLC-APCI-MS. Negative ion APCI-MS provided a pseudomolecular ion of *m/z* 477 $[M-H]^-$ and a second fragment of *m/z* 315 evidently produced by the loss of a hexose moiety $[M-162-H]^-$. Collision-induced

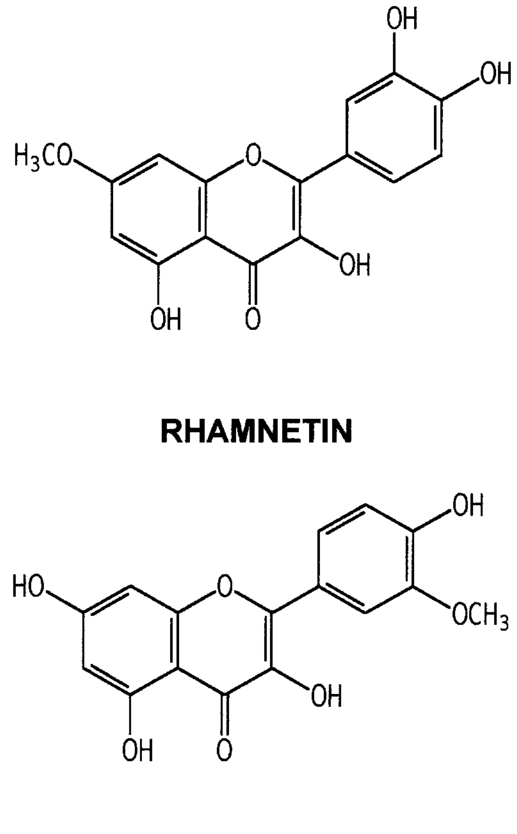

Figure 3. Structure of rhamnetin and isorhamnetin.

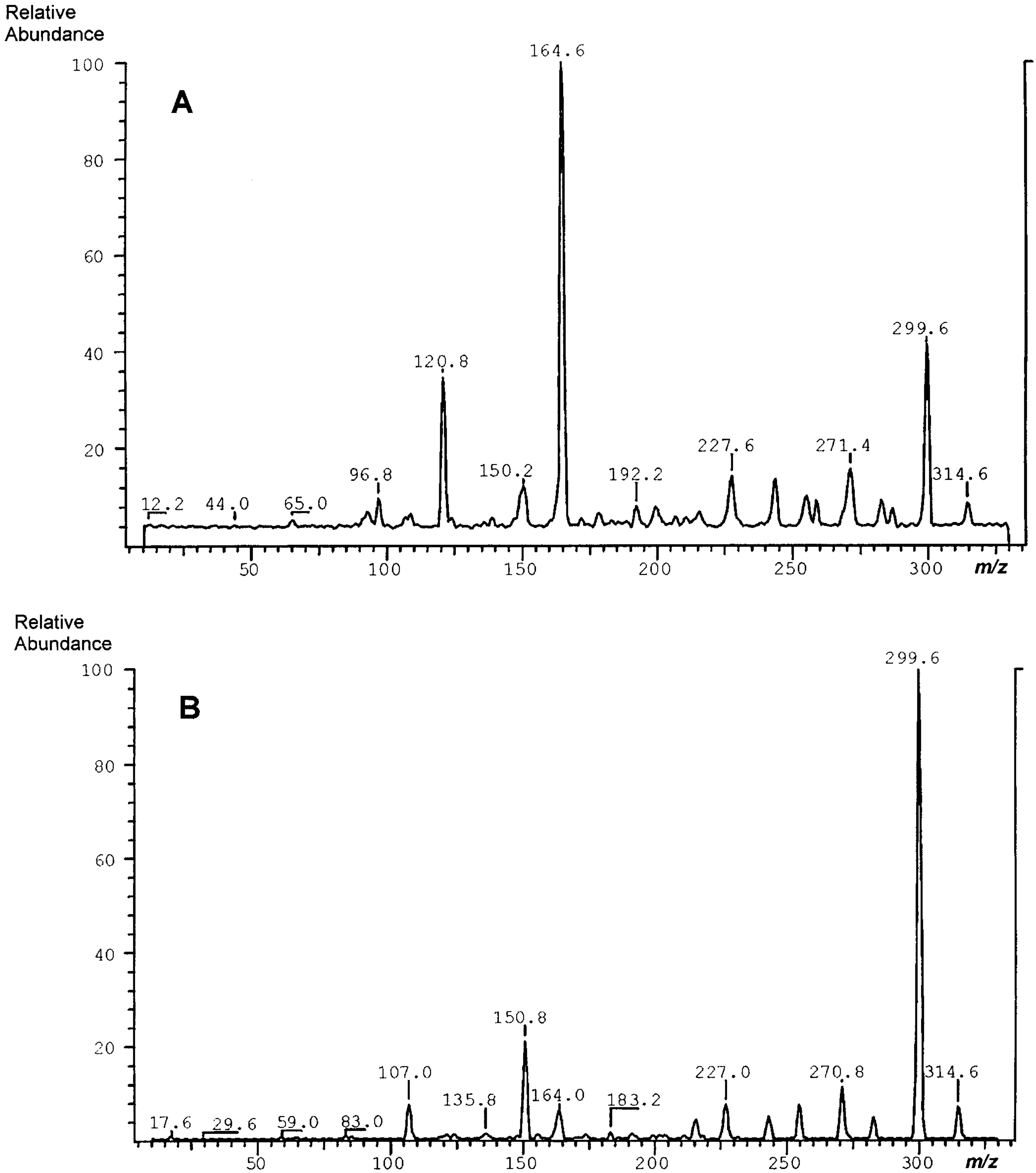

Figure 4. See opposite for caption.

dissociation of the pseudomolecular ion caused a characteristic fragment ion of the aglycone with *m/z* 315 which was assigned to isorhamnetin (Fig. 2) since analysis of the reference compound provided identical fragmentation.

Isorhamnetin and rhamnetin (Fig. 3) could be differentiated by performing MS^2 and MS^3 product ion analysis on the standard compounds isorhamnetin 3-*O*-glucoside and rhamnetin, respectively. As can be seen from Fig. 4(A) and (B), collision-induced dissociation of the aglycone yielded ions at *m/z* 300 which were evidently produced by the loss of the methyl group. In the case of isorhamnetin, this was the most prominent fragment, while rhamnetin yielded an intense fragment at *m/z* 165. MS^3 product ion analysis of peak **5** [Fig. 4(C)] and peak **6** [Fig. 4(D)] revealed that these compounds were isorhamnetin glycosides. Based on retention time, UV spectra, and MS analyses, the presence of isorhamnetin 3-*O*-glucoside in extracts of "Brettacher" apples could thus be confirmed unambiguously.

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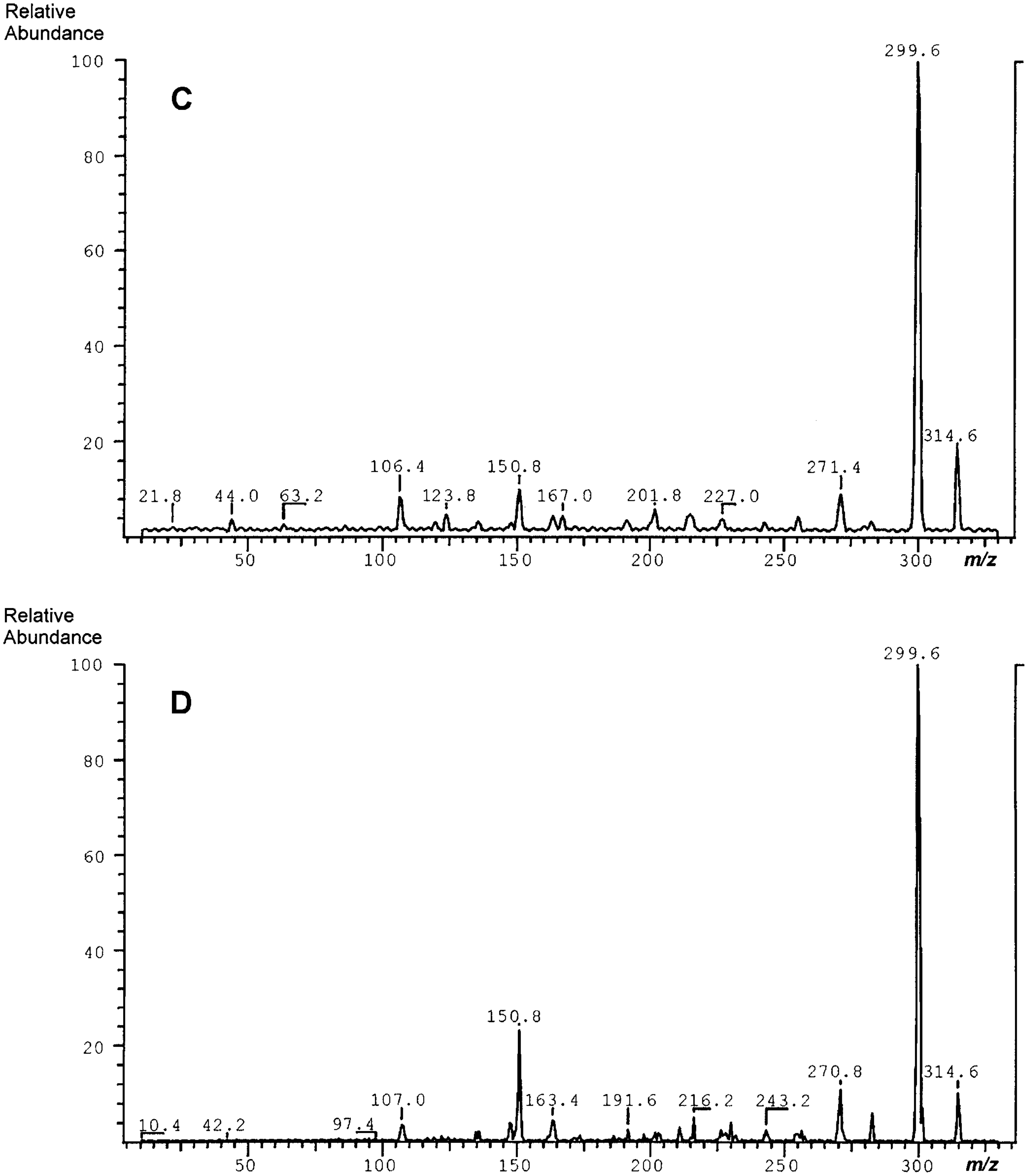

Figure 4. (A) MS^2 (m/z 315) product ion analysis of rhamnetin, and (B) MS^3 (m/z 477 $\rightarrow$ 315) product ion analysis of isorhamnetin 3-*O*-glucoside. The corresponding MS^3 product ion spectra of (C) peak **5** (Fig. 1), and (D) peak **6** (Fig. 1) are shown for comparison.

The second isorhamnetin glycoside co-eluted with quercetin 3-rhamnoside and could therefore only be detected by tandem MS (Fig. 5). Since the galactoside of a given flavonol aglycone elutes prior to the glucoside (Schieber *et al.*, 2001), this compound was tentatively assigned as isorhamnetin 3-*O*-galactoside. The identity of the quercetin aglycone could be confirmed by performing MS^2 and MS^3 product ion analysis. However, no major differences in the fragmentation of the quercetin glycosides were observed. UV spectral data and characteristic ions of flavonoids are shown in Table 1.

Quantification was based on external standard calibration curves. It was found that the amounts of isorhamnetin 3-*O*-glucoside ranged from 2.4 to 4.8 mg/kg fresh weight. In order to determine the origin of the isorhamnetin glycosides, a differential extraction of peels, flesh and core was performed. Peels were found to contain 10.4 mg/kg isorhamnetin 3-*O*-glucoside on a

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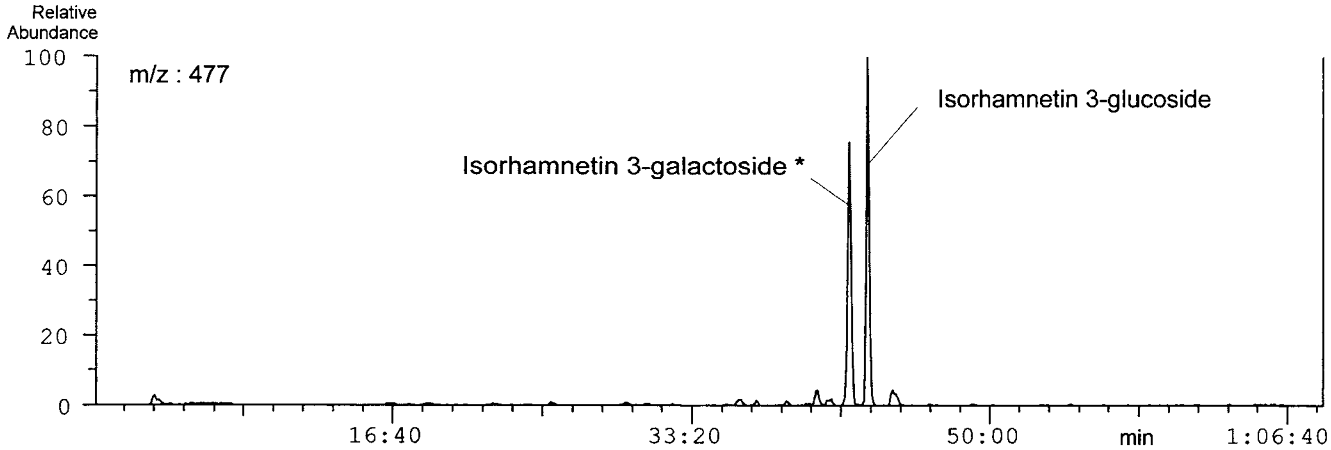

Figure 5. HPLC-APCI-MS analysis (in the negative ion mode) of flavonol glycosides of "Brettacher" apples (extracted trace of *m/z* 477) displaying isorhamnetin 3-*O*-galactoside (tentatively identified) and isorhamnetin 3-*O*-glucoside.

fresh weight basis, while the compound could not be detected in the flesh; only trace amounts were found in the core.

Esters of caffeic and *p*-coumaric acids together with quinic acid, catechins, procyanidins, and quercetin and dihydrochalcone glycosides, respectively, are the predominant phenolic compounds of apples (Spanos *et al.*, 1990; Spanos and Wrolstad, 1992; Tomás-Barberán *et al.*, 1993). Chlorogenic, caffeic, *p*-coumaroyl quinic, and *p*-coumaric acids as well as quercetin and isorhamnetin glycosides and procyanidins have been found in pear fruits (Spanos and Wrolstad, 1992; Oleszek *et al.*, 1994). According to Spanos and Wrolstad (1992), the major differences between the phenolic profiles of pear and apple fruit are the presence of arbutin and the lack of phloretin derivatives in pears. Although a large number of studies on the phenolic composition of apple cultivars has been conducted (e.g. Burda *et al.*, 1990; Delage *et al.*, 1991; Pérez-Ilzarbe *et al.*, 1991; Picinelli *et al.*, 1997; Escarpa and González, 1998; Price *et al.*, 1999; Awad *et al.*, 2000; Podsedek *et al.*, 2000), isorhamnetin glycosides have never been found in apple fruits. Therefore, the presence of isorhamnetin 3-*O*-glucoside has been taken as a marker for the detection of pear juice in admixture with apple juice (Wald and Galensa, 1989; Fernández de Simón *et al.*, 1992). Our recent investigations on the phenolic composition of apple and pear fruits revealed that arbutin could not be detected in all pear fruits examined, whereas isorhamnetin 3-*O*-glucoside was present in all cultivars, in even higher amounts than arbutin (Schieber *et al.*, 2001). Therefore, isorhamnetin 3-*O*-glucoside has been suggested as a more suitable marker for the detection of pear juice admixture to apple juices. However, we have now demonstrated for the first time the presence of isorhamnetin glycosides in extracts of apple fruits. These findings are of particular interest with respect to the control of authenticity of apple juices, especially since "Brettacher" apples are used both as a dessert fruit and for juice production (Bitsch *et al.*, 2000), and also since the quantities of isorhamnetin 3-glucoside found in "Brettacher" apples are comparable with those found in pear fruit extracts (Schieber *et al.*, 2001). During the industrial production of apple juice, flavonol glycosides are partially extracted from the peels; therefore, processing of "Brettacher" apples would inevitably lead to the presence of isorhamnetin glycosides in the juice. Furthermore, the possible occurrence of isorhamnetin glycosides in other apple cultivars must also be taken into consideration.

It has been shown that pear fruits contain both quercetin and isorhamnetin glycosides (Nortje and Koeppen, 1965; Duggan, 1969; Wald and Galensa, 1989; Schieber *et al.*, 2001). With respect to the authenticity of apple products, investigations should therefore be extended to isorhamnetin glycosides other than isorhamnetin 3-*O*-glucoside. Isorhamnetin diglycosides have so far not been detected in apples and especially not in cv. "Brettacher". Since acylated isorhamnetin glycosides, which are also found in pear fruits, are prone to degradation during juice processing, they are less suitable for authenticity control (Wald and Galensa, 1989). The method recently established also allows the separation of flavonol diglycosides and is therefore expected to be a helpful tool for authenticity control (Schieber *et al.*, 2001).

The stationary phase with hydrophilic endcapping used in this study was specifically developed for the separation of very polar analytes which are not sufficiently retained on conventional reversed-phase systems. It proved to be excellent for the quantitative determination of phenolic compounds, especially of flavonol glycosides, in extracts of apple pomace and pear fruits, respectively (Schieber *et al.*, 2001). In this study, a quercetin with an attached pentose moiety was detected but could not be assigned to one of the commercial standards. Lommen *et al.* (2000) reported that a commercial reference compound erroneously labelled as avicularin (quercetin 3-*O*-α-arabinofuranoside) was in reality guajaverin (quercetin 3-*O*-α-arabinopyranoside). Since we purchased quercetin 3-*O*-arabinoside (without further specification) from the same source, we conclude that peak **4** should be assigned to avicularin, whereas our standard was also guajaverin.

The successful application of LC-MS to polyphenol analysis has been demonstrated (Wolfender *et al.*, 1994, 1995; Grayer *et al.*, 2000). In the present study, PAD and APCI-MS were employed for the characterisation of flavonol glycosides in extracts of "Brettacher" apples. Since volatile eluents were used, coupling to an MS was possible without changing the chromatographic conditions. Using this methodology, the presence of two

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Table 1. UV spectra and characteristic ions of flavonoids from *Malus domestica* cv. "Brettacher" detected by HPLC-PAD (see Fig. 1; data of selected standard compounds are also included)

Peak	Identity	HPLC-PAD UV spectrum[a] λ_{max} (nm)	$[M-H]^-$ *m/z*	HPLC-APCI-MS MS experiment[b] *m/z* (% base peak)
1	Quercetin 3-*O*-galactoside	257, 267sh, 295sh, 355	463	-MS^2 [463]: 463 (22), 301 (100), 271 (8), 255 (5) -MS^3 [463 → 301]: 301 (76), 273 (15), 151 (59), 121 (100)
2	Quercetin 3-*O*-glucoside	257, 267sh, 295sh, 355	463	-MS^2 [463]: 463 (18), 301 (100), 271 (9), 255 (3) -MS^3 [463 → 301]: 301 (74), 273 (10), 151 (69), 121 (100)
3	Quercetin 3-*O*-xyloside	257, 267sh, 295sh, 355	433	-MS^2 [433]: 433 (13), 301 (100), 271 (13), 255 (8) -MS^3 [433 → 301]: 301 (71), 273 (16), 151 (54), 121 (100)
4	Quercetin 3-*O*-arabinoside[c]	257, 267sh, 295sh, 355	433	-MS^2 [433]: 433 (11), 301 (100), 271 (12), 255 (9)
5	Quercetin 3-*O*-rhamnoside	257, 267sh, 295sh, 355	447	-MS^2 [447]: 447 (24), 301 (100), 271 (17), 255 (19) -MS^3 [447 → 301]: 301 (61), 273 (10), 151 (51), 121 (100)
	Isorhamnetin 3-*O*-galactoside		477	-MS^2 [477]: 477 (18), 315 (100), 300 (16), 285 (24), 271 (21), 243 (29) -MS^3 [477 → 315]: 315 (21), 300 (100), 271 (8), 151 (11), 107 (3)
6	Isorhamnetin 3-*O*-glucoside	255, 267sh, 297sh, 352	477	-MS^2 [477]: 477 (13), 315 (100), 300 (6), 285 (29), 271 (37), 243 (29) -MS^3 [477 → 315]: 315 (8), 300 (100), 271 (11), 151 (23), 107 (4)
Standard	Isorhamnetin 3-*O*-glucoside	255, 267sh, 297sh, 352	477	-MS^2 [477]: 477 (8), 315 (100), 300 (10), 285 (30), 271 (36), 243 (30) -MS^3 [477 → 315]: 315 (7), 300 (100), 271 (11), 151 (18), 107 (9)
Standard	Rhamnetin	nd	315	-MS^2 [315]: 315 (8), 300 (37), 271 (12), 165 (100), 121 (37)

[a] sh = shoulder; nd = not determined.
[b] For mass spectrometric conditions see Experimental section.
[c] Reliable MS^3 data could not be obtained for quercetin 3-*O*-arabinoside owing to insufficient amounts of sample.

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isorhamnetin glycosides in extracts of apple fruits cv. "Brettacher" was demonstrated for the first time. These unexpected findings are of considerable importance not only from a chemotaxonomic point of view, but also with respect to authenticity control of apple-derived food products. Extended screening of the phenolic composition of apples, however, is still necessary in order to clarify whether isorhamnetin glycosides are present in further cultivars. In addition, more data on pear polyphenolics are urgently needed in order to establish reliable markers for authenticity control studies. These investigations are a subject of our current research programme.

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