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Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection

Ulla Justesen*, Pia Knuthsen, Torben Leth

Danish Veterinary and Food Administration, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

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Abstract

A high-performance liquid chromatographic (HPLC) separation method with photo-diode array (PDA) and mass spectrometric (MS) detection was developed to determine and quantify flavonols, flavones, and flavanones in fruits, vegetables and beverages. The compounds were analysed as aglycones, obtained after acid hydrolysis of freeze-dried food material. Identification was based on retention time, UV and mass spectra by comparison with commercial standards, and the UV peak areas were used for quantitation of the flavonoid contents. Examples of HPLC–MS analyses of orange pulp, tomato, and apple are presented. The method has been used to screen foods on the Danish market, and the contents of flavones, flavonols, and flavanones were measured. © 1998 Elsevier Science B.V.

Keywords: Food analysis; Flavonols; Flavones; Flavanones

1. Introduction

The large group of plant polyphenols attracts major interest because of the potential anti-atherogenic properties presumably based on their function as natural antioxidants. Of the plant polyphenols, the flavonoids are of particular interest because of their high prevalence in foods such as fruits, vegetables and tea. In a cohort study performed by Hertog et al. [1], it was found that flavonoid intake was inversely related to mortality from coronary heart disease among middle-aged Dutch men. Another cohort study [2], performed in Finland, also indicated an inverse relationship between flavonoid intake, especially from apples and onions, and coronary heart disease. Although an inverse relationship has also been observed between the intake of fruits and vegetables, and cancers at various sites [3], it has not been possible, in epidemiological studies, to establish correlation of individual flavonoids with decreased cancer risks [4]. Animal and cell culture studies suggest, however, that flavonoids might be protective against cancers at various sites [5].

The number of flavonoids in plants is large, and complexed by the occurrence mostly in O-glycosidic forms with a number of sugars such as glucose, galactose, rhamnose, arabinose, xylose and rutinose.

*Corresponding author.

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Glycosylation increases the polarity of the flavonoids and thereby the water solubility, which is necessary for storage in plant cell vacuoles. The flavonoid functions in plants are believed to be as protective agents against UV radiation and also against microorganisms [6].

The physiological effects in humans are not known, but the theories are based on the function of flavonoids as antioxidants and free radical scavengers. In this respect, it is speculated that the groups (catechins, anthocyanins, flavones, flavonols, flavanones etc.) differ in their properties, dependent on number and position of hydroxyl groups and sugar substitutions. It has been shown in oxygen radical absorbance assays [7] that the antiperoxyl radical activities increase with the number of hydroxyl groups. Using an in vitro lipoprotein oxidation model, Vinson et al. [8] showed that flavanols and flavonols were the most effective, and flavones and flavanones the least effective as antioxidants. The experiments have mainly been performed using the aglycones.

Little is known about the absorption, distribution, metabolism, and excretion of flavonoids in humans. It has been accepted until recently, that only the aglycones are able to pass the gut wall, but recent papers by Hollman et al. [9] and Paganga and Rice-Evans [10] argue that the flavonoids may be absorbed as glycosides and present as such in plasma. Other studies of the metabolism of rutin in rats [11] or diosmetin in human volunteers [12] did not indicate the presence of glycosides in plasma.

Methods for quantitation of flavonoids in food samples have been published [13,14]. These methods did not include determination of flavanones. Bronner and Beecher [15] presented a method for the quantitation of the flavanone-glycosides hesperidin, naringin and narirutin in orange and grapefruit juice.

For the purpose of screening fruits, vegetables and beverages to estimate the flavonoid intake in Denmark, we have developed an HPLC method with photo-diode array (PDA) and mass spectrometric (MS) detection for quantitation of flavonols, flavones and flavanones in foods. The extraction and acid hydrolysis conditions are based on the method reported by Hertog et al. [13]. In the present study, we have included analysis of the compounds listed in Table 1. The structures are schematised in Fig. 1. The UV peak areas of the compounds were used for quantitation, and the MS detection was used to increase the specificity of the method. The number of flavonoids present in foods is high, and therefore interference with each other is a possibility in any chromatographic system. For the number of flavonoid aglycone compounds we have as standards, we observe coelution of some of the flavonoids. We have included the MS detection in addition to PDA detection to exclude the possibility of interference in such cases, and also to be able to verify the glycosidic structure. Examples of HPLC–MS analyses of flavonoid aglycones in orange pulp and tomato are presented, as is the detection of phloretin-glycosides in apple peel.

2. Materials and methods

2.1. Sample preparation

Whole fruits and vegetables were purchased at

Table 1
HPLC retention times, UV maxima and pseudomolecular ions of flavone, flavonol and flavanone aglycones

	t_R (min)	UV max (nm)	$[M-H]^-$ or M^-
Apigenin	24.2	341	269
Eriodictyol	13.0	289	288
Hesperitin	18.5	289	301/302
Isorhamnetin	24.0	372	315
Kaempferol	22.9	366	285
Luteolin	18.5	351	285
Myricetin	11.5	375	317
Naringenin	17.5	292	272
Phloretin	18.4	287	273
Quercetin	16.6	374	301

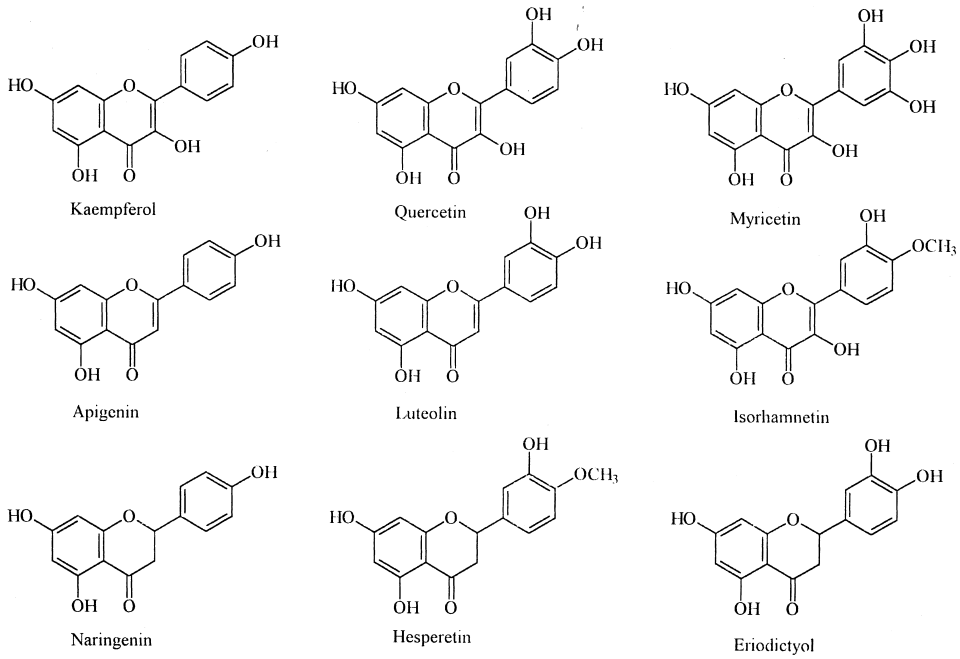

Fig. 1. Structures of the flavones, flavonols, and flavanones used in the standard mixtures (Fig. 2).

local grocery stores, cleaned and, when necessary, divided into edible and non-edible parts, which were weighed separately. Apples, oranges and pears were peeled (as standardised as possible), and the peel was analysed separately from the pulp. The edible parts of the foods were chopped in a food processor, lyophilised 24–48 h and kept at −18°C until further analysis. The percentage of moisture was measured by difference in weight before and after lyophilisation.

2.2. Extraction and hydrolysis

The solid samples were prepared as follows: 40 ml of 62.5% aqueous methanol containing BHA (2 g/l) was added to 0.500 g of freeze-dried sample material. To this, 10 ml of 6 *M* HCl was added carefully to give a total volume of 50 ml. The extraction mixture thus consisted of 1.2 *M* HCl in 50% aqueous methanol. For Brassica samples, 17 ml of 6 *M* HCl was added to the 33 ml of 62.5% aqueous methanol. The extraction mixture was thereafter heated to 90°C on a steam bath and refluxed for 2 h, allowed to cool in the refrigerator, diluted to 100 ml with methanol and sonicated for 5 min to form the final extract.

The procedure for liquid samples such as tea, fruit juices and wine was: 40 ml of 62.5% aqueous methanol (containing 2 g/l BHA) was added to 15 ml of the liquid sample. To this, 10 ml of 6 *M* HCl was added and mixed carefully. The rest of the procedure was as described above, except that red wine samples were hydrolysed for 4 h at 90°C.

Approximately 3 ml of the final extract were filtered through a 0.45-μm filter (Sartorius Minisart) prior to 20 μl injection into the HPLC system. The samples were prepared and analysed in duplicate.

The procedure for extraction of flavonoid-glycosides was as follows: 0.5 g freeze-dried sample was pulverised in a mortar and extracted with 20 ml 62.5% aqueous methanol using BHA as antioxidant. After sedimentation, a 2-ml aliquot was added to 2 ml water, pH 2.5 and the samples were filtered (as above) before injection of 20-μl aliquots to the HPLC.

2.3. Standards

Flavonoid standards were purchased from Apin (Oxon, UK) and Sigma (St. Louis, MO, USA). The standards were dissolved in DMSO to a concen-

tration of 0.1 g/l and kept protected from light at −18°C for up to 3 months. Working solutions were made up each day by diluting 0.50 ml standard stock solution with 10 ml 62.5% aqueous methanol containing BHA (2 g/l), and 2.5 ml 6 *M* HCl. Chromatograms of standard mixtures are presented in Fig. 2A and B.

2.4. HPLC

The HPLC system consisted of a Waters system (Milford, MA, USA) 717 autoinjector, 616 pump, and 996 PDA detector. For the analysis of the acid hydrolysed food samples, a Phenomenex (Torrance, CA, USA) RP C_{18} column (250×4.6 mm, 5 μm) protected by a guard column (LC_{18}) was used. The mobile phase consisted of methanol–water (30:70, v/v) with 1% formic acid (A) and 100% methanol (B). The gradient was 25–86% B in 50 min at a flow-rate of 1 ml/min. UV spectra were recorded from 220–450 nm at a rate of 1.00 spectrum/s and a resolution of 1.2 nm. HPLC grade methanol was purchased at Rathburn (Walkerburn, UK).

For analysis of glycosides, an Hewlett-Packard (Palo Alto, CA, USA) Purospher RP C_{18} 250×4.6 mm, 5 μm) column was used, with a mobile phase consisting of 1% formic acid in water (A) and 100% acetonitrile (B). The gradient was 5–60% B in 60 min at a flow-rate of 1 ml/min.

2.5. Mass spectrometry

MS was performed on a VG Platform II quadrupole instrument (Micromass, Chesire, UK) equipped with an atmospheric pressure ionisation (API) source using the APcI inlet. The characteristic pseudomolecular anions of the compounds are listed in Table 1. The probe and ion source parameters were: source temperature 150°C, probe temperature 450°C, cone voltage −30 eV, and corona discharge 1.6–1.9 kV. Negative ion mass spectra of the aglycones and glycosides were acquired from 120 to 450 a.m.u. and 120 to 650, respectively, at a scan rate of one scan/s. The HPLC was connected to the probe of the mass spectrometer via the UV cell outlet, using PEEK tubing.

2.6. Quantitation

For each compound, peak areas were determined at the wavelength providing maximal UV absorbance (see Table 1) at a minimal signal-to-noise criterion of 5 (peak height), corresponding approximately to 1 ng injected on the column and to 0.1 mg/100 mg fresh weight of sample at a drying factor of 0.1. Calibration curves of the standards ranging from 2.5 to 25 mg/ml (10 levels) revealed good linearity with R^2 values exceeding 0.99 (peak areas vs. concentration). Quantitation was performed based on external standards with a mixture of standards of known concentration that were analysed in duplicate before and after the batch of samples, and the peak areas were used to calculate the sample contents of the compounds listed in Table 1.

2.7. Validation

A series of sample analyses were performed to investigate the performance of the method. Apple peel, broccoli, and orange peel samples were selected for reproducibility. Individual analysis of these samples were repeated in duplicate for 5 days. For recovery, apple peel, onion, orange pulp, and tea samples were selected. Three repeated analyses of 100% addition of the individual components were performed on the same day. The aglycone standards were added before acid hydrolysis. The data from the validation experiments are seen in Table 2.

3. Results and discussion

3.1. Quantitative and identification procedure by HPLC and MS

Using the present method, we isolated and quantified the flavones, flavonols, and flavanones present in Danish foods. HPLC with PDA detection was used to separate and quantify the flavonoids, and MS was used for unambiguous detection of flavonoid aglycones in the hydrolysed samples, to eliminate misidentification of coeluting compounds with similar UV spectra (see Fig. 2).

The method validation (Table 2) indicates good performance of the method — the day-to-day varia-

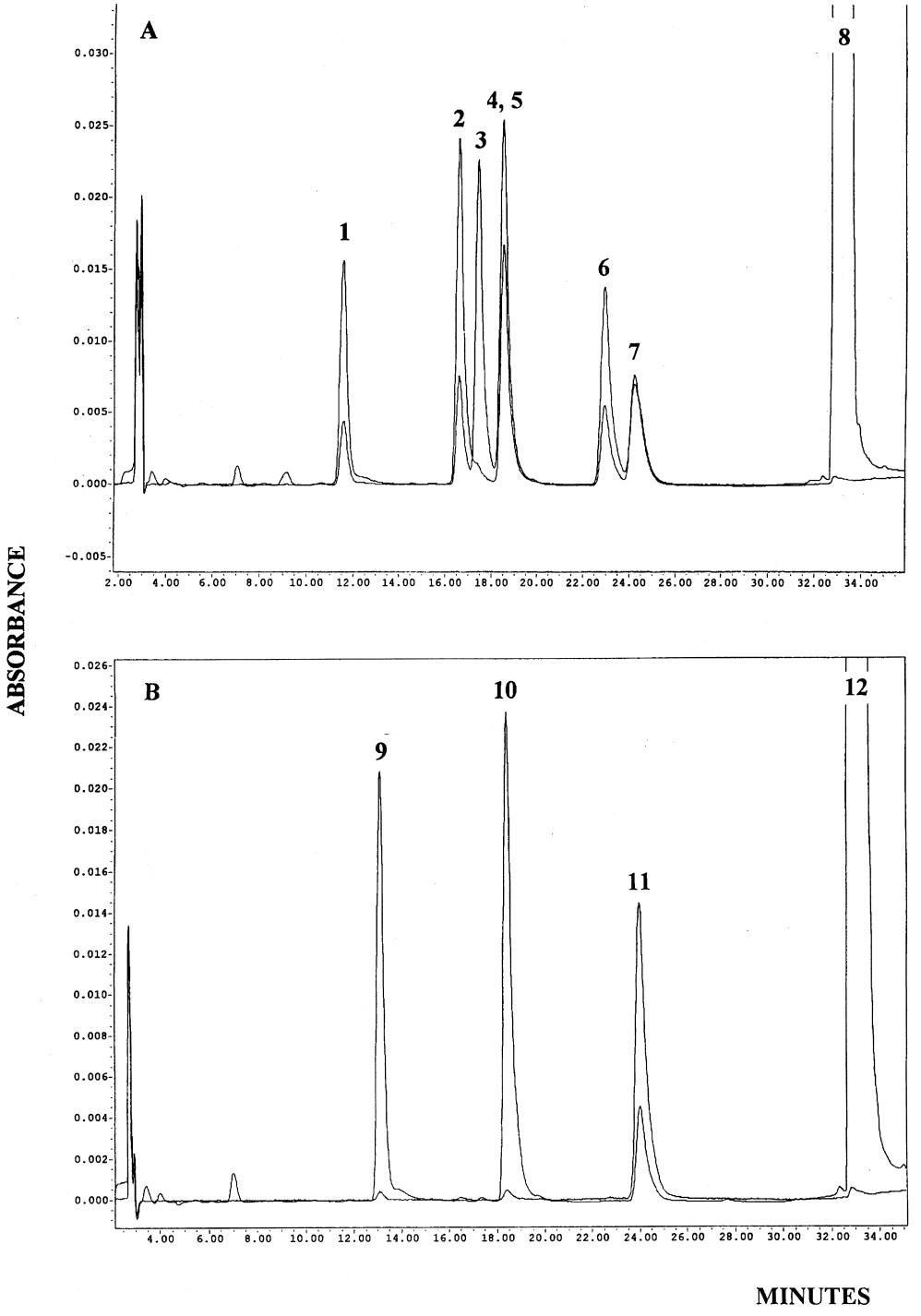

Fig. 2. HPLC chromatograms of the standard mixtures recorded at 290 and 365 nm. (A) 1, Myricetin; 2, quercetin; 3, naringenin, 4, luteolin; 5, hesperetin; 6, kaempferol; 7, apigenin; 8, BHA (antioxidant): (B) 9, eriodictyol; 10, phloretin; 11, isorhamnetin; 12, BHA.

Table 2
Mean content (mg/100 g fresh weight) of five samples, reproducibility of five components in three samples, recovery as 100% addition of six individual components to four samples

Food	Component	Average content (mg/100 g)	Reproducibility C.V.[a] (%)	Recovery[b] (%)
Broccoli	Quercetin	6.8	6	
	Kaempferol	10	13	
Orange peel	Hesperetin	21	11	
	Naringenin	375	4	
Apple peel	Quercetin	23	3	103
Orange pulp	Hesperetin	56.0±0.7[c]		78
	Naringenin	11.3±0.1		68
Onion	Quercetin	34.8±1.0		95
Tea	Quercetin	1.6±0.1		81
	Kaempferol	1.1±0.1		97

[a] Between-day variations, performed duplicate analyses for 5 consecutive days.
[b] Performed as 100% addition of the average contents of the individual component (as aglycone). The addition was performed in triplicate on the same day.
[c] Triplicate analyses performed on the same day (mean±standard deviation).

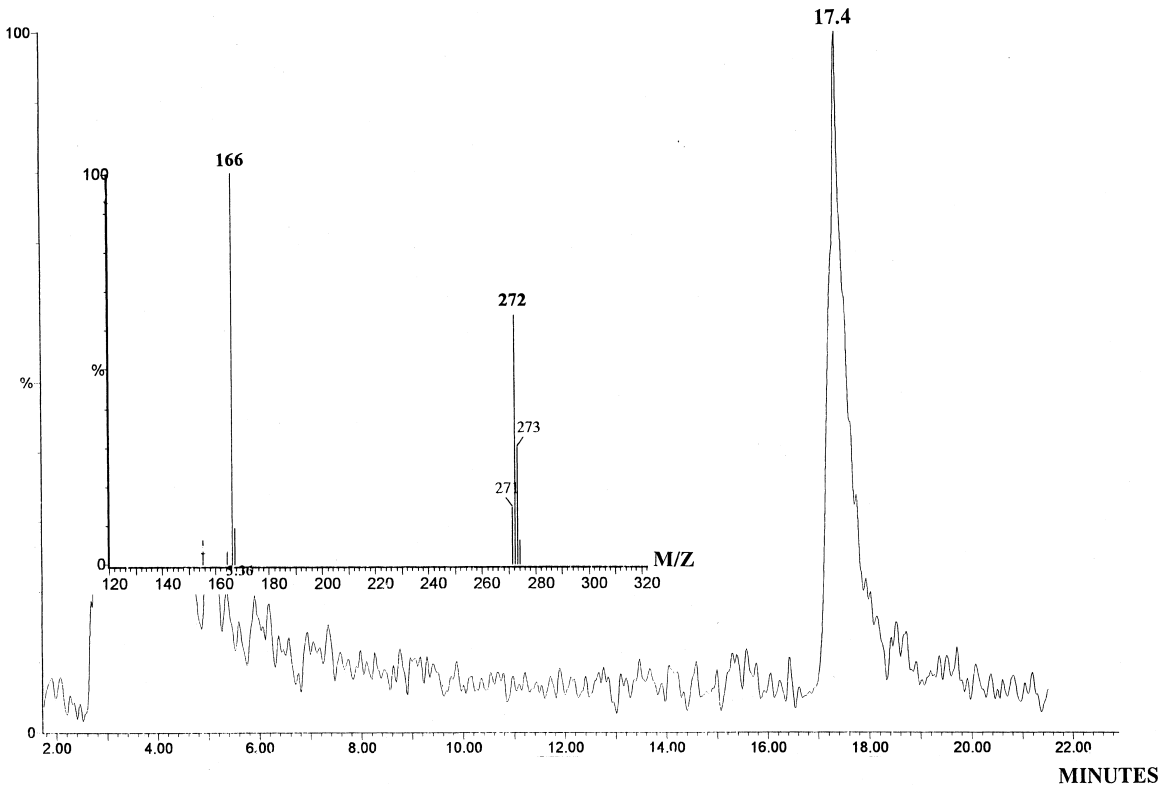

Fig. 3. HPLC–MS analysis of a hydrolysed tomato sample. Ion chromatogram of m/z 272 and mass spectrum at t_R 17.4 min. Identified as naringenin by comparison with commercial standard.

tions were below 13%. The recoveries of the added aglycone standards were in the range of 68–103%, except for the recovery of myricetin standard (30%). The low recovery of myricetin aglycone standard may be explained by degradation in the acid hydrolysis process. A more correct estimate of myricetin recovery should be performed using a myricetin-glycoside standard.

It has been reported in the literature that myricetin is difficult to quantify, due to problems with either instability [13] or with interference from other compounds [16]. We did not observe the interference described by Shepherd and Ibe [16], which may be explained by our 50-min gradient in the chromatographic system, allowing the very polar compounds to elute prior to myricetin. We agree that myricetin is less stable than the other flavonoid aglycones. By taking precautions such as using freshly made up standard mixtures, cooling of the autosampler, and protecting the vials from UV light, the degradation of the myricetin standard is within acceptable limits (approximately 15%). We have therefore included the quantitation of myricetin in the present study since it contributes significantly to the flavonoid levels in tea, which is important due to the high average daily intake in Denmark.

The levels of the flavones, flavonols and flavanones were as expected according to previous investigations of these compounds in foods [15,17,18]. Neither group, however, included the quantitation of flavanones in orange, orange juice or tomatoes. We found significant amounts of flavanones in these foods. The naringenin and hesperetin contents of orange pulp were found to average 11 and 31 mg/100 g fresh weight, respectively, and the naringenin content of tomatoes was calculated to 1.5 mg/100 g fresh weight, equalling the levels of quercetin in the tomato samples ana-

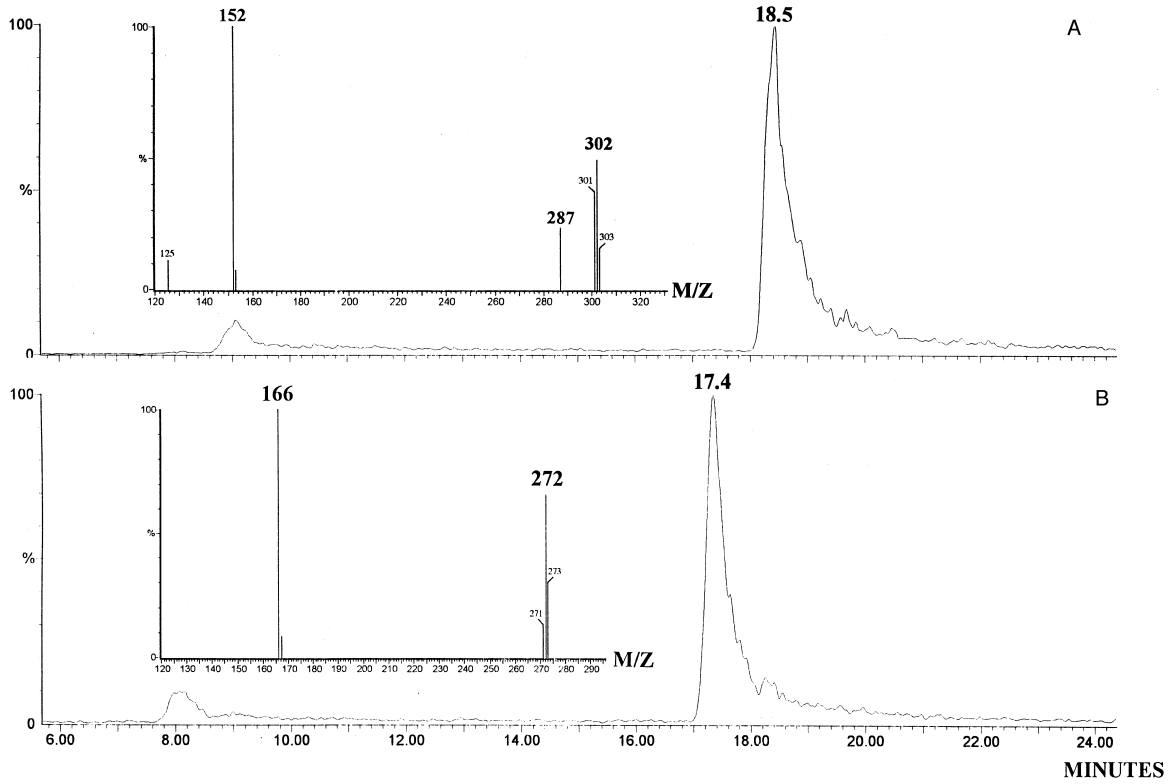

Fig. 4. Mass spectra of the two major peaks in the orange pulp sample. (A) Ion chromatogram of the ion m/z 302. Inserted is the spectrum of the compound eluting at t_R 18.5 min. Identified as hesperetin. (B) Ion chromatogram of the ion m/z 272. Inserted is the spectrum of the compound eluting at t_R 17.4 min. Identified as naringenin.

lysed (Table 3). Fig. 3 shows the mass chromatogram of a tomato sample, mass spectrum and retention time of the compound eluting at 17.4 min being close to identical to the data obtained from naringenin standard.

Fig. 4A and B presents the mass spectra of hesperetin and naringenin obtained from HPLC–MS of hydrolysed orange pulp sample. Fig. 4A shows the spectrum of hesperetin, the pseudomolecular ion 302 and fragment 287 (loss of methyl) is indicative of hesperetin in the orange sample, as compared to a commercial standard of hesperetin. The spectrum in Fig. 4B, the pseudomolecular ion m/z 272 and fragment ions m/z 166 and 152, is close to identical to a standard spectrum of naringenin.

Although the presence of phloretin-glycosides (phloridzin and phloretin-xyloglucoside) in apple flesh, seed and skin have been reported [19], we were not able to quantify phloretin in 18 apple skin or flesh samples using the present method. By extraction of apple peel and pulp before hydrolysis, we used HPLC–MS to verify the presence of phloretin-glycosides in apple samples. The ion chromatogram of m/z 273 and the mass spectra inserted (Fig. 5) indicates the presence of two phloretin-glycosides, a diglycoside m/z 567 and a monoglycoside m/z 435. The monoglycoside is suggested to be phloridzin (the difference of 162 corresponds to one glucose molecule) and the diglycoside to be phloretin-xyloglucoside (the difference of 294 corresponds to one glucose and one xylose molecule). The detection of phloretin-conjugates by this procedure but not using the acid hydrolysis procedure suggests that phloretin is degraded by the acid hydrolysis due

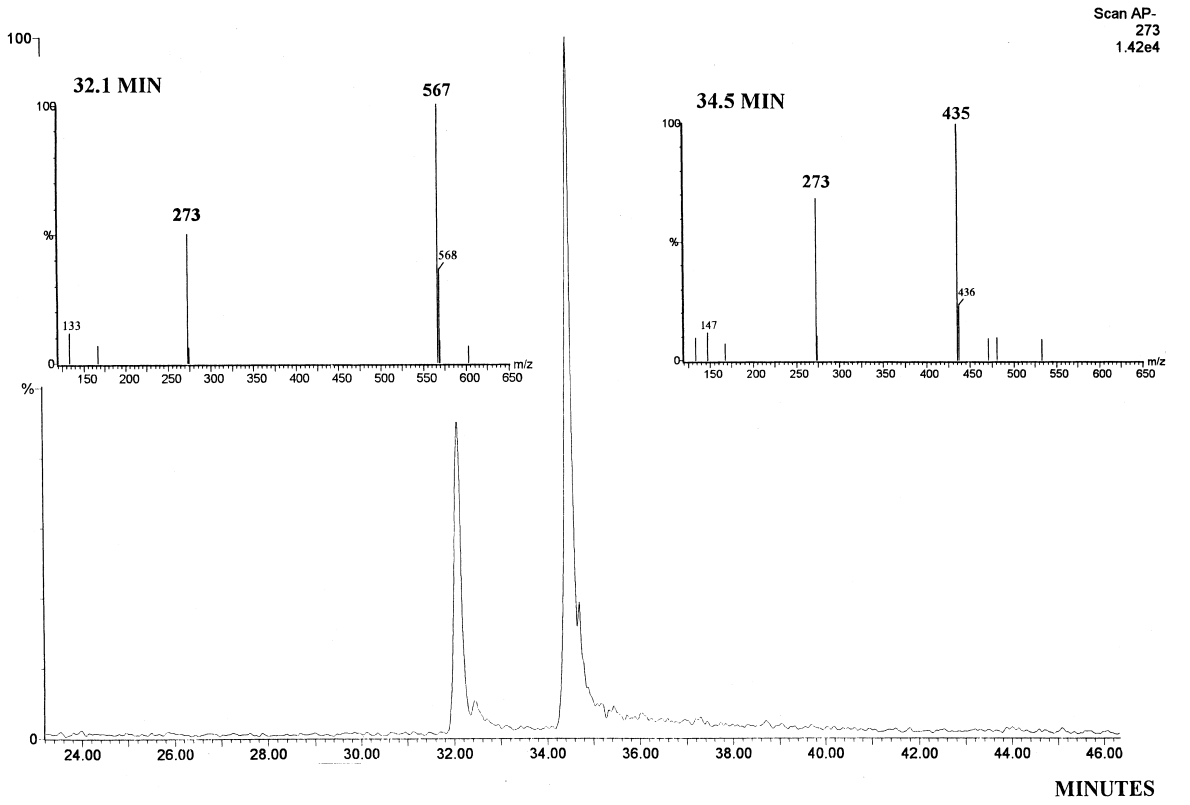

Fig. 5. HPLC–MS analysis of apple peel extract. Ion chromatogram of mass 273 and the mass spectrum obtained at 32.0 min. The pseudomolecular ion m/z 567 and the fragment ion m/z 273 indicates the xyloglucoside of phloretin. The mass spectrum at 34.5 min indicates the phloretin–glucoside phloridzin, as the retention time, pseudomolecular ion m/z 435 and fragment ion at m/z 273 were found to be similar to a commercial standard.

to the less stable open-ring dihydrochalcone structure.

3.2. Quantitative analysis of flavones, flavonols, and flavanones in foods

The results from quantitation of flavonols, flavones and flavanones in a number of fruits, vegetables and beverages are seen in Table 3. The data presented here are average values of duplicate analyses of one or a number of samples of the particular food. For foods of limited interest in this study, due to low average intake in Denmark, we analysed only one sample. As expected from the data presented by Hertog et al. [17], quercetin is overall the major flavonol, followed by kaempferol. The

Table 3
Flavone, flavonol and flavanone contents in fruits, vegetables and beverages presented as average values±standard deviation (mg/100 g fresh weight)

Food subject	n[a]	Quercetin	Kaempferol	Hesperetin	Naringenin	Myricetin	Apigenin	Luteolin
Apple	18	2.0±0.4						
Apricot	1	2.6						
Bean, green	2	1.6±0.6						
Blackcurrant	3	3.7±0.1	0.1±0.1					
Blueberry	1	7.3						
Broccoli	5	3.7±2.5	6.0±3.4					
Brussels sprouts	2	0.6±0.1	0.9±0.1					
Celery, leaf	3						75±28	20±8
Celery, stalk	4						1.6±0.9	0.5±0.3
Cherry	1	1.0						
Cowberry	1	21	0.5					
Cranberry	1	16				23		
Grapefruit, pulp	2	0.5±0.1	0.4±0.1	1.5±0.3	53±6			
Grapes, blue	2	3.7±3.0						
Grapes, green	1	0.2						
Kale	4	12±2	47±3					
Leek	2		3.1±1.3					
Lemon, pulp	1			17	0.5			
Lime, pulp	1	0.4		43	3.4			
Onion, red	3	45±21						
Onion, spring	2	18±12	0.6±0.6					
Onion, yellow	5	34±7						
Orange, pulp	5			31±2	11±2			
Orange, juice	3			9.0±1.0	0.8			
Parsley	2		1.1				185±5	1.1±1.1
Pear, peel	2	4.5±2.0						
Plum, blue	1	1.5						
Red currant	2	0.8±0.1						
Red raspberry	1	0.5						
Red wine	21	0.8±0.5				1.0±0.6		
Rosebud	1	2.6	1.5					
Salads[b]	5	<1.0						
Strawberry	4	0.6±0.5	0.5±0.3					
Sweet pepper, green	2	0.5						0.5±0.1
Sweet pepper, red	2							0.1±0.1
Sweet pepper, yellow	2							0.2±0.2
Tea	8	1.4±0.5	1.6±0.5			0.4±0.2		
Tomato	5	1.4±0.8			1.5±0.4			

[a] Number of samples analysed.

[b] Five different salads (cabbage) were analysed: cabbage lettuce, China cabbage, oxheart cabbage, iceberg salad, savoy. Duplicate analyses of two individual samples of each type of salad were performed, each with a content below 1.0 mg/100 g fresh weight.

flavanones hesperetin and naringenin are present in citrus fruits, naringenin also in tomatoes. The flavones apigenin and luteolin were found present only in a limited number of foods such as celery, sweet peppers (luteolin only), and parsley (apigenin only). In celery, apigenin and luteolin contents in the leaves are approximately 40 times higher than the levels in the stalks. In other foods we also observed differences in the levels of flavonoids in the various parts of the plant. For example in 18 apple samples we found quercetin to be located in the skin only (average value 16 mg/100 g fresh weight). No quercetin (or below detection limits of the method) was found in the flesh of apple.

We found low levels (<0.1 mg/100 g fresh weight) of flavonoids in various lettuces and cabbages. Other foods which are consumed in considerable amounts in Denmark such as potatoes, carrot, peas and cauliflower were found not to contain flavones, flavonols, or flavanones.

4. Conclusion

The method for extraction, hydrolysis, and analysis by HPLC–MS presented here has proved specific and sensitive for quantitation of flavonols, flavones, and flavanones in selected fruits, vegetables and beverages consumed in Denmark. The data will be used to estimate the average Danish intake of flavones, flavonols, and flavanones. The results will be presented elsewhere.

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