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Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis

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Abstract

For the quantitative determination of individual flavonoid glycosides in plant materials, the glycosides are normally hydrolysed and the resulting aglycones are identified and quantified. However, the hydrolysis conditions which result in optimal breakdown of glycosides are too harsh for some of the other phenolic compounds present in the same plant material. Therefore, the effects of different hydrolysis conditions and different antioxidants on pure flavonoid glycones and aglycones were studied. On the basis of the results obtained with standards, suitable hydrolysis methods for red spring onion and spinach were developed. The best results from these vegetables were obtained by refluxing at 80 °C for 2 h with 1.2 M HCl in 50% aqueous methanol with addition of 2 mg ascorbic acid as an antioxidant. The method developed in this study is suited to the screening of flavonoids in vegetables and leafy vegetables.

Keywords: Flavonoids; HPLC; Hydrolysis; Onion; Phenolic acids; Spinach

1. Introduction

Flavonoids, a large group of plant polyphenols, are present in plant tissues in relatively high concentrations, either as sugar conjugates or as aglycones (Markham, 1982). Although flavonoids have generally been considered to be non-nutritive agents, in recent years the health effects of flavonoids present in human diet have attracted much attention. Studies suggest that they act as antioxidants (Burns et al., 2000; Kaneko & Baba, 1999), and there is also some evidence from epidemiological and in vivo studies that their consumption is associated with a reduced risk of certain cancers and cardiovascular diseases (Avila, Velasco, Cansado, & Notario, 1994; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Knekt, Järvinen, Reunanen, & Maatela, 1996; Piantelli et al., 1994). In addition to the proposed beneficial effects of flavonoids, mutagenicity and carcinogenicity have also been reported (Cross, Tilby, Chipman, Ferry, & Gescher, 1996; Gaspar, Laires, Monteiro, Laureano, Ramos, & Rueff, 1993).

In plants, flavonoids occur mainly in leaves and in the outer parts of the plants. In vegetables, quercetin and its glycosides predominate but glycosides of kaempferol, luteolin and apigenin are also present (Hertog, Hollman, & Katan, 1992a; Hertog, Hollman, & Venema, 1992b). Onions have been shown to contain large amounts of flavonoids, especially quercetin and its glycosides, and therefore consitute one of the major sources of flavonoids in western diets (Knekt et al., 1996). Spinach has also been recognised as a good source of flavonoids (Goldbohm, Hertog, Brants, van Poppel, & ven den Brandt, 1998).

Quantitative determination of individual flavonoid glycosides in plant materials is difficult, due to their large number. Therefore, the glycosides are normally hydrolysed and the resulting aglycones are identified and quantified. Methods for acid hydrolysis of flavonoids from vegetables and berries have been published earlier by Hertog et al. (1992b) and by Häkkinen, Kärenlampi, Heinonen, Mykkänen, and Törrönen (1998). Usually, hydrolysis of flavonoid glycosides requires relatively high concentrations (1–2 M) of mineral acids under refluxing conditions (Hertog et al., 1992b; Merken & Beecher, 2000). However, the conditions resulting in optimal breakdown of glycosides are too

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harsh for some of the other phenolic compounds present in the same plant material. Catechins are degraded and myricetin is also partially destroyed (Häkkinen et al., 1998; Merken & Beecher, 2000).

In order to develop sample preparation procedures for the formation of aglycones of flavonoids without degrading the aglycones themselves, the effects of different hydrolysis conditions and different antioxidants on pure flavonoid glycones and aglycones were studied. On the basis of the results obtained with standards, hydrolysis methods for red spring onion and spinach were developed.

2. Materials and methods

2.1. Standards

The HPLC-grade flavonoid standards, purchased from Extrasynthese were apigenin (1102S), caffeic acid (6018), (+)-catechin (0976S), chlorogenic acid (6019), trans-cinnamic acid (6024), 3-coumaric acid (6030), ferulic acid (6077), isoquercitrin (1119S), kaempferol (1124S), luteolin (1125S), myricetin (0055), quercetin (1135S), and rutin (1139S). The standard stock solutions were prepared by dissolving standards in methanol containing 0.2% phosphoric acid to a concentration of 200 μg ml^{-1}. For the calibration curves, ranging from 0.2 to 20 μg ml^{-1}, the standard stock solutions were diluted to final concentrations with 0.6 M HCl in 75% aqueous methanol.

2.2. Hydrolysis of standard mixture

In a preliminary experiment the hydrolysis methods of Hertog et al. (1992b) and Häkkinen et al. (1998) were tested. The standard mixture (20 μg ml^{-1} of each flavonoid) was hydrolysed, either by refluxing at 80 °C for 2 h in 1.2 M HCl in 50% aqueous methanol (Hertog et al., 1992b), or at 35 °C for 16 h in 1.2 M HCl in 50% aqueous methanol (Häkkinen et al. 1998). The 16 h hydrolysis was performed either in air or under N_2-atmosphere. The effects of different antioxidants were also tested. Prior to hydrolysis, 10 mg *tert*-butylhydroquinone (TBQH; Hertog et al., 1992b), 8 mg ascorbic acid (Häkkinen et al., 1998), or no antioxidant were added to the mixture.

2.3. Hydrolysis of pure standards

On the basis of the preliminary experiment, the effects of two different hydrolysis conditions were tested on selected pure standards: refluxing at 80 °C for 2 h in 1.2 M HCl in 50% aqueous methanol (Hertog et al., 1992b), and 16 h at 35 °C in 1.2 M HCl in 50% aqueous methanol (Häkkinen et al., 1998). The hydrolysis were performed in duplicate. No antioxidants were added. The selected standards represented different chemical groups: phenolic acids, flavonoid glycosides and aglycones. Quercetin was used as a model of an acid hydrolysis-resistant aglycone and myricetin as a model of an acid hydrolysis-sensitive aglycone.

2.4. Hydrolysis of plant material

The effects of antioxidants (ascorbic acid and TBHQ) in the hydrolysis of plant material were tested using early red onion samples. The plant material was freeze-dried and ground. Fifty milligrams of the ground plant material were hydrolysed in 5 ml of 1.2 M HCl in 50% aqueous methanol. To the hydrolysis mixture, 10 mg TBHQ, 8 mg ascorbic acid or no antioxidant were added. The hydrolysis were performed in triplicate. After refluxing at 80 °C for 2 h, the extract was allowed to cool and was made up to 10 ml and sonicated. The extract was filtered through a 0.45-μm filter for organic solvents, prior to injection. The amount of the antioxidant ascorbic acid was optimised further in an experiment in which red onion and spinach samples were used. The plant samples were hydrolysed as described above, and the hydrolysis were performed in duplicate. Ascorbic acid was added prior to hydrolysis at concentrations of 0, 1, 2, 3, 4, 5, 10 or 15 mg.

2.5. HPLC separations

The hydrolysed samples were analysed using a Waters HPLC system, comprising a Millenium^{32} (Version 3.05.01) chromatography manager, a Waters 712 WISP automatic sample injector, a Waters 2487 Dual Wavelength Absorbance Detector and two Waters 6000A pumps. Reversed phase separations were carried out at room temperature using a 150 × 3.9 mm i.d., 5 μm C_{18} Symmetry column (Waters), fitted with a 20 × 3.9 mm i.d., 5 μm C_{18} Symmetry guard column (Waters). The mobile phase was a 25-min, 20–60% gradient of methanol in water with 300 μl l^{-1} trifluoroacetic acid, eluted at a flow rate of 0.8 ml min^{-1}. After each analysis, the column was washed with 100% methanol for 2 min, returned to 20% methanol and re-equilibrated for 10 min before the next analysis. The eluted components were monitored at 280 and 340 nm (Fig. 1).

3. Results and discussion

3.1. Hydrolysis of the standard mixture

The complete hydrolysis of the flavonoid glycosides (isoquercitrin and rutin) was achieved only by refluxing at 80 °C (Table 1.). The amount of quercetin in the mixture increased due to its formation from its glycosides.

Table 1
Hydrolysis of the standard mixture at different temperatures and with different antioxidants. Recoveries presented as percentage (%) of the amount (20 μg ml^{-1}) in the original standard mixture

	No antioxidant addition			TBHQ addition			Ascorbic acid addition		
	80 °C 2 h	35 °C 16 h	35 °C + N_2/16 h	80 °C 2 h	35 °C 16 h	35 °C + N_2/16 h	80 °C 2 h	35 °C 16 h	35 °C + N_2/16 h
Phenolic acids									
Caffeic acid	45	54	54	49	69	65	49	73	56
Chlorogenic acid	45	53	-	15	39	36	-	132	280
trans-Cinnamic acid	85	64	65	90	90	87	115	114	60
3-Coumaric acid	49	58	58	53	67	63	83	90	55
Ferulic acid	38	53	54	46	70	68	65	101	61
Flavonols (aglycones)									
Kaempferol	83	68	68	104	94	91	99	69	85
Myricetin	32	57	26	95	92	89	111	32	61
Quercetin	175	97	85	226	145	140	204	29	115
Flavones (aglycones)									
Apigenin	93	71	72	106	101	99	104	67	89
Luteolin	101	75	47	115	103	100	111	73	94
Glycosides									
Isoquercitrin/rutin	–	53	45	–	60	57	26	45	69
Catechins									
Catechin	150	85	126	11	71	69	–	346	90

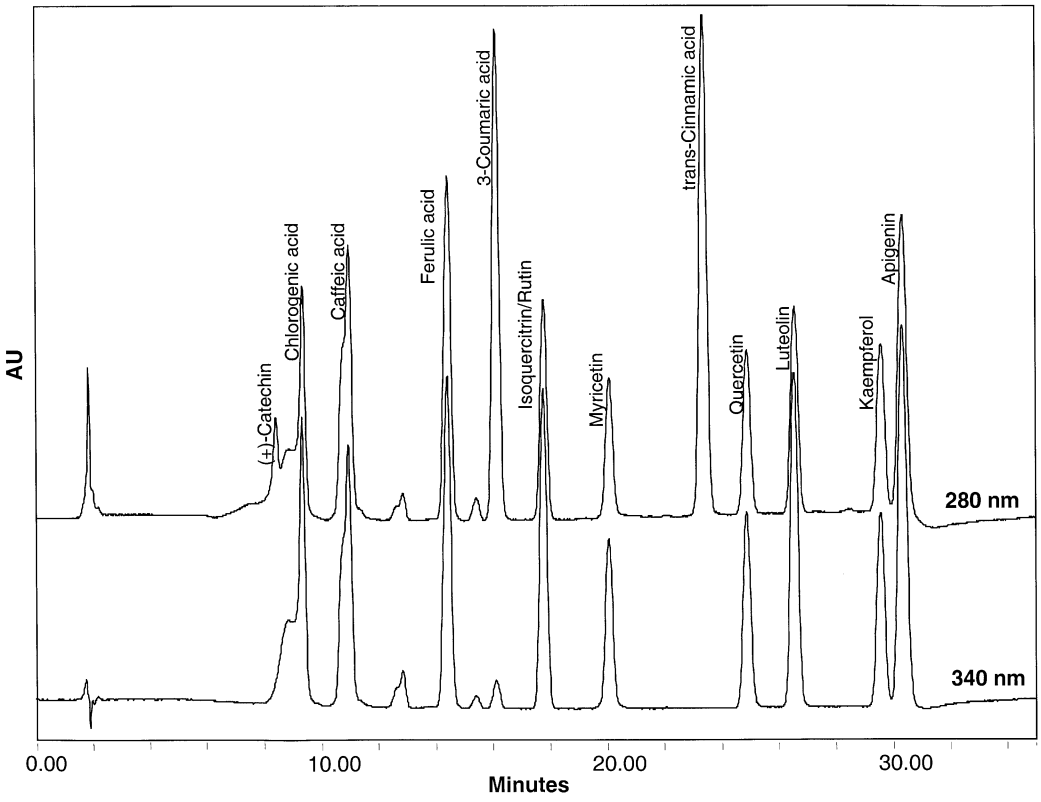

Fig. 1. HPLC chromatogram of the standards monitored at 280 and 340 nm.

For most of the flavonoid aglycones, the 2 h refluxing at 80 °C with 1.2 M HCl with no antioxidant addition gave the best results. However, if no antioxidant was added to the mixture, myricetin was extensively degraded under these conditions (recovery 32%). The phenolic acids were degraded, regardless of whether antioxidants were used or not. These results are in accordance with results of Häkkinen et al. (1998). Ascorbic acid also appeared to interfere with the detection of phenolic compounds eluting during the first eleven minutes (e.g chlorogenic acid and catechin, Fig. 1). TBHQ on the other hand interfered with the detection of quercetin, depending on the elution profile. Hydrolysis under N_2-atmosphere did not appear to offer any advantages, except in the case of quercetin, for which the extended exposure time (16 h) to HCl and ascorbic acid caused degradation (Table 1). Degradation of quercetin, due to increasing reaction time, has also been reported by Hertog et al. (1992b).

3.2. Hydrolysis of pure flavonoids

On the basis of the first results, a second experiment was planned on which the hydrolysis efficacies with 1.2 M HCl at 80 or at 35 °C were compared using selected pure standards (20 μg ml^{-1}) separately (Table 2). The hydrolysis for 16 h at 35 °C was not efficient enough to produce aglycones from glycosides (isoquercitrin and rutin), but the hydrolysis for 2 h at 80 °C with 1.2 HCl efficiently produced aglycones from glycosides, which were no longer detectable after the 2 h hydrolysis (Table 2). However, the hydrolysis for 2 h at 80 °C began to degrade even quercetin slightly, which in general is rather resistant to acid hydrolysis (Table 2). The degradation of the more sensitive compounds (e.g. myricetin, *trans*-cinnamic acid, ferulic acid, 3-coumaric acid) was increased at the higher temperature.

Table 2
Comparison of hydrolysis at 35 and at 80 °C with individual pure standards[a]

Compound	35 °C 16 h 1.2 M HCl	80 °C 2 h 1.2 M HCl	Detected aglycone
Phenolic acids:			
Caffeic acid	57	33	
Chlorogenic acid	92	75	
trans-Cinnamic acid	88	49	
3-Coumaric acid	85	53	
Ferulic acid	104	35	
Glycosides:			
Isoquercitrin	58	n.d.	Quercetin
Rutin	90	n.d.	Quercetin
Aglycones:			
Myricetin	121	15	
Quercetin	136	73	

[a] Recovery of compounds as percentage of the original amount (20 μg ml^{-1}). n.d., not detected.

3.3. Hydrolysis of plant material with antioxidants

Due to the chemical complexity of any given plant material, it is necessary to determine the necessity for the use of antioxidants in each case, separately. Skinned red storage onion was used as a model to compare the two antioxidants, TBQH and ascorbic acid. Only the results for the detected flavonoids are shown (Table 3). The use of antioxidant clearly enhanced the amount of quercetin that was detected from red onion extracts. Although TBQH gave slightly higher concentrations of quercetin than ascorbic acid, it caused some interference with the detection and we therefore decided to optimise the concentration of ascorbic acid further.

Red spring onion bulbs (skinned), red spring onion leaves and spinach were used as experimental material. These plant materials were of interest since they are major sources of flavonoids in western diets. Onion bulbs are high in quercetin but leafy vegetables (onion leaves and spinach) also contain considerable amounts of kaempferol. Only the results for the flavonoids detected are shown in Table 4. In all of the cases, 2 mg of ascorbic acid gave the best yield of quercetin and kaempferol. No other flavonoids were detected. When using higher concentrations of ascorbic acid, the detected amounts of flavonoids decreased. This was most likely due to the fact that, at high concentrations, antioxidants start acting as a pro-oxidants. Since both ascorbic acid and flavonoids have antioxidant properties, it appears that higher addition of ascorbic acid than 2 mg will act as a pro-oxidant rather than as an antioxidant.

Table 3
Hydrolysis of red onion with different antioxidants

Sample	Quercetin			
	mg/kg d.w.[a]	± S.D.	mg/kg f.w.[b]	± S.D.
Red onion	1449	± 5.2	348	± 1.2
Red onion with TBHQ	2160	± 117.2	518	± 28.1
Red onion with ascorbic acid	1866	± 163.8	448	± 39.3

[a] Lyophilized dry weight.
[b] Fresh weight.

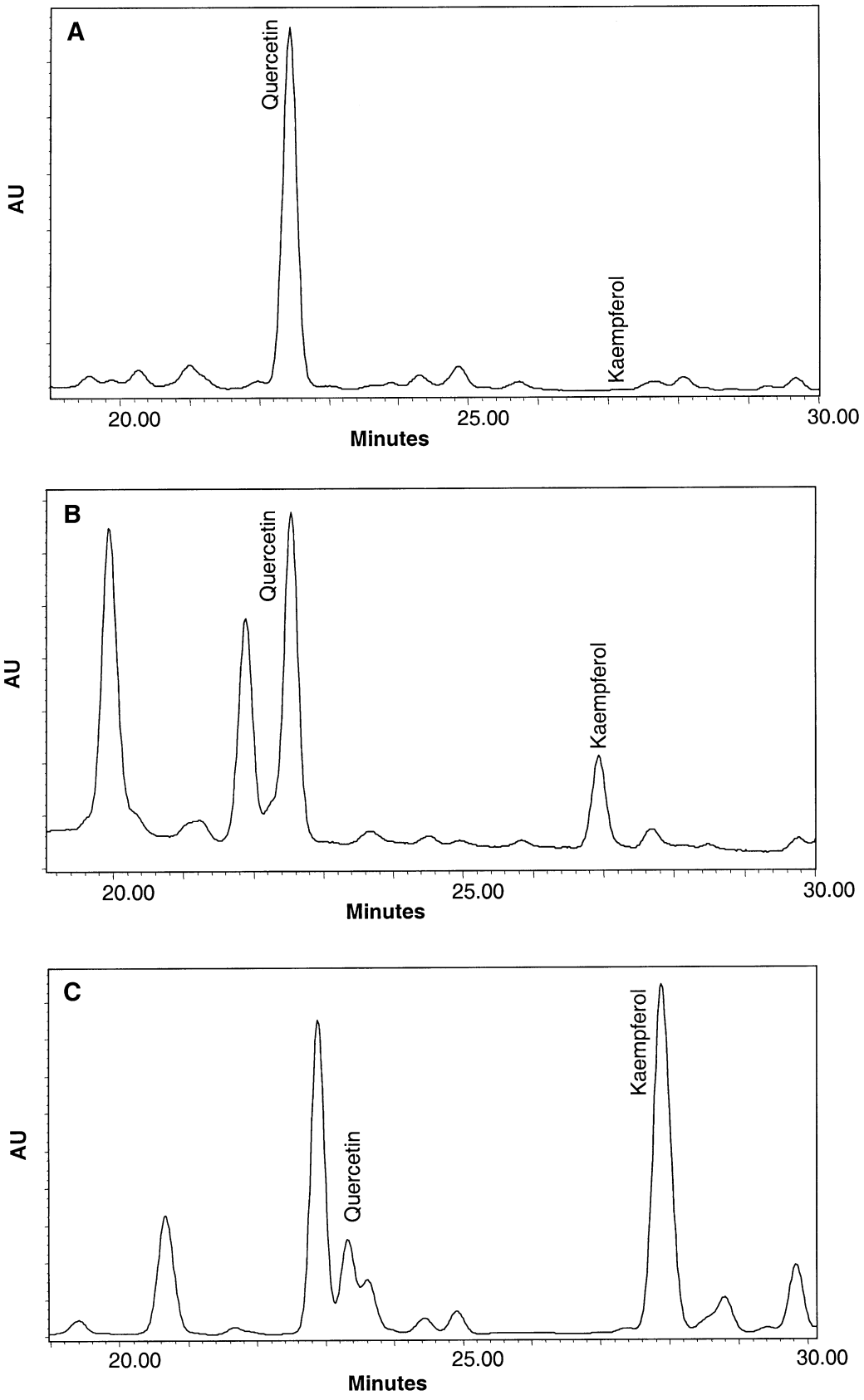

Fig. 2. HPLC chromatograms of (A) red spring onion bulb, (B) red spring onion leaf and (B) spinach monitored at 340 mn.

Table 4
Hydrolysis of plant material in the presence of different levels of ascorbic acid

Sample	Ascorbic acid addition (mg)	Quercetin mg/kg d.w.[a]	Kaempferol	Quercetin mg/kg f.w.[b]	Kaempferol
Red spring onion/bulb	15	940	–	261	–
	10	750	–	208	–
	5	848	–	236	–
	4	964	–	268	–
	3	951	–	264	–
	2	1100	–	306	–
	1	726	–	202	–
	0	980	–	273	–
Red spring onion/leaf	15	378	126	90	30
	10	324	112	77	27
	5	357	124	85	30
	4	407	138	97	33
	3	462	151	110	36
	2	526	171	126	41
	1	354	135	85	32
	0	13	122	3	29
Spinach	15	597	3039	74	379
	10	858	4084	107	509
	5	925	4234	115	528
	4	874	4016	109	501
	3	684	3414	85	426
	2	953	4414	119	550
	1	736	3559	92	444
	0	766	3667	96	457

[a] Lyophilized dry weight.
[b] Fresh weight.

The amount of quercetin detected by the best method in the red spring onion bulb (outer skin removed) was 1100 mg/kg dry weight (306 mg/kg fresh weight; Table 4). No kaempferol was detected (Fig. 2a). Comparable amounts of quercetin have been reported earlier (Crozier, Lean, McDonald, & Black, 1997; Ewald, Fjelkner-Modig, Johansson, Sjöholm, & Åkesson, 1999; Hertog et al., 1992b), although much wider ranges of quercetin levels in onion bulbs have been reported in the literature (Bilyk, Cooper, & Sapers, 1984; Chu, Chang, & Hsu, 2000; Patil & Pike 1995). The absence of kaempferol is also in agreement with literature, where in general very low levels or no kaempferol have been detected in the edible part of onion (Bilyk et al., 1984; Hertog et al., 1992a, 1992b; Ewald et al., 1999).

The leaves of red spring onion contained lower levels of quercetin than the edible part but considerable amounts of kaempferol (Fig. 2b; Table 4). There are no earlier reports on the flavonoid content of spring onion leaves, but leek has been reported to contain comparable amounts of kaempferol (Hertog et al. 1992a; Hóvári, Lugasi, & Dworschák, 1999). Kaempferol was clearly the main flavonoid in fresh spinach (Fig. 2c). With the best method, 4414 mg/kg dry weight (550 mg/kg fresh weight) of kaempferol was detected in spinach (Table 4). The quercetin levels were comparable to levels in red spring onion leaves, which is slightly lower than the level reported by Hóvári et al. (1999). The kaempferol levels are higher than those reported in the literature (Hertog et al., 1992a; Hóvári et al., 1999; Chu et al., 2000) but are in accordance with the general notion that leafy vegetables are good sources of kaempferol (Goldbohm et al., 1998). These differences in reported kaempferol levels might well be due to different hydrolysis methods used.

4. Conclusions

In recent years, reports suggesting beneficial nutritional and physiological effects of flavonoids have increased interest in vegetables and berries as an important source of bioactive plant phenolics. A vast amount of literature has emerged, comparing different vegetables and berries as sources of these compounds. Unfortunately, in most cases, not enough attention has been paid to the method of analysis used in evaluation of the flavonoid contents of these different plant materials.

Several reports have been published on the levels of different flavonoids in vegetables and other foodstuffs and, on the basis of the available literature, it seems clear that there is no single suitable method for

hydrolysis and analysis of flavonoids from any plant material. The method of choice is always a compromise between efficient production of aglycones from the plant material and degradation of aglycones. Different plant materials also contain different flavonoids and phenolic compounds in different forms, resulting in variable susceptiblity to degradation. For the samples used in this study (onion bulb, onion leaves, and spinach), the 2 h refluxing at 80 °C with 1.2 M HCl and 2 mg ascorbic acid addition gave the best results. This method is suitable for screening vegetables and leafy vegetables but would probably not be suitable for samples, which have higher concentrations of phenolic acids and myricetin. However, the method is still a compromise between efficient production of aglycones and degradation of aglycones, and thus not suitable for obtaining accurate quatitative data.

On the basis of the present study, it is evident that the optimal hydrolysis conditions for the analysis of flavonoids and phenolic compounds should be determined separately for each plant material studied. In cases for which very high accuracy is required, it might even be necessary to use different hydrolysis methods for different compounds from the same plant material. Therefore, extreme caution should be used in comparing the flavonoid contents of different plant materials (e.g. vegetables, leafy vegetables, berries) reported in the literature, since the results from different research groups using different hydrolysis and analysis methods may vary considerably.

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