Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1054 (2004) 181–193
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Characterisation of flavonols in broccoli (*Brassica oleracea* L. var. *italica*) by liquid chromatography–UV diode-array detection–electrospray ionisation mass spectrometry

F. Vallejo, F.A. Tomás-Barberán, F. Ferreres*

Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, P.O. BOX 164, E-30100 Espinardo, Murcia, Spain

Available online 28 July 2004

Abstract

The flavonoid composition of broccoli inflorescences has been studied by LC/UV-DAD/ESI-MS^n. A large number of hydroxycinnamic acid esters of kaempferol and quercetin glucosides has been characterised. The structures of the flavonoid glycosides were analysed after alkaline hydrolysis, and were identified as 3-sophoroside/sophorotrioside-7-glucoside/sophoroside of kaempferol, quercetin and isorhamnetin (this last found in trace amount). These complex quercetin and isorhamnetin glucosides have not been previously characterised in nature. In addition, several less complex glucosides based on the same aglycones have been identified. The effect of sugar substitution and acylation on chromatographic mobility and ESI ionisation and fragmentation are discussed.

Keywords: Food analysis; Broccoli; Flavonoids; Acylated glycosides

1. Introduction

Recent studies have shown the relevant biological activities of flavonoids against various types of cancer and cardiovascular diseases [1–3]. Broccoli inflorescence is a good source of health promoting compounds since it contains glucosinolates, flavonoids and hydroxycinnamic acids, vitamin C and other minor compounds.

Scarce information is available regarding broccoli polyphenols identification. Several studies have previously reported on broccoli flavonoids but none has described more than five flavonol glycosides and also without identifying the combinations with hydroxycinnamic acids and with glycosylations up to two glucoses [5,6]. A recent study on cauliflower flavonoids showed that Brassicaceae species contain very complex flavonoids with up to five sugar residues on the flavonol nucleus [7]. Although broccoli has been reported to be one of the main flavonol sources in the diet, very little is known about the structures, as they are combination of hydroxycinnamic acids with highly glycosylated flavonoids, that make their identification very difficult by classical methods [4]. HPLC coupled to MS–MS–DAD has shown to be a very useful method in the identification of these complex flavonoids, as have been recently reported in cauliflower leaves [7].

The purpose of this work was to identify the polyphenols from broccoli inflorescences by high-performance liquid chromatogram coupled with on-line mass spectrometry with electrospray ionisation source (LC/UV-DAD/ESI-MS^n).

2. Experimental

2.1. Plant material

The broccoli (*Brassica oleracea* L. var. *italica*) (Marathon cv.) florets used for the assays were obtained from the Instituto Murciano de Investigacion y Desarrollo Agraroalimentario (IMIDA, Murcia, Spain). At optimum commercial

* Corresponding author. Tel.: +34 9683 963 24; fax: +34 9683 962 13.
E-mail address: federico@cebas.csic.es (F. Ferreres).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.05.045

maturity, uniform size plants, free from insect and/or mechanical damage, were selected at random and immediately transported to the laboratory where the edible portions were cut. Samples of 20 g from each plant per replicate were combined, weighed, frozen at −70 °C and freeze-dried. This tissue was ground into a fine powder and stored at −20 °C for further analysis.

2.2. Phenolic extraction

It was carried out according to methodology previously described for cauliflower [7]. Thus, freeze-dried samples (70 g) were extracted by boiling with 3 L of distilled water for 60 min. This aqueous extract was then mixed with Amberlite XAD-2 particles (Supelco, Hellefonte, PA) in sufficient amount to fill a column of 55 cm × 4 cm and stirred for 4 h at room temperature to retain the phenolic compounds on the surface of the non-ionic Amberlite particles [8]. The Amberlite particles were packed into a chromatography column, washed with distilled water (5 L), and the absorbed phenolics eluted with methanol (1 L). The methanol extract was then taken to dryness and redissolved in methanol/water (1:1, v/v) and the phenolic fraction purified in a Sephadex LH-20 column (40 cm × 3 cm). The phenolic fraction was followed under UV light (254 and 360 nm). This phenolic fraction was freeze-dried and used for the HPLC and hydrolysis analyses.

2.3. Alkaline and acid hydrolysis

This was achieved according to previously reported methodology described for cauliflower with slight changes [7]. This was performed by adding 1 mL 4N NaOH to the hydroalcoholic phenolic extract purified as described above (1 mL) and keeping the mixture for 16 h at room temperature in a stoppered test tube under N_2 atmosphere. After this step, the alkaline hydrolysis products were acidified with concentrated HCl (up to pH 1–2) and directly analysed by LC/UV-DAD/ESI-MS^n. Total acid hydrolysis was carried out by adding 1 mL 4N HCl to 1 mL of the hydroalcoholic phenolic extract and this solution was kept in a stoppered test tube, incubated for 30 min at 85 °C and directly analysed by LC/UV-DAD/ESI-MS^n.

2.4. LC/UV-DAD/ESI-MS^n analyses

Chromatographic analyses were carried out on a LiChroCART column (250 mm × 4 mm, RP-18, 5 μm particle size, LiChrospher® 100 stationary phase, Merck, Darmstadt, Germany) protected with a LiChroCART guard column (4 mm × 4 mm, RP-18, 5 μm particle size, Merck, Darmstadt, Germany). The mobile phase consisted of two solvents: water–formic acid (0.1%) (A) and methanol (B). For studying both free flavonol glycosides and the corresponding acylated derivatives a linear gradient starting with 20% B was installed to reach 50% B at 35 min and 80% B at 37 min. On the other hand, for the analysis of the acids and the aglycones obtained after acid hydrolysis, a linear gradient starting with 15% B and reaching 65% B at 50 min was used to reach 80% B at 52 min. The flow rate was 1 mL min^{-1}, and the injection volume ranged 10–50 μL depending on the compound and extract assayed. Spectral data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 340 nm for glycosides and acylated derivatives, and at 330 and 360 nm for hydroxycinnamic acids and flavonoid aglycones, respectively. The LC/UV-DAD/ESI-MS^n analyses were carried out in an Agilent HPLC 1100 series equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by a ChemStation software (Agilent, v. 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionisation interface and was controlled by LCMSD software (Agilent, v. 4.1). The ionisation conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebuliser pressure and flow rate of nitrogen were 65.0 psi and 11 L min^{-1}, respectively. The full scan mass covered the range from *m/z* 200 up to 2000 for free glycosides and acylated derivatives and from *m/z* 90 up to 400 for acids and aglycones. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionisation mode. MS^n is carried out in the automatic mode on the more abundant fragment ion in MS^{n-1}.

Tables 1–4 show the most frequent ions which characterise the fragmentation of these flavonoid *O*-glycosides. Other ions were found but they have not been included due to their low significance on the MS behaviour ions. The classical nomenclature [9] for glycoconjugates was adopted to designate the fragment ions. Ions $^{k,l}X_j$, Y_j^n, Z_j^n represent those fragments still containing the flavonoid aglycone, where *j* is the number of the interglycosidic bond broken, counted from the aglycone, *n* represents the position of the phenolic hydroxyl where the oligosaccharide is attached, and the *k* and *l* denote the cleavage within the carbohydrate rings.

The ions obtained as a consequence of a second oligosaccharide fragmentation have been labelled according to previous reports [10,11]. Thus, ions obtained from the ion Y_0^{7-} ($-MS^3[(M-H) \rightarrow Y_0^7]^-$) have been labelled starting with the ion Y_0^{7-} and followed by the resultant MS^3 ion, e.g. the ion $Y_0^7Y_2^{3-}$ (MS^3 of compound **3**) (Table 1, Fig. 3, Scheme 1) denotes the loss of terminal sugar of the triglycoside in the position 3 (Y_2^{3-}) from the fragmentation of ion Y_0^{7-} (total loss of a glycosylation in the position 7). The losses indicated in the MS^3 scan show that the fragment

Table 1
R_t, UV, –MS: $[M-H]^-$, $-MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow Y_0^7]\ (-162/-324)]^-$ data of flavonoid-3-*O*-Soph/Sophtr-7-*O*-Glc/Soph

Compounds	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$-MS^2[M-H]^-$ (*m/z*) (%)	$-MS^3[(M-H) \rightarrow Y_0^7]^-$ (*m/z*) (%)				
				Y_0^{7-}	$Y_0^{70,2}X^-$	$Y_0^7Y_2{}^{3-}$	$Y_0^7Y_1^{3-}$	$Y_0^7Z_1^{3-}$	$Y_0^7Y_0^{3-}$
				Flavonoid-3-*O*-sophorotrioside-7-*O*-glucoside/sophoroside					
				(−162/−324)	(−120)	(−162)	(−324)[a]	(−342)	(−486)
1 Querc-3-Sophtr-7-Glc	8.1	256, 266sh, 354	949.8	787 (100)	667 (100)		463 (50)[a]	445 (89)	301 (30)
3 Querc-3-Sophtr-7-Soph	9.0	256, 266sh, 352	1111.8	787 (100)	667 (100)	625 (22)	463 (16)[a]	445 (83)	301 (68)
4 Kaempf-3-Sophtr-7-Glc	9.6	266, 316sh, 348	933.8	771 (100)		609 (33)		429 (38)	285 (100)
5 Kaempf-3-Sophtr-7-Soph	10.4	266, 316sh, 349	1095.9	771 (100)		609 (18)	447 (32)[a]	429 (59)	285 (100)
7 Isorhmnt-3-Sophtr-7-Glc[b]	10.9		963.5	801 (100)	681 (37)			459 (66)	314 (100)[c]
9 Isorhmnt-3-Sophtr-7-Soph[b]	11.9		1125.5	801 (100)	681 (90)	639 (60)		459 (80)	315 (100)
				Flavonoid-3-*O*-sophoroside-7-*O*-glucoside/sophoroside					
				(−162/−324)	(−120)		(−162)	(−180)	(−324)
2 Querc-3-Soph-7-Glc	8.8	coelution with **3**	787.7	625 (100)			463 (52)	445 (60)	300 (100)[c]
6 Kaempf-3-Soph-7-Glc	10.5	coelution with **5**	771.6	609 (100)			489 (28)	429 (25)	284 (100)[c]
8 Kaempf-3-Soph-7-Soph	11.2	266, 318sh, 352	933.8	609 (100)	489 (29)			429 (18)	285 (100)

[a] The ion $[Y_0^7Y_1^3]^{-\bullet}$ (Y_0^7-324) has not been observed in the attached chromatograms, however it has been observed as an abundant peak in other chromatograms.
[b] Compounds in trace amounts and hidden by other. Their UV spectra have not been observed property.
[c] Fragments from homolytic cleavage of the glycosidic bond ($[Y_0^7Y_0^3 - H]^{-\bullet}$) [16].

Table 2
R_t, UV, –MS: $[M-H]^-$, $-MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow Y_0^3]^-$ data of flavonoid-3-*O*-Glc-7-*O*-Soph, -3,7-di-*O*-Glc, -3-*O*-Soph and -3-*O*-Glc

Compounds	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$-MS^2[M-H]^-$ (*m/z*) (%)				$-MS^3[(M-H) \rightarrow Y_0^3]^-$ (*m/z*) (%)	
				Flavonoid-3-*O*-glucoside-7-*O*-sophoroside					
					Y_0^{3-} (−162)	Y_0^{7-} (−324)	[Aglic−H]$^-$ (−486)	$Y_0^3Y_1^{7-}$ (−162)	$Y_0^3Y_0^{7-}$ (−324)
11 Querc-3-Glc-7-Soph	14.6	257, 266sh, 354	787.9		625 (26)	463 (100)	301 (14)	463 (12)	301 (100)
13 Kaempf-3-Glc-7-Soph	17.7	266, 320sh, 350	771.5		609 (26)	447 (100)	285 (30)		
15 Isorhmn-3-Glc-7-Soph	18.7	255, 266sh, 350	801.9		639 (39)	477 (100)	315 (13)		
				Flavonoid-3,7-di-*O*-glucoside					
				$^{0,2}X^-$ (−120)	Y_0^{7-}/Y_0^{3-} (−162)		[Aglic−H]$^-$ (−324)		
10 Querc-3,7-diGlc	13.8	255, 266sh, 294sh, 354	625.7	505 (9)	463 (100)		301 (34)		
12 Kaempf-3,7-diGlc	17.1	266, 318sh, 349	609.9	489 (24)	447 (100)		285 (33)		
14 Isorhmn-3,7-diGlc	18.2	255, 268sh, 354	639.7	519 (11)	477 (100)		315 (17)		
				Flavonoid-3-*O*-sophoroside					
				$^{0,2}X^-$ (−120)	Y_1^{3-} (−162)	Z_1^{3-} (−180)	Y_0^{3-} (−324)		
16 Querc-3-Soph[a]	21.6	257, 267sh, 293, 355	625.6	505 (4)	463 (17)	445 (54)	300 (100)[b]		
17 Kaempf-3-Soph	25.4	266, 294sh, 350	609.7	489 (2)	447 (21)	429 (55)	285 (100)		
				Flavonoid-3-*O*-glucoside					
							Y_0^{3-} (−162)		
18 Querc-3-Glc	28.7	255, 266sh, 355	463.9				301 (100)		
19 Kaempf-3-Glc	33.2	266, 294sh, 349	447.9				285 (100)		

[a] Compounds in trace amounts and hidden by other. UV spectra from deacylated extract chromatogram.
[b] Fragments from homolytic cleavage of the glycosidic bond ($[Y_0^7Y_0^3 - H]^{-\bullet}$) [16].

came from the trapped and fragmented ion (Y_0^{7-}) and not from the deprotonated molecular ion.

3. Results and discussion

The broccoli phenolic fraction was analysed by HPLC/UV-DAD and the characteristic sinapoyl/feruloyl gentiobiosides were detected [4] (Fig. 1). In addition a large number of flavonoid compounds was detected in the extract and their UV spectra suggested that they were mainly acylated derivatives in which sinapic, ferulic, caffeic and *p*-coumaric acids were linked to the flavonoid-glycoside molecules. Their characteristic UV spectra showed a flavonol spectrum overlapped with a hydroxycinnamic acid spectrum with a broad maximum around 330 nm, and the structural information obtained by these spectra was much smaller than that obtained from the UV spectra of non-acylated flavonoids or hydroxycinnamic acid derivatives. After alkaline hydrolysis the flavonoid pattern was much simpler (Fig. 2) indicating that the naturally occurring flavonoids were different acylated derivatives of the same flavonol glycosides. By this reason it was decided to start the structural study with the analysis of the aglycones and the deacylated glycosides.

3.1. Flavonoid aglycones and hydroxycinnamic acids

After acid hydrolysis of the broccoli phenolic extract the LC/UV-DAD/ESI-MS^n study, showed that the main acids present were sinapic, *p*-coumaric, caffeic and ferulic, and that the flavonoid aglycones were kaempferol as the main compound (3,5,7,4′-tetrahydroxyflavone), quercetin (3,5,7,3′,4′-pentahydroxyflavone) and isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone), this last in trace amounts. They were identified by their UV spectrum, MS analysis and co-chromatography with standards.

3.2. Deacylated glycosides

After saponification (Fig. 2), a large number of flavonoid hexosides (most probably glucosides due to filogenetic similitude with the cauliflower compounds) have been characterised. Glycosides of quercetin, isorhamnetin and kaempferol, with the following glycosylation patterns: 3-glucoside; 3,7-diglucoside; 3-sophoroside; 3-sophoroside-7-glucoside; 3-glucoside-7-sophoroside; 3-sophoroside-7-sophoroside; 3-sophorotrioside-7-glucoside and 3-sophorotrioside-7-sophoroside.

The tentative assignation of the glycosylation positions over the flavonol nucleus has been achieved taking into account that the hydroxyl at 3 position was always blocked (UV spectra, band I ≅ 348–355 nm) (Tables 1–2) and released after acid hydrolysis (aglycones UV spectra, band I ≅ 367–371 nm); and that glycosylation on the hydroxyl at 7 position once the hydroxyl at 3 position is substituted, is

Table 3
R_t, and –MS: $[M-H]^-$, $-MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow (M-H-162)]^-$ data of acylated derivatives from flavonoid-3-*O*-sophoroside/sophorotrioside-7-*O*-glucoside

Compounds[a]	R_t (min)	$[M-H]^-$ (*m/z*)	$-MS^2[M-H]^-$ (*m/z*) (%)												$-MS^3[(M-H) \rightarrow (M-H-162)]^-$ (*m/z*) (%)									
			−146 (-*p*.Coum)	−162 (-Glc)	−176 (-Fer)	−206 (-Sinp)	−308 (-G-*p*C)	−324 (-G-C)	−338 (-G-F)	−354 (-G-MC)	−368 (-G-S)	−470 (-G-C-*p*C)	−486 (-G-C-C)	−530 (-G-C-S)	−544 (-G-F-S)	−574 (-C-S-S)	−146 (-*p*.Coum)	−162 (-Caf)	−176 (-Fer)	−192 (-MeOCaf)	−206 (-Sinp)	−308 (-C-*p*C)	−324 (-C-C)	−368 (-C-S)	−382 (-F-S)	−412 (-S-S)
Acylated derivatives from **1**: quercetin-3-*O*-sophorotrioside-7-*O*-glucoside																									
20 1-Caf	9.2	1111.7		949 (100)				787 (59)										787 (100)							
21 1-diCaf	9.5	1273.6		1111 (100)				949 (30)				787 (94)										787 (100)			
22 1-Sinp	12.0	1155.6		993 (100)		949 (49)														787 (100)					
23 1-Fer	12.4	1125.5		963 (100)	949 (37)				787 (71)			787 (61)							787 (100)						
24 1-*p*.Coum	12.8	1095.7	949 (57)	933 (100)			787 (84)										787 (100)								
25 1-Caf/Sinp	26.0	1317.6		1155 (100)				993 (73)			949 (4)		787 (20)					993 (100)					787 (24)		
26 1-diSinp	27.8	1361.9		1199 (100)		1155 (31)					993 (90)				787 (30)						993 (100)				787 (41)
27 1-Fer/Sinp	28.7	1331.8		1169 (100)	1155 (29)	1125 (7)			993 (65)		963 (15)			787 (9)						993 (100)	963 (31)			787 (32)	
Acylated derivatives from **4**: kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside																									
28 4-MeOCaf	9.9	1125.7		963 (100)						771 (26)											771 (100)				
29 4-Sinp	13.3	1139.9		977 (100)							771 (7)										771 (100)				
30 4-Fer	13.9	1109.5		947 (100)					771 (7)											771 (100)					
31 4-Caf/Sinp	27.4	1301.6		1139 (100)				977 (12)			933 (2)		771 (3)					977 (100)			933 (17)		771 (30)		
32 4-diSinp	29.1	1345.9		1183 (100)							977 (7)				771 (2)						977 (100)				771 (16)
Acylated derivatives from **2**: quercetin-3-*O*-sophoroside-7-*O*-glucoside																									
33 2-Caf	8.9	949.6		787 (100)				625 (52)										625 (100)							
34 2-Fer	12.0	963.7		801 (100)	787 (25)				625 (23)										625 (100)						
35 2-*p*.Coum	12.4	933.6	787 (8)	771 (100)			625 (27)										625 (100)								
Acylated derivatives from **6**: kaempferol-3-*O*-sophoroside-7-*O*-glucoside																									
36 6-Caf	10.8	933.9		771 (100)														609 (100)							
37 6-Caf/*p*.Coum	14.8	1079.6		917 (100)			771 (3)	755 (86)				609 (7)					771 (100)	755 (7)				609 (5)			

1: quercetin-3-*O*-sophorotrioside-7-*O*-glucoside. **2**: quercetin-3-*O*-sophoroside-7-*O*-glucoside. **4**: kaempferol-3-*O*-sophorotrioside-*O*-7-glucoside. **6**: kaempferol-3-*O*-sophoroside-7-*O*-glucoside.
[a] G (Glc): Glucosyl. *p*C (*p*.Coum): *p*.Coumaroyl. F (Fer): Feruloyl. S (Sinp): Sinapoyl. MC (MeOCaf): MethoxyCafeoyl.

Table 4
R_t, –MS: $[M-H]^-$, $-MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow (M-H\text{-}324)]^-$ data of acylated derivatives from **5**: kaempferol-3-*O*-sophorotrioside-7-*O*-sophoroside

Compounds[a]	R_t (min)	$[M-H]^-$ (*m/z*)	$-MS^2[M-H]^-$ (*m/z*) (%)									$-MS^3[(M-H) \rightarrow (M-H\text{-}324)]^-$ (*m/z*) (%)								
			−324 (-Soph)	−470 (-Soph-pC)	−486 (-Soph-C)	−500 (-Soph-F)	−516 (-Soph-MC)	−530 (-Soph-S)	−692 (-Soph-C-S)	−706 (-Soph-F-S)	−722 (-Soph-MC-S)	−736 (-Soph-S-S)	−146 (-p.Coum)	−162 (-Caf)	−176 (-Fer	−192 (-MeOCaf)	−206 (-Sinp)	−368 (-C-S)	−382 (-F-S)	−398 (-MC-S)	−412 (-S-S)
38 5-MeOCaf	10.4	1287.6	963 (100)				771 (89)									771 (100)					
39 5-Caf	11.3	1257.7	933 (100)		771 (67)									771 (100)							
40 5-Sinp	12.8	1301.5	977 (100)					771 (49)									771 (100)				
41 5-Fer	14.4	1271.7	947 (100)			771 (53)									771 (100)						
42 5-*p*.Coum	15.3	1241.6	917 (100)	771 (39)							771 (14)		771 (100)							771 (62)	
43 5-MeOCaf/Sinp	25.7	1493.6	1169 (100)				977 (39)									977 (100)	963 (32)				
44 5-Caf/Sinp	26.8	1463.7	1139 (100)		977 (19)			933 (4)	771 (12)					977 (100)			933 (16)	771 (38)			
45 5-diSinp	28.2	1507.7	1183 (100)					977 (47)				771 (14)					977 (100)				771 (25)
46 5-Fer/Sinp	29.1	1477.7	1153 (100)			977 (24)		947 (7)		771 (4)					977 (100)		947 (90)		771 (32)		

5: kaempferol-3-*O*-sophorotrioside-7-*O*-sophoroside.
[a] G (Glc): Glucosyl. *p*C (*p*.Coum): *p*.Coumaroyl. F (Fer): Feruloyl. S (Sinp): Sinapoyl. MC (MeOCaf): MethoxyCafeoyl.

usually found in cauliflower flavonoids [7,12–14] are different glucosidic combinations of kaempferol on the hydroxyls at 3 and 7 positions, and it is not unexpected that the broccoli glycosides have the same glycosylation pattern.

The assignation of different sugar substitutions (mono-, di- or triglicosides) on the flavonoid hydroxyls has been carried out in accordance to previous studies focused on the MS analyses of similar compounds from cauliflower [7] and other plant species [11]. These studies showed that the first fragmentation of the $[M-H]^-$ ion is always due to the breakdown of the *O*-glycosidic bond at 7 position leading to a base peak ion Y_0^{7-} $[M-H\text{-}162]^-$ for monohexosides or $[M-H\text{-}324]^-$ for dihexosides, and the remaining sugars on the flavonoid molecule should be linked to the hydroxyl at the 3 position of the flavonol nucleus.

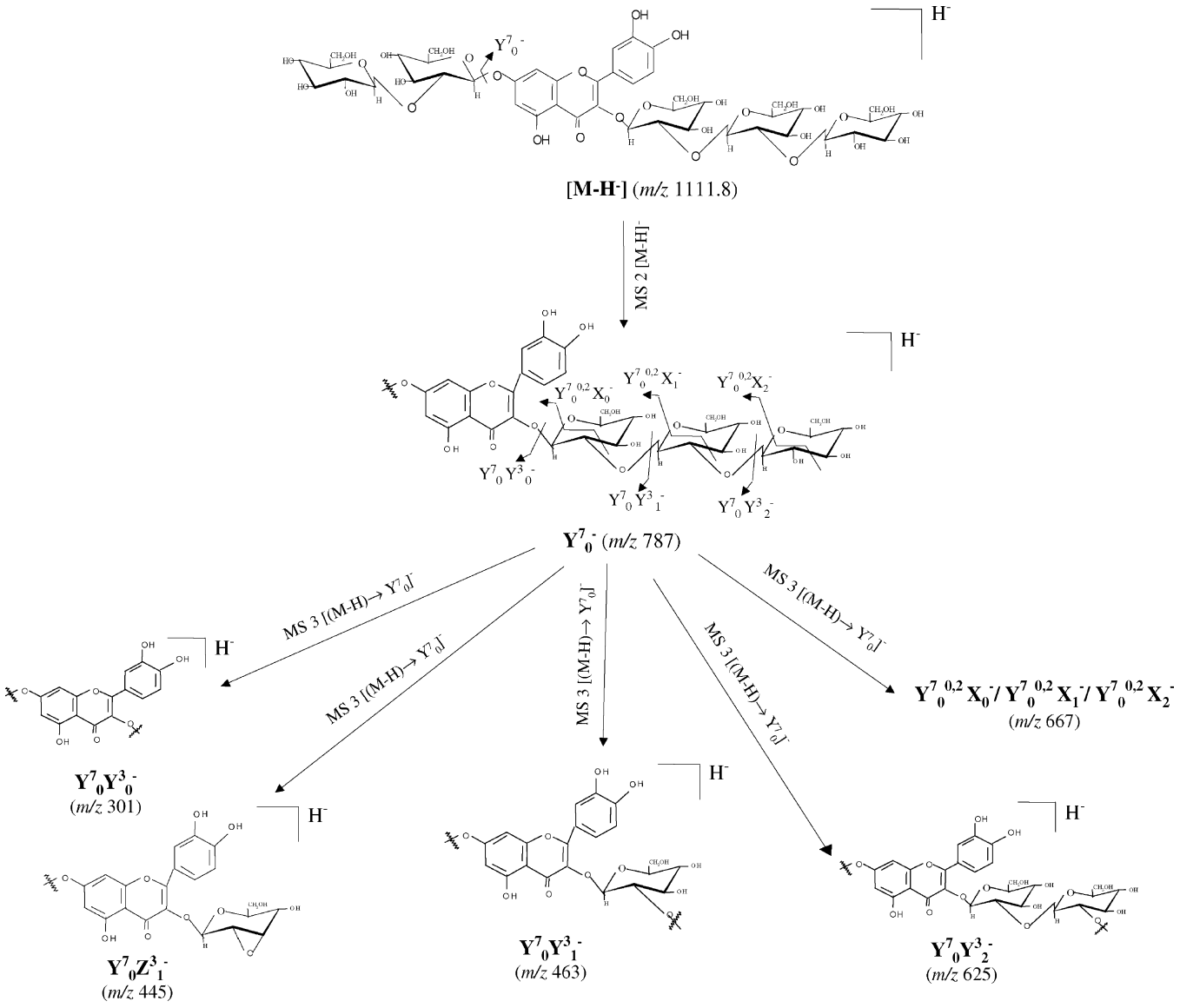
The interglucosidic linkages were identified in all cases as $1 \rightarrow 2$ by the MS fragmentation. Thus, in glycosides showing this interglycosidic linkage a gradual oligosaccharide breakdown yielding Y_n^- or Z_n ions was observed, while in $1 \rightarrow 6$ linkages these intermediate ions were not detected [11,15].

In broccoli flavonoids this intermediate fragmentation has been observed in all cases showing that the interglycosidic linkage was $1 \rightarrow 2$ in the glycosides at the 3 position. However, in the oligosaccharides at 7 position the presence of the characteristic $1 \rightarrow 2$ fragments could not always be observed due to the high lability of the glycosidic linkage at this position. Nevertheless, the $-MS^2$ analysis of compound **11** (quercetin-3-Glc-7-Soph) shows that the interglycosidic linkage of the oligosaccharide at 7 position is also $1 \rightarrow 2$ as it is demonstrated below. This data together with the glycosides fragmentation at position 3 of the flavonoids and the comparison with cauliflower compounds [12–14] suggests that both interglycosidic linkages of the sugar residues at 3 and 7 positions, would be assigned as $1 \rightarrow 2$.

3.2.1. Flavonoid-3-O-sophoroside/sophorotrioside-7-O-glucoside/sophorosides

In the $-MS^2[M-H]^-$ of flavonoid compounds with glucose or sophorose substitutions at position 7, and with sophorose or sophorotriose substitutions at position 3 (Table 1), only the peak produced by the total loss of the sugar residues at 7 (Y_0^{7-}**)** ($[M-H\text{-}162]^-$ or $[M-H\text{-}324]$) was observed (Table 1, Fig. 3, Scheme 1).

In the $-MS^3[(M-H) \rightarrow Y_0^7]^-$ the ions produced by the fragmentation of the oligosaccharides at the position 3 are clearly observed (Scheme 1). This was due to the partial loss of sugars $[Y_0^{70,2}X]^-$ (−120 amu) or due to losses of one or more glucose residues, which could lead to different ions (Table 1): $[Y_0^7Y_2^3]^-$ (−162 amu), $[Y_0^7Y_1^3]^-$ (−324 amu), $[Y_0^7Z_1^3]^-$ (−342 amu) and also $[Y_0^7Y_0^3]^-$ (−486 amu) the total loss of the glycosidic fraction leading to the aglycone ion, which are characteristic of the interglycosidic linkage $1 \rightarrow 2$ [11]. In Fig. 3 the MS spectra of compound **3** is shown. In the $-MS^2$, both the loss of the sugar residue at 7, and the ions of the partial fragmentation of the sophorotriose at 3



Scheme 1. ESI-MS^n fragmentation pathway of quercetin-3-sophorotrioside-7-sophoroside (**3**).

position in the –MS^3 have been observed. The structures of the different characterised flavonols are shown in Table 1.

As far we are aware, compounds **1**, **3**, **7** and **9** have never been characterised in nature. Similar compounds with kaempferol as aglycone (**4** and **5**) have recently been characterised in cauliflower [7].

3.2.2. *Flavonoid-3-O-glucoside-7-O-sophorosides*

In the $-MS^2[M-H]^-$ of this group of compounds (Table 2), in addition to the presence of the base peak Y_0^{7-} ($M-$H-324) (loss of glycosidic residue at 7 position), the ion produced by the loss of 162 amu from the $[M-H]^-$ ion was observed (Fig. 4). This ion will be mainly produced from the fragmentation of the glycosidic residue at 3 ($Y_{0^3}{}^-$), since the contribution of the ion $Y_{1^7}{}^-$ will be rather irrelevant. The fragmentation of this ion ($-MS^3[(M-H) \rightarrow Y_0^3]^-$) in compound **11** (Querc-3-Glc-7-Soph) yielded the ion $[Y_0^3Y_1^7]^-$ (*m*/*z* 463, 12%) (Table 2, Fig. 4, Scheme 2) which indicated an interglycosidic bond 1 → 2 in the oligosaccharide at the 7 position.

Regarding the rest remaining compounds (**13** and **15**) and the oligosaccharides at 7 (described above), it could not be possible to confirm the nature of the interglycosidic bond as 1 → 2 under the MS–MS conditions, due to the lability of the *O*-glycosidic bond at the 7 position. However, as we indicated before by comparison with the cauliflower compounds, we expect that the oligosaccharides at 7 have the same interglycosidic linkage than the oligosaccharides at 3 position. In this group the following compounds have been characterised: quercetin-3-*O*-glucoside-7-*O*-sophoroside (**11**), kaempferol-3-*O*-glucoside-7-*O*-sophoroside (**13**) and isorhamnetin-3-*O*-glucoside-7-*O*-sophoroside (**15**).

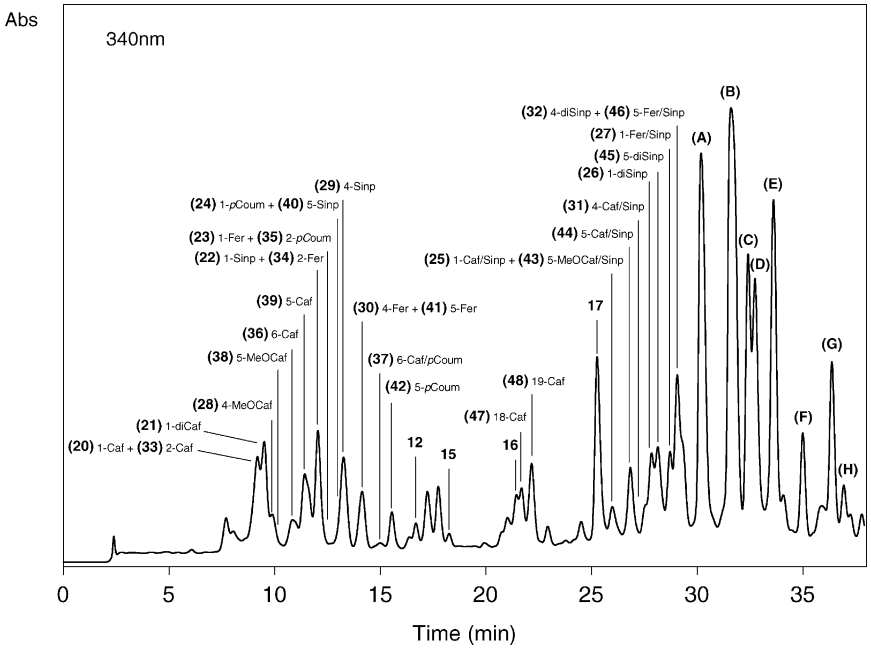


Fig. 1. HPLC chromatogram of broccoli native flavonoids and glycosyl hydroxycinnamic acid compounds. Flavonoids acylated with hydroxycinnamic acid derivatives have been labelled according to their original flavonoid glycoside number (see Fig. 2) followed by the attached acid. Sinapoyl/feruloyl gentiobioside compounds. (A) 1,2-Disinapoylgentiobiose; (B) 1-sinapoyl-2-feruloylgentiobiose; (C) 1,2-diferuloylgentiobiose; (D) 1,2,2′-trisinapoylgentiobiose; (E) 1,2′-disinapoyl-2-feruloylgentiobiose; (F) 1-sinapoyl-2,2′-diferuloylgentiobiose; (G) 1,2,2′-trisinapoylgentiobiose; (H) 1,2,2′-triferuloylgentiobiose. Compounds numbered according to HPLC eluction order [4].

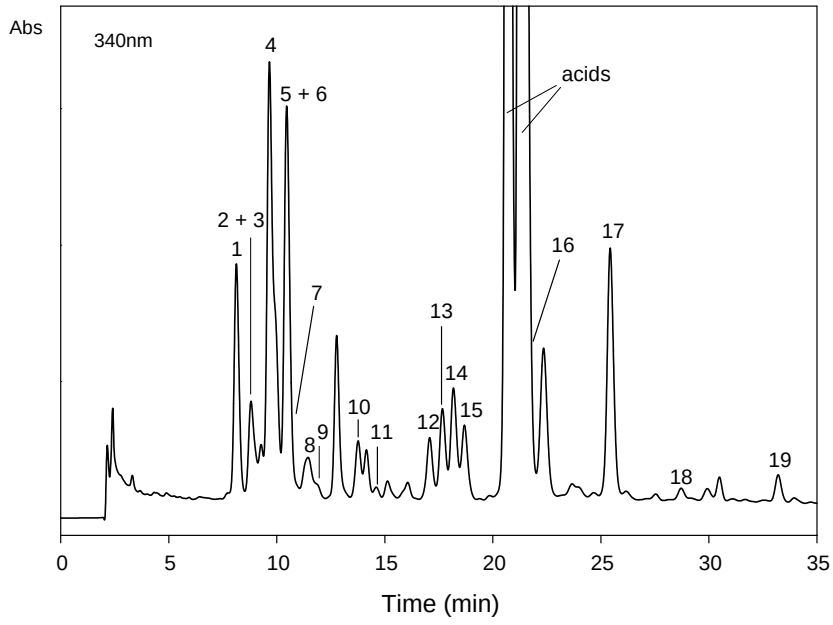


Fig. 2. HPLC chromatogram of broccoli flavonols after alkaline hydrolysis. (1) Quercetin-3-*O*-sophorotrioside-7-*O*-glucoside; (2) quercetin-3-*O*-sophoroside-7-*O*-glucoside; (3) quercetin-3-*O*-sophorotrioside-7-*O*-sophoroside; (4) kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside; (5) kaempferol-3-*O*-sophorotrioside-7-*O*-sophoroside; (6) kaempferol-3-*O*-sophoroside-7-*O*-glucoside; (7) isorhamnetin-3-*O*-sophorotrioside-7-*O*-glucoside; (8) kaempferol-3-*O*-sophoroside-7-*O*-sophoroside; (9) isorhamnetin-3-*O*-sophorotrioside-7-*O*-sophoroside; (10) quercetin-3,7-di-*O*-glucoside; (11) quercetin-3-*O*-glucoside-7-*O*-sophoroside; (12) kaempferol-3,7-di-*O*-glucoside; (13) kaempferol-3-*O*-glucoside-7-*O*-sophoroside; (14) isorhamnetin-3,7-di-*O*-glucoside; (15) isorhamnetin-3-*O*-glucoside-7-*O*-sophoroside; (16) quercetin-3-*O*-sophoroside; (17) kaempferol-3-*O*-sophoroside; (18) quercetin-3-*O*-glucoside; (19) kaempferol-3-*O*-glucoside.

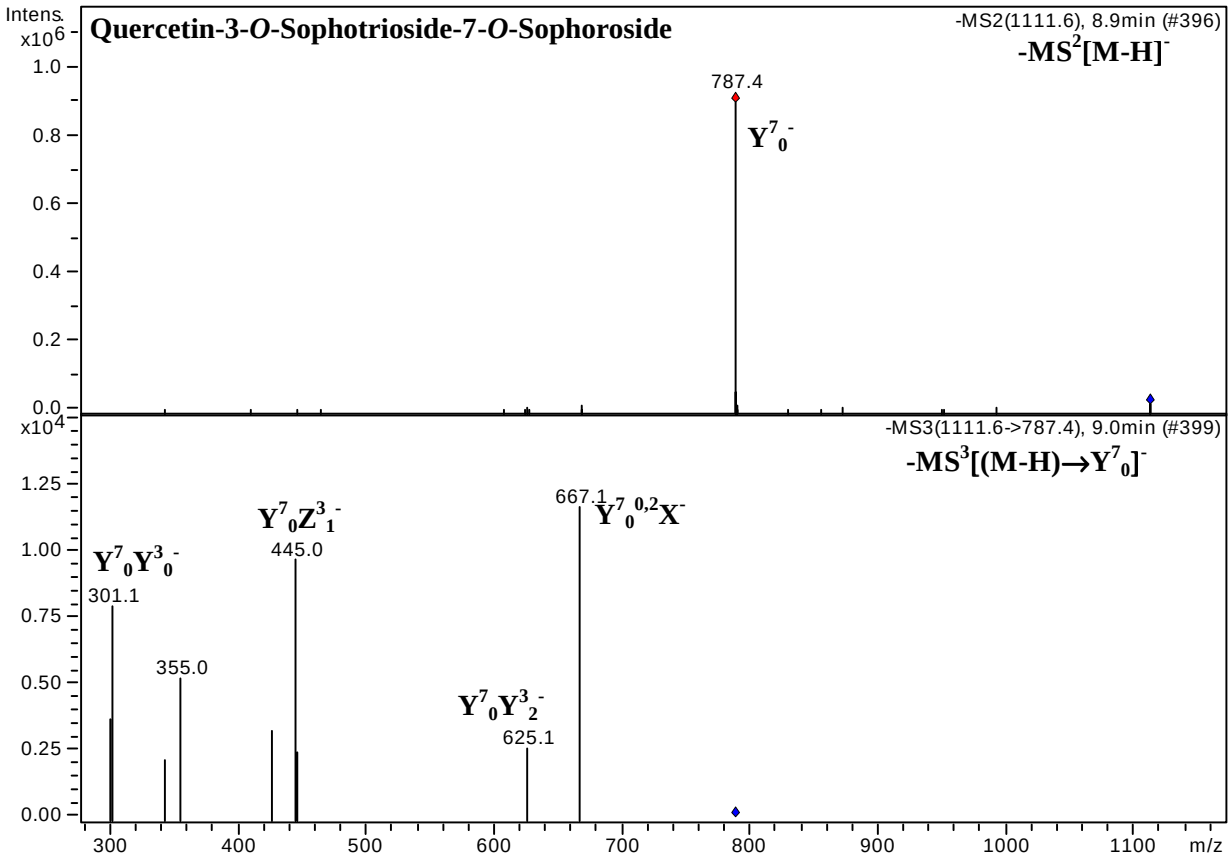


Fig. 3. $MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow Y_0^7]^-$ analysis of quercetin-3-*O*-sophorotrioside-7-*O*-sophoroside (**3**).

3.2.3. Flavonol 3,7-di-O-glucosides, 3-O-sophorosides and 3-O-glucosides

According to Ferreres et al. [11], in 3,7-diglucosides a base peak ion at $[M-H\text{-}162]^-$ in the $-MS^2[M-H]^-$ mode is always observed (Table 2). This ion was obtained as a result of the loss of the glucose at 3 position ($Y_{0^3}{}^-$) and mainly due to the loss of the glucose at 7 position ($Y_{0^7}{}^-$, the easiest breakdown position). Moreover, the occurrence of the fragment ion ${}^{0,2}X^-$ ($M-H\text{-}120$) by partial sugar breakdown, and the ion which characterises the aglycone ($M-H\text{-}324$) were also detected. In this group, the 3,7-di-*O*-glucosides of quercetin, kaempferol and isorhamnetin were identified (compounds **10**, **12** and **14**, respectively).

The $-MS^2[M-H]^-$ fragmentation of 3-sophorosides (Table 2) is the typical for diglycosides at 3 position with an interglycosidic linkage (1 → 2) [11]. The characteristic ions $Y_{1^3}{}^-$ and $Z_{1^3}{}^-$, and the base peak ion $Y_{0^3}{}^-$ ($M-H\text{-}324$) due to the aglycone were observed. Their high relative abundance (base peak) differentiates this group from the group described above (flavonoid-3,7-di-*O*-glucoside). In this group the 3-*O*-sophoroside derivatives of quercetin and kaempferol (**16** and **17**) were identified.

The 3-glucosides of quercetin (**18**) and kaempferol (**19**) (Table 2) have also been characterised. These are rather common flavonoids in nature.

3.3. Acylated glycosides

Naturally occurring broccoli flavonoid compounds (Fig. 1) are shown in Tables 3 and 4. All of them are acylated with hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic and sinapic). In the MS fragmentation of compounds **28**, **38** and **43**, the losses corresponding to an unidentified acid residue with 30 amu higher than caffeic acid has also been detected, and this has been tentatively identified as methoxycaffeic acid (−192 amu). Furthermore, compounds **12** and **15**–**17** without acylation have also been characterised. Acylated compounds have been grouped in Tables 3 and 4 depending on their glycosidic combinations: **1**, **2** and **4**–**6** (Table 1). They have been labelled by the number of this glycoside followed by the acid or acids which are linked to the flavonoid molecule. The UV spectra of the glycosides are not included in the tables as this information is not relevant because the flavonoid glycoside spectrum is overlapped with the acid spectrum. They are characterised by a maximum with a high absorption ≅330 nm (325–335 nm), and eventually can show a little maximum that coincide with the flavonoid band II (≅255–268 nm). Furthermore, several compounds co-elute in the same peak, and therefore their UV spectra are not very useful from the identification point of view.

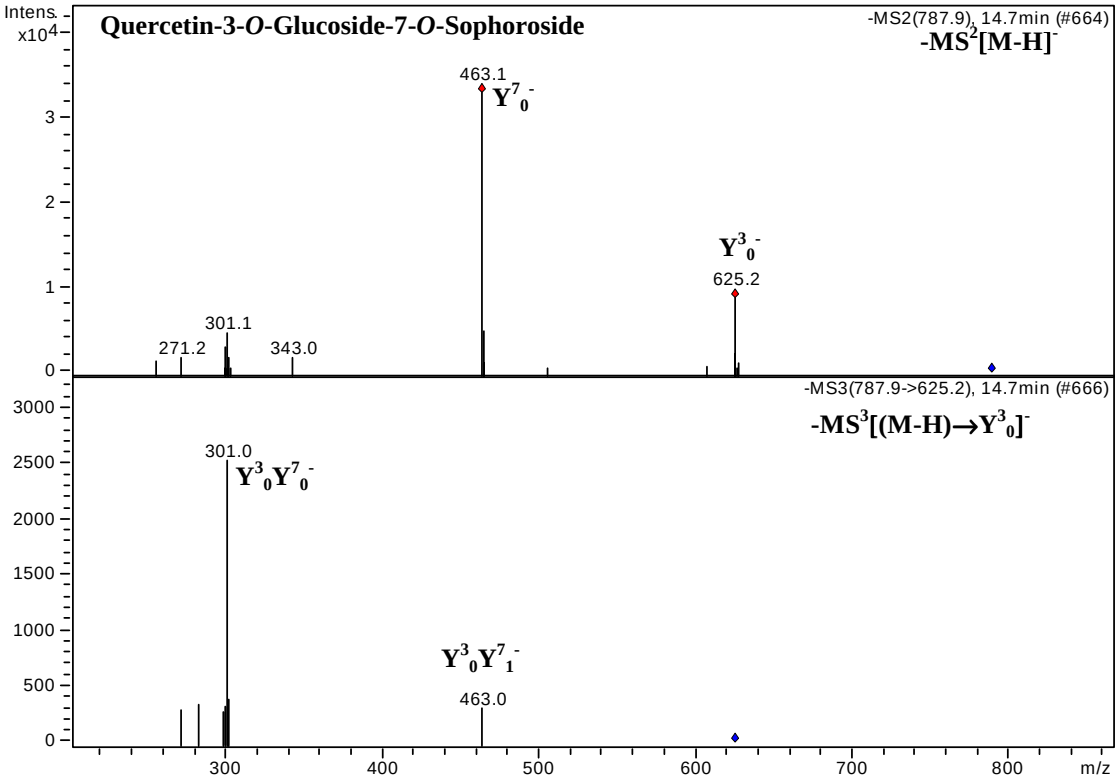


Fig. 4. $MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow Y_0^3]^-$ analysis of quercetin-3-*O*-glucoside-7-*O*-sophoroside (**11**).

The MS data (Tables 3 and 4) include the $-MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow (M-H\text{-}162)]^-$ (Table 3)/$-MS^3[(M-H) \rightarrow (M-H\text{-}324)]^-$ (Table 4) spectra. These spectra show fragments which correspond to losses of both sugar at the 7 position and acyl residues (Figs. 5 and 6). Thus, among different ions, the corresponding to the aglycone glycosylated at 3 was also observed. The fragmentation of this ion (Figs. 5c and 6c) is similar to that shown before (Tables 1 and 2) and it is not shown in Tables 3 and 4.

The data show the preferential fragmentation of the glycosyl derivatives at the 7 position compared to the glycosylation at 3, as well as the presence of acylations. Thus, all acylated compounds studied in the negative mode MS^2 (Tables 3 and 4), showed the presence of the base peak at (*M*−H-162) corresponding to the 7-glucosyl derivatives (Table 3) and also (*M*−H-324) corresponding to the 7-sophorosyl derivatives (Table 4).

In addition to the ion produced by the loss of the glycosyl residue at 7 (base peak), the MS^2 analysis of acylated derivatives, showed losses of the respective acids and/or the sugar and acids from the $[M-H]^-$ ion (Tables 3 and 4, Figs. 5 and 6).

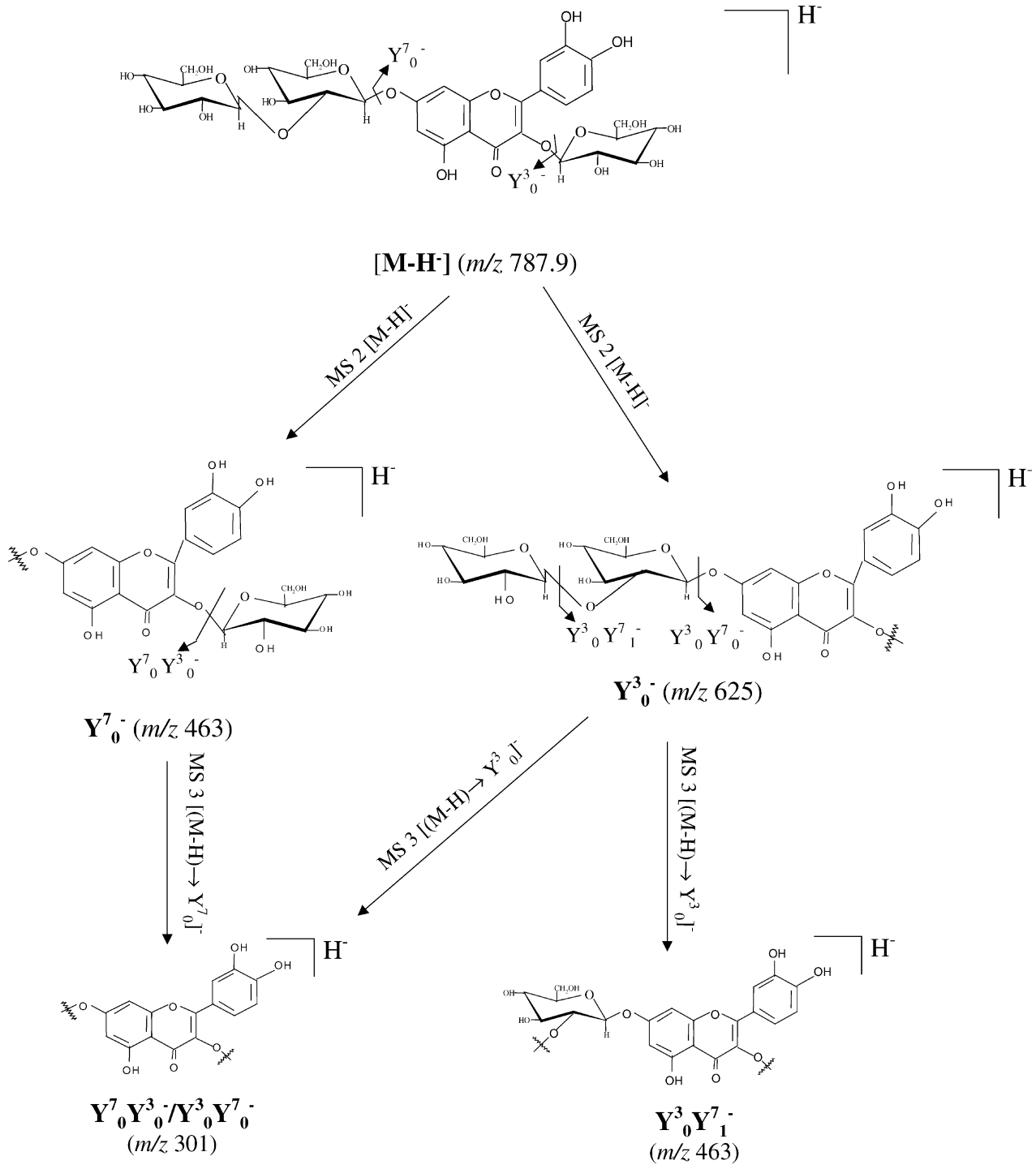
In the case of the caffeoyl derivatives from 7-glucosylflavonoids (compounds **20**, **21**, **25**, **31**, **33**, **36** and **37**) the caffeoyl loss coincided with that due to the glucose loss at 7 and therefore also contributed to the ion [*M*−H-162].

As in the case described above, in the MS–MS analysis of the ions produced after the loss of the sugar residues at 7 position ($-MS^3[(M-H) \rightarrow (M-H\text{-}162/324)]^-$) (Tables 3 and 4), the acid loss was easily detected showing that the acylation is always present on the sugars at 3 position in these compounds. In the case of monoacylated derivatives the ion corresponding to the flavonoid 3-*O*-glycoside is always the base peak. Regarding the diacylated derivatives, the base peak usually corresponds to the loss of one of the acid residues.

The structures of the different acylated flavonols characterised are shown in Tables 3 and 4.

To the best of our knowledge, the characterisation of these acylated derivatives has not been previously reported in nature, with the exception of cauliflower compounds (**29**, **32**, **36**, **40** and **45**) recently characterised [7].

Besides the acylated compounds showed in Tables 3 and 4, the caffeoyl derivatives from **18** (compound **47**) and **19** (compound **48**) were also detected (Fig. 2), where **47**: quercetin-3-*O*-(caf)-glucoside (R_t 21.8 min, –MS: 625.7 ($[M-H]^-$), $-MS^2[M-H]^-$: 463 (100%) [*M*−H-162, (–Caf)]$^-$; 301 (31%) [*M*−H-324(–Caf-Glc)]$^-$); **48**: kaempferol-3-*O*-(caf)-glucoside (R_t 22.2 min, –MS: 609.6 ($[M-H]^-$), $-MS^2[M-H]^-$: 447 (44%) [*M*−H-162, (–Caf)]$^-$; 285 (100%) [*M*−H-324 (–Caf-Glc)]$^-$).



Scheme 2. ESI-MS^n fragmentation pathway of quercetin-3-glucoside-7-sophoroside (**11**).

3.4. Chromatographic behaviour

Regarding the elution order of the deacylated flavonoids, the results previously reported for cauliflower flavonoids have been confirmed [7]. Thus, in some cases an increase in the number of glucose residues on the flavonoid nucleus has produced and increase in the HPLC retention time. This fact was due to the introduction of a second glucose residue on the glucose at the 7 position, which increases the retention time, i.e. compound couples **1**/**3**, **4**/**5**, **7**/**9**, **6**/**8**, **10**/**11**, **12**/**13** and **14**/**15**.

On the other hand, the chromatographic elution order of the free hydroxycinnamic acids does not always follow the same chromatographic behaviour as the corresponding

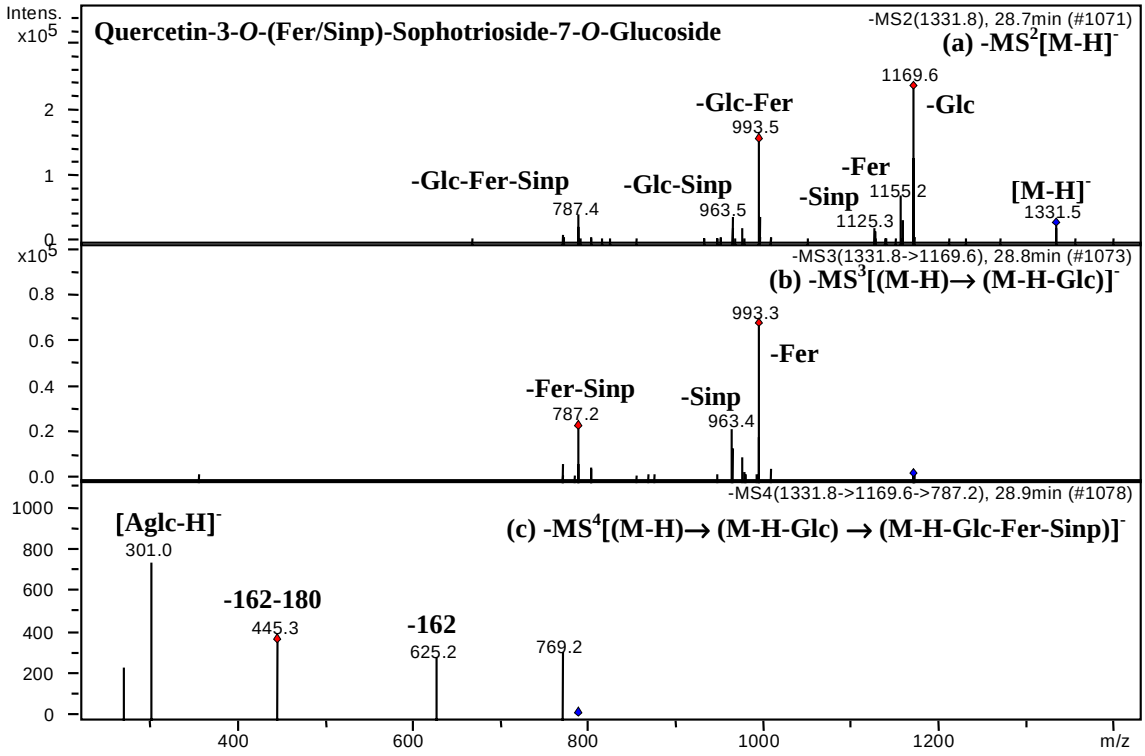


Fig. 5. MS^n analysis of quercetin-3-*O*-(feruloyl/sinapoyl)-sophorotrioside-7-*O*-glucoside (**27**). (a) $-MS^2[M-H]^-$; (b) $-MS^3[(M-H) \rightarrow (M-H\text{-Glc})]^-$; (c) $-MS^4[(M-H) \rightarrow (M-H\text{-Glc}) \rightarrow (M-H\text{-Glc-Fer-Sin})]^-$.

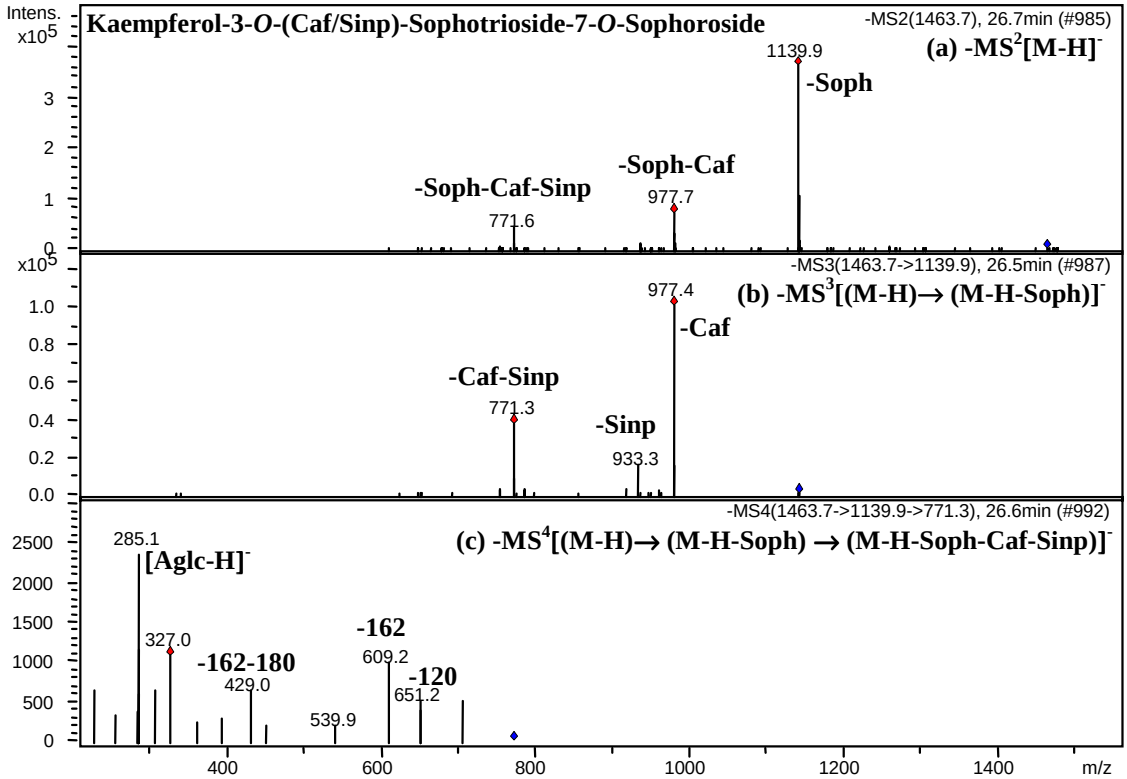


Fig. 6. MS^n analysis of kaempferol-3-*O*-(caffeoyl/sinapoyl)-sophorotrioside-7-*O*-sophoroside (**44**). (a) $-MS^2[M-H]^-$; (b) $-MS^3[(M-H) \rightarrow (M-H\text{-Soph})]^-$; (c) $-MS^4[(M-H) \rightarrow (M-H\text{-Soph}) \rightarrow (M-H\text{-Soph-Caf-Sin})]^-$.

acylated flavonoids, considering that acylations will always be at the same positions. Thus, the elution order of free acids at RP-HPLC is, from shorter to longer, caffeic < *p*-coumaric < ferulic < sinapic, while in the studied acylated flavonoids the order is caffeoyl < sinapoyl < feruloyl < *p*-coumaroyl.

Another unexpected behaviour in the chromatographic mobility at RP-HPLC has been observed when comparing the elution order of flavonoid glycosides and their corresponding acylated derivatives, considering that acylation is always at the same position. Thus, compound **1 (**quercetin-3-*O*-sophorotrioside-7-*O*-glucoside) elutes with a R_t at 8.1 min while **2** (quercetin-3-*O*-sophoroside-7-*O*-glucoside) elutes slightly later with a R_t of 8.8 min. However, the elution order is inversed when these compounds are acylated. Thus, the acylated derivatives of **2** elute before the corresponding counterparts of **1**: 2-caffeoyl (R_t 8.9 min)/1-caffeoyl (R_t 9.2 min); 2-feruloyl (R_t 12.0 min)/1-feruloyl (R_t 12.4 min); 2-*p*-coumaroyl (R_t 12.4 min)/1-*p*-coumaroyl (R_t 12.8 min).

Acknowledgements

The authors are grateful to the Spanish Comisión Interministerial de Ciencia y Tecnología (CICYT/AGL2001-1125) for their financial support of this work.

References

[1] M.G.L. Hertog, E.J.M. Feskens, P.C.H. Hollman, M.B. Katan, D. Kromhout, Lancet 342 (1993) 1007.
[2] S.O. Keli, M.G.L. Hertog, E.J.M. Feskens, D. Kromhout, Arch. Intern. Med. 156 (1996) 637.
[3] P. Knekt, R. Jarvinen, R. Seppänen, M. Heliövaara, L. Teppo, E. Pukkala, A. Aromaa, Am. J. Epidemiol. 146 (1997) 223.
[4] F. Vallejo, F.A. Tomás-Barberán, C. García-Viguera, J. Agric. Food Chem. 51 (2003) 3776.
[5] M.G.L. Hertog, P.C.H. Hollman, M.B. Katan, J. Agric. Food Chem. 40 (1992) 2379.
[6] K.R. Price, F. Casuscelli, I.J. Colpuhoun, M.J.C. Rhodes, J. Sci. Food Agric. 77 (1998) 468.
[7] R. Llorach, A. Gil-Izquierdo, F. Ferreres, F.A. Tomás-Barberán, J. Agr. Food Chem. 51 (2003) 3895.
[8] M.I. Gil, F. Ferreres, F.A. Tomás-Barberán, J. Agric. Food Chem. 46 (1998) 2007.
[9] B. Domon, C. Costello, Glycoconj. J. 5 (1988) 397.
[10] M. Stobiecki, Phytochem. 54 (2000) 237.
[11] F. Ferreres, R. Llorach, A. Gil Izquierdo, J. Mass Spectrom. 39 (2004) 312.
[12] J.K. Nielsen, C.E. Olsen, K.P. Marianne, Phytochemistry 34 (1993) 539.
[13] L.C. Olson, M. Veit, G. Weinssenböck, F. Bornman, Phytochemistry 49 (1998) 1021.
[14] K.E. Wilson, M.I. Wilson, B.M. Greenberg, Photochem. Photobiol. 67 (1998) 547.
[15] F. Cuyckens, R. Rozenberg, E. Hoffmann, M. Claeys, J. Mass Spectrom. 36 (2001) 1203.
[16] E. Hvattum, D. Ekeberg, J. Mass Spectrom. 38 (2003) 43.