

## HPLC-DAD-MS/MS ESI Characterization of Unusual Highly Glycosylated Acylated Flavonoids from Cauliflower (*Brassica oleracea* L. var. *botrytis*) Agroindustrial Byproducts

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In a recent program to investigate the possible use of vegetable handling and commercialization wastes and residues as a source of potentially bioactive extracts, we have studied cauliflower byproducts. A total of twenty-eight compounds were characterized by HPLC-DAD-MS, of these, twenty-two are produced naturally by the plant. The main compounds found were kaempferol 3-diglucoside-7-glucoside and its combinations with different hydroxycinnamic acids. Moreover, some flavonoids with an unusual high grade of glycosylation have been isolated and tentatively identified for the first time. To our knowledge, the characterization of flavonoids with more than four sugars has not been previously reported. The new products isolated were tentatively identified as kaempferol 3-diglucoside-7-diglucoside, kaempferol 3-triglucoside-7-diglucoside, kaempferol 3-feruloyldiglucoside-7-diglucoside, kaempferol 3-sinapoyltriglucoside-7-glucoside, kaempferol 3-disinapoyltriglucoside-7-glucoside, kaempferol 3-sinapoyltriglucoside-7-diglucoside and kaempferol 3-disinapoyltriglucoside-7-diglucoside.

**KEYWORDS:** Flavonoids; phenolics; hydroxycinnamic acid derivatives; HPLC-DAD-MS/MS ESI; Brassica; cauliflower byproducts; acylated kaempferol tetra- and pentaglucosides

### INTRODUCTION

In the past few years, there has been a renewed interest in the study of flavonoids from plant sources and especially plant food products due to their antioxidant properties and other biological activities. In fact, epidemiological studies have stressed the capacity of *Brassica* species to prevent cardiovascular diseases as well as their activity against some type of cancers (1, 2). The substances that seem to be responsible for these properties are the glucosinolates and their derived products (3), as well as the flavonoids and other phenolic compounds (4, 5). The role of flavonoids in the prevention of these diseases has been suggested to be mainly related to the prevention of the LDL and DNA oxidation through a scavenging activity against peroxy and hydroxyl radicals (6).

Research on *Brassica* vegetables has been focused on the edible parts. However, scarce information is available regarding their corresponding byproducts. In fact, recent work in our laboratory has shown that cauliflower byproducts are a good source of phenolic compounds, mainly flavonol derivatives (7). Some of the polyphenols present in leaves from different *Brassica* species have been characterized previously. These polyphenols are different combinations of kaempferol and quercetin with glucose and different hydroxycinnamic acids (8–10), as well

as other phenolics compounds such as a number of sinapic acid derivatives that have also been identified previously (11).

The purpose of this work was to identify the polyphenols from cauliflower byproducts by high-performance liquid chromatograph coupled with on-line mass spectrometry with electrospray ionization source (HPLC-DAD-MS/MS ESI) as a way to propose these byproducts as a source of new polyphenols with possible use as dietary or food antioxidants.

### MATERIALS AND METHODS

**Plant Material.** The typical cauliflower byproducts (*Brassica oleracea* L. var. *botrytis*) mainly consist of leaves. The byproduct was supplied by Agrosol Cooperative (Lorca, Murcia, Spain). Before freeze-drying, fresh cauliflower byproducts were weighed and chopped with a sharp stainless steel knife in small pieces.

**Phenolics Extraction, Isolation and Purification.** Freeze-dried byproducts (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, Bellefonte, PA) in sufficient amount to fill a column of 55 × 4 cm and stirred for 4 h at room temperature to retain the phenolic compounds on the surface of the nonionic Amberlite particles (12). The Amberlite particles were packed into the chromatography column, washed with distilled water (5 L), and the absorbed phenolics eluted with methanol (1 L). The methanol extract was taken to dryness and redissolved in methanol/water (1:1, v/v) for chromatography on a 40 × 3 cm Sephadex LH-20 column. The elution of the different

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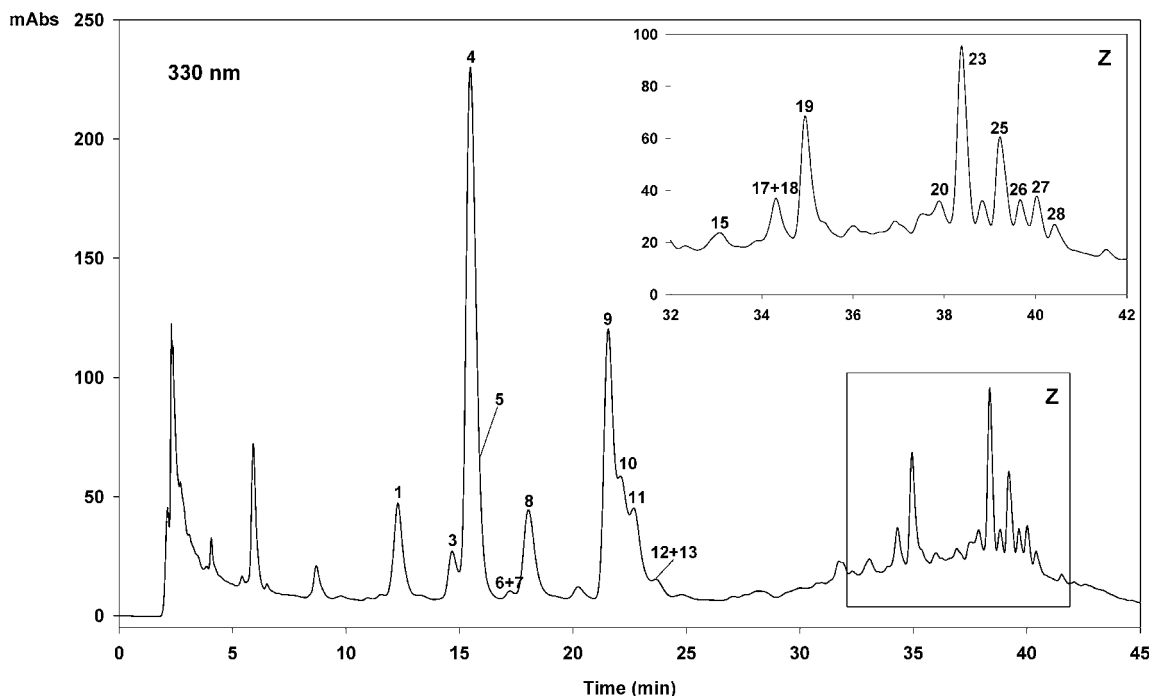


Figure 1. HPLC profile of phenolic compounds from cauliflower byproducts recorded at 330 nm. Identifications of the peaks: see Table 1.

phenolic fractions was followed under UV light (254 and 360 nm). The first eluting fractions contained the larger flavonoid molecules (compounds 3–7, 9–13, 20 and 23), followed by those of intermediate size (17–19), and ending with the smaller natural compounds (15).

The fractions eluted under these conditions were then chromatographed by semipreparative HPLC to isolate the different constituents. This was performed on a  $25 \times 1$  cm i.d.,  $5 \mu\text{m}$  Sherisorb ODS-2 column (Teknokroma, Barcelona, Spain) isocratically, with different mixtures of methanol/water. The purity of the isolated compounds was followed by analytical HPLC, using a  $25 \times 0.4$  cm i.d.,  $5 \mu\text{m}$  LiChroCART C18 column (Merck, Darmstadt, Germany) and as mobile phases water + 5% HCOOH (A) and methanol (B) with a solvent flow rate of 1 mL/min, starting with 10% B to reach 20% B after 25 min, 50% B at 40 min, 50% B at 45 min, 90% B at 46 min, and 90% B at 50 min. The UV chromatograms were recorded at 330 nm. The isolated compounds were freeze-dried for storage.

**Alkaline and Acid Hydrolysis.** Alkaline hydrolysis was achieved, adding 1 mL 4N NaOH to freeze-dried products (<1 mg) and keeping them for 16 h in a stoppered test tube under  $\text{N}_2$  atmosphere. The alkaline hydrolysis products were acidified with concentrated HCl (color change,  $\text{pH} \approx 1$ ) and directly analyzed by HPLC-DAD-MS/MS. This solution was used for partial (mild) acid hydrolysis by keeping it in a stoppered test tube for 30 min in an oven adjusted to  $75^\circ\text{C}$ . Then, it was directly analyzed by HPLC-DAD-MS/MS. Total acid hydrolysis was carried out by adding 1 mL 4N HCl to a methanol solution of the different isolated products (v/v) in a stoppered test tube for 45 min at  $90^\circ\text{C}$ .

**Study of Hydrolysis Products.** The different organic acids released after hydrolysis were identified by HPLC-DAD-MS against authentic markers. The sugars were identified by TLC using different sugars as standards (13). The flavonoid aglycones were identified by spectroscopic UV analysis, using alkaline and metal reagents as described previously (14) and HPLC-DAD-MS.

**HPLC-DAD-MS/MS ESI.** The system used for qualitative analyses was an Agilent HPLC equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (G1312A), an autosampler (G1313A), a degasser (G1322A), and a photodiode array detector (G1315B). The HPLC system was controlled by a ChemStation software (Agilent, v. 08.03).

The mass detector was an ion trap spectrometer (G2445A) with electrospray ionization system. It was controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted at  $350^\circ\text{C}$  and 4kV for capillary temperature and voltage, respectively. The

nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full scan mass ranged from  $m/z$  200 up to  $m/z$  2000. Helium was used as collision gas for fragmentation experiments and the collision energy was adjusted at 100%. All mass spectrometry data were recorded in the negative mode. Total ion chromatograms were recorded as two alternating automatic scan events: full scan mass spectra (MS) and MS/MS for fragmentation of the most abundant pseudomolecular ions.

**Qualitative MS Analyses of the Isolated Compounds.** The mass detector for the analyses was an Agilent ion trap spectrometer (Agilent Technologies, Waldbronn, Germany). MS analyses of isolated compounds were carried out by constant infusion to the electrospray ionization system. The isolated compounds were dissolved in water/methanol (50:50, v/v). The flow rate of the constant infusion was 10  $\mu\text{L}/\text{min}$ . The capillary voltage and temperature were 4kV and  $300^\circ\text{C}$ , respectively. The pressure of the nebulizer was 15 psi, and the nitrogen flow was 5 L/min. The isolated compounds were subjected to up to five sequential manual events, depending on the molecular weight of the compounds: (MS) to find pseudomolecular ion, (MS/MS) for fragmentation of the pseudomolecular ion, and ( $\text{MS}^n$ ,  $n = \text{up to } 5$ ) in order to fragment the major ions obtained in every step.

## RESULTS AND DISCUSSION

The cauliflower byproduct extracts were analyzed by HPLC, using a reversed phase C18 column, and the chromatogram profile is shown in Figure 1. A total of twenty-eight compounds were identified. Twenty-two of them were naturally produced by the plant, and six of them were only produced after alkaline and acid hydrolyses (Table 1). After acid hydrolysis all the flavonoids in this work gave glucose as the only sugar and kaempferol as the aglycone, with the exception of compounds 1 and 8 that gave quercetin.

**Identification of Flavonoids from Cauliflower Byproducts.** The compounds were tentatively identified by means of a combination of the UV and mass spectra obtained by HPLC-DAD-MS/MS ESI and the results of alkaline and acid hydrolysis.

**Compound 4.** Mild acid hydrolysis of 4 lead to the aglycone kaempferol (24) and several compounds of partial hydrolysis, including kaempferol 7-glucoside (21), kaempferol 3-diglucoside (19), and another kaempferol diglucoside ( $R_t$  28.0 min, MS [ $\text{M}-\text{H}$ ] $^-$  at  $m/z$  609) with the hydroxyl at the 3 position blocked

Table 1. Flavonoid Glycosides and Hydroxycinnamic Acid Derivatives from Cauliflower

peak	R <sub>t</sub> (min)	UV (nm)	[M-H] <sup>-</sup> (m/z)	MS/MS (m/z)	structure assignment
1 <sup>a</sup>	12.3	255, 268sh, 351	787	625, 462, 300	quercetin 3-diglucoside-7-glucoside
2 <sup>b</sup>	14.0	267, 395sh, 286sh, 348	933	771, 609, 429, 285	kaempferol 3-triglucoside-7-glucoside
3	14.7	251, 267, 285sh, 329	963	801, 609, 446, 284	kaempferol 3-acyldiglucoside-7-glucoside (unidentified acyl, m/z 192)
4	15.5	267, 293sh, 318sh, 348	771	610, 429, 285	kaempferol 3-diglucoside-7-glucoside
5	16.0	268, 298sh, 318sh, 334	933	771, 609, 429, 285	kaempferol 3-caffeoyldiglucoside-7-glucoside
6	17.4	267, 294sh, 317sh, 347	1095	933, 771, 609, 429, 284	kaempferol 3-triglucoside-7-diglucoside
7	17.6	267, 294sh, 317sh, 347	933	771, 609, 429, 284	kaempferol 3-diglucoside-7-diglucoside
8 <sup>a</sup>	18.0	252, 269sh, 301sh, 338	993	831, 625, 445, 300	quercetin 3-sinapoyldiglucoside-7-glucoside
9	21.5	269, 333	977	815, 609, 429, 284	kaempferol 3-sinapoyldiglucoside-7-glucoside
10	22.1	249, 269, 331	947	785, 609, 429, 284	kaempferol 3-feruloyldiglucoside-7-glucoside
11	22.7	252, 269, 321, 356sh	1139	977, 771, 609, 429, 284	kaempferol 3-sinapoyltriglucoside-7-glucoside
12	23.4	253sh, 269, 300sh, 333	1301	1139, 977, 771, 609, 447, 285	kaempferol 3-sinapoyltriglucoside-7-diglucoside
13	23.7	253sh, 269, 300sh, 333	1109	947, 785, 609, 447, 285	kaempferol 3-feruloyldiglucoside-7-diglucoside
14 <sup>b</sup>	28.0	267, 294sh, 317sh, 347	609	447, 285	kaempferol 3-glucoside-7-glucoside
15	33.2	261, 296, 338sh	771	609, 429, 284	kaempferol 3-acyldiglucoside (unidentified acyl, m/z 162))
16 <sup>b</sup>	33.8	265, 299sh, 347	771	609, 447, 285	kaempferol 3-triglucoside
17	34.2	251sh, 267, 333	977	771, 609, 429, 284	kaempferol 3-sinapoyltriglucoside
18	34.4	249, 267, 333	815	609, 429, 284	kaempferol 3-sinapoyldiglucoside
19	34.9	268, 298sh, 346	609	429, 284	kaempferol 3-diglucoside
20	38.0	269, 330	1507	1183, 977, 771, 609, 284	kaempferol 3-disinapoyltriglucoside-7-diglucoside
21 <sup>b</sup>	38.1	255sh, 265, 319sh, 367	447	285	kaempferol 7-glucoside
22 <sup>b</sup>	38.2	255sh, 266, 319sh, 367	609	285	kaempferol 7-diglucoside
23	38.4	269, 330	1345	1183, 977, 771, 609, 284	kaempferol 3-disinapoyltriglucoside-7-glucoside
24 <sup>b</sup>	47	253sh, 266, 294sh, 322sh, 367	285		kaempferol
25 <sup>a</sup>	39.2	227, 240, 262, 330	754	530	1,2-disinapoyldiglucoside
26 <sup>a</sup>	39.7	223, 241, 330	725	500	1-sinapoyl-2-feruloyldiglucoside
27 <sup>a</sup>	40.1	223, 240, 330	960	736, 530	1,2,2'-trisinapoyldiglucoside
28 <sup>a</sup>	40.4	240, 262, 330	930	706, 482	1,2'-disinapoyl-2-feruloyldiglucoside

<sup>a</sup> Compounds not isolated. Analyzed using HPLC-DAD-MS/MS. <sup>b</sup> Products obtained after alkaline or acid hydrolysis.

(UV spectrum) that was tentatively identified as kaempferol 3-glucoside-7-glucoside. The UV study in methanol and after the addition of alkaline and metal reagents (14) showed that **4** was a kaempferol derivative, with the hydroxyls at the 7 and 3-positions blocked. The UV analyses (14) of one product of the partial hydrolysis (**21**) showed that this was a kaempferol derivative with a free hydroxyl at the 3 position and blocked at the 7 position. Its mass spectrum suggested that this compound was kaempferol 7-glucoside. Acid hydrolysis of compound **4** only released glucose and kaempferol.

The MS analysis of compound **4** (Table 1) showed a pseudomolecular ion at *m/z* 771 (kaempferol trihexoside). MS/MS analysis of this ion produced an ion at *m/z* 610, showing the loss of a hexose, and MS/MS of 771 → 610 led to a *m/z* 429 ion for the loss of another hexose to end with the loss of another hexose to lead to kaempferol (*m/z* 285).

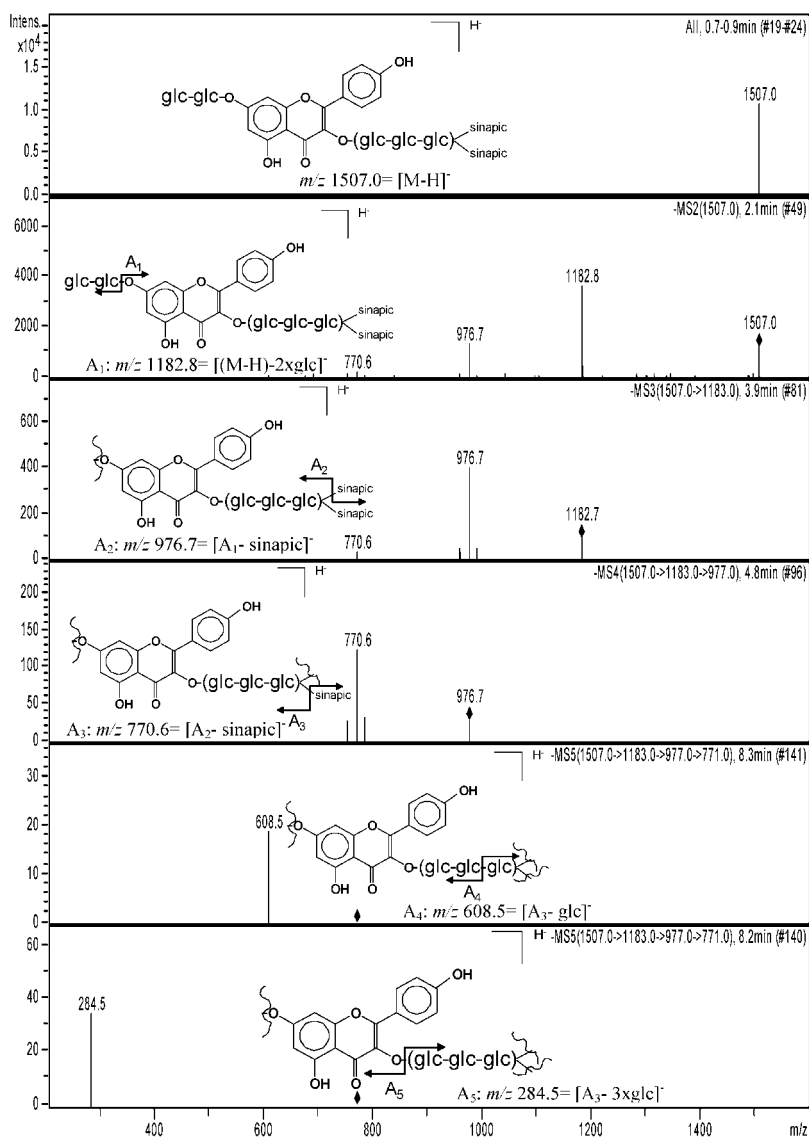
After alkaline hydrolysis, UV, MS spectra, and the HPLC retention time remained unchanged, showing that this treatment had no effect on this compound and only produced a small amount of another flavonoid glycoside that coincided with kaempferol 3-diglucoside (**19**) in retention time, UV, and MS spectra. Thus, this was identified as kaempferol 3-diglucoside-7-glucoside.

Compounds **10** and **11** were isolated as a single fraction, although they were identified by HPLC-MS with different retention times (22.1 and 22.7 min, respectively) when the ions at *m/z* 947 and 1139 were extracted and plotted. The UV spectroscopic analysis of these compounds showed that they were flavonoids acylated with hydroxycinnamic acid derivatives as revealed a very broad Band I shifted to shorter wavelengths compared to a nonacylated flavonol glycoside (**10**, 249, 269, 331 nm; **11**, 252, 269, 321, 356sh). MS/MS of the pseudomolecular ion of **10**, at *m/z* 947 produced an ion at *m/z* 785, which coincided with the loss of a glucose from position 7. MS/MS of the daughter ion at *m/z* 785 produced an ion at *m/z* 609, coincident with a kaempferol diglucoside produced

by the loss of the feruloyl residue. The aglycone kaempferol was detected after the sequential loss of two glucoses from position 3.

The MS/MS analysis of compound **11** showed that the pseudomolecular ion at *m/z* 1139 lost a glucose from position 7, to give a fragment at *m/z* 977. Then MS/MS analysis of this ion showed the sequential loss of sinapic acid (*m/z* 771) followed by the loss of three glucoses from position 3 to obtain the kaempferol aglycone (Table 1). When the fraction containing **10** and **11** was subjected to alkaline hydrolysis, compound **4** was obtained (kaempferol 3-diglucoside-7-glucoside) as a saponification product of compound **10**, and an additional compound (**2**) with a mass at *m/z* 933 corresponding to a kaempferol tetraglucoside as a product of compound **11**. Ferulic and sinapic acids were also detected (UV, MS, and chromatographic comparison with authentic markers). Mild acid hydrolysis produced only kaempferol 7-glucoside as an intermediate, as well as the acids and the aglycone kaempferol. Therefore, **10** was tentatively assigned as kaempferol 3-feruloyldiglucoside-7-glucoside and **11** as kaempferol 3-sinapoyltriglucoside-7-glucoside.

Compound **20** showed a pseudomolecular ion at *m/z* 1507, consistent with a kaempferol pentaglucoside acylated with two sinapic residues (Figure 2). The saponification led to an ion at *m/z* 1095 (kaempferol pentaglucoside) and R<sub>t</sub> 17.4 min that coincided with compound **6** and sinapic acid. Mild acid hydrolysis led to kaempferol, sinapic acid, and a flavonol glucoside (R<sub>t</sub> 38.2 min, UV 255sh, 266, 319sh, 367, and [M-H]<sup>-</sup> *m/z* 609) in accordance to a kaempferol 7-diglucoside (**22**). The UV spectroscopic study of **20** showed that it was an acylated derivative of kaempferol, with substituted hydroxyls at the 3 and 7 positions. The MS/MS analysis showed that the pseudomolecular ion at *m/z* 1507 suffered a first loss of two glucose residues from the 7 position to give a fragment ion at *m/z* 1183 that could be assigned to a kaempferol triglucoside acylated with two sinapic acid residues (Figure 2). From this ion, the sequential loss of two sinapoyl residues to give kaempferol 3-triglucoside was produced (Figure 2). Thus,



**Figure 2.** MS/MS (negative ion mode) of kaempferol 3-disinapoyltrigluco-7-diglucoside. Sequential fragmentation,  $MS^n$  ( $n=2-5$ ), of the ion at  $m/z$  1507 and the derived major fragment ions by constant infusion to the ion trap mass spectrometer.

compound **20** was characterized as kaempferol 3-disinapoyltrigluco-7-diglucoside.

The remaining compounds were characterized in a similar way. Compounds **1** and **8** were not isolated by semipreparative chromatography, and their structures were tentatively assigned by their UV spectra and their MS/MS fragmentation that was similar to those of compounds **4** and **9**, respectively. Compounds **3**, **5**, and **9** had a similar behavior to that of **10**, and after alkaline hydrolysis led to compound **4**, therefore, they were identified as different acylated derivatives of **4**. Regarding compound **5**, its acyl residue was unstable under alkaline conditions and was only studied after acid hydrolysis.

The pairs **6-7**, **12-13**, and **17-18** were isolated as single fractions and analyzed in a similar way to the pair **10-11**. Alkaline hydrolysis of **12** and **13** led to compounds **6** and **7**; therefore, they were characterized as the corresponding acylated derivatives.

The unidentified acid of compound **15** was unstable in alkaline media, as in the case of compound **5**, and it was necessary to perform a direct acid hydrolysis without a previous alkaline treatment to analyze it properly. Alkaline hydrolysis of **23** gave a similar saponification product to compound **11**, and its UV study was very similar to that of compound **20**.

In addition to these flavonol glycosides, four hydroxycinnamic acid digluco-7-diglucosides (**25-28**) were also detected in the extracts and identified by HPLC-MS analyses by comparison with the reported data for hydroxycinnamic acid esters in broccoli florets (11). They were identified as 1,2-disinapoyldiglucoside, 1-sinapoyl-2-feruloyldiglucoside, 1,2,2'-trisinapoyldiglucoside, and 1,2'-disinapoyl-2-feruloyldiglucoside.

The HPLC/MS analysis of cauliflower byproducts revealed the presence of flavonols and hydroxycinnamic acid derivatives. The main compound was kaempferol 3-diglucoside-7-glucoside (**4**) (Figure 1), and its combinations with glucose, sinapic, caffeic, and ferulic acid were the most important phenolic compounds identified. The process of extraction and isolation of these compounds could cause deacylations, which could contribute to increase the relative abundance of compound **4**. Some of the flavonoids reported here have been previously described in different *Brassica* species, such as oil seed rape (*Brassica napus* L.) (9) and cabbage (*Brassica oleracea* L.) (10).

The unusual high grade of glycosylation of some flavonoids, such as the tetragluco-7-diglucosides **7**, **11**, **13**, and **23**, and pentagluco-7-diglucosides **6**, **12**, and **20**, should be noted. To the best of our knowledge, these compounds have never been reported before (15).



Moreover, flavonoid glycosides with more than four sugar moieties have not been previously reported in nature (16).

The new tentatively identified naturally occurring flavonoids are kaempferol 3-diglucoside-7-diglucoside (7), kaempferol 3-triglucoside-7-diglucoside (6), kaempferol 3-feruloyldiglucoside-7-diglucoside (13), kaempferol 3-sinapoyltriglucoside-7-glucoside (11), kaempferol 3-disinapoyltriglucoside-7-glucoside (23), kaempferol 3-sinapoyltriglucoside-7-diglucoside (12), and kaempferol 3-disinapoyltriglucoside-7-diglucoside (20).

Regarding the disaccharide form, publications on *Brassica* flavonoids (8–10) suggests that these disaccharides should be sophorosides (1 → 2 linkage), as these have been reported for several *Brassica* flavonoids using <sup>1</sup>H-<sup>1</sup>H-COSY and <sup>13</sup>C NMR analysis. In the case of the sinapic acid derivatives, the gentiobiose form (1 → 6 linkage) was detected instead and demonstrated by <sup>13</sup>C NMR (11).

After mild acid hydrolysis, the different compounds can be grouped into three groups: Group A, which yields kaempferol 7-diglucoside (compounds 6, 7, 12, 13, and 20); group B, which yields kaempferol 7-glucoside (compounds 3, 4, 5, 9, 10, 11, and 23); and group C, which only yields kaempferol (compounds 15, 17, 18, and 19).

**MS/MS Fragmentation Behavior.** The MS fragmentation of the compounds present in these groups showed a common pattern (Table 1). In those included in the groups A and B, the first loss was the sugar in position 7, followed by the acyl residues, and then the loss of other sugars from position 3. This clearly indicates that the removal of the glucose or diglucose residues from the hydroxyl in position 7 is much more favored in ESI-MS than the loss of an acyl residue or the glucose or diglucose residues from position 3. This special sensitivity of the glycosidic linkage at the 7 position is also shown by the general direct loss of the diglucose residue from this position rather than the sequential loss of two glucoses, as in the case of flavonol 7 diglucosides (i.e., compound 20). This MS pattern contrasts with the chemical sensitivity of these glycosidic linkages, as mild acid hydrolysis always releases first the sugars at the 3 position, and the 7-glycoside intermediates can be isolated and analyzed.

**Chromatographic Behavior.** The chromatographic behavior of these flavonoid compounds under reversed-phase HPLC shows that, in the same conditions, a higher degree of glycosylation lead to a shorter retention time. As a general rule, the introduction of a glucose on the hydroxyl at the 7 position dramatically reduces the retention time. Thus, kaempferol 3-triglucoside (16, *R*<sub>t</sub> 33.8 min) elutes much earlier when an additional glucosyl residue is introduced on the hydroxyl at the 7 position (2, *R*<sub>t</sub> at 14.0 min). The same is observed within the pair kaempferol 3-diglucoside (19, *R*<sub>t</sub> 34.9 min) and kaempferol 3-diglucoside-7-glucoside (4, *R*<sub>t</sub> 15.5 min). However, the introduction of a second glucosyl residue on the glucose at the 7 position increases the retention time. Thus, kaempferol 3-diglucoside-7-glucoside (4) elutes earlier (15.5 min) than kaempferol 3-diglucoside-7-diglucoside (7) (17.6 min) and even earlier than kaempferol 3-triglucoside-7-diglucoside (6) (17.4 min). The same is observed for kaempferol 3-triglucoside-7-glucoside (2) that elutes with shorter retention time (14.0 min) than kaempferol 3-triglucoside-7-diglucoside (6) (17.4 min). Something similar is also observed with acylated derivatives (see pairs 11–12 and 10–13). Because ferulic acid elutes earlier than sinapic acid, it is unusual that compound 9 (kaempferol 3-sinapoyldiglucoside-7-glucoside, *R*<sub>t</sub> 21.5 min) elutes earlier than the corresponding feruloyl derivative 10 (kaempferol 3-feruloyldiglucoside-7-glucoside, *R*<sub>t</sub> 22.1 min).

The acylation with hydroxycinnamic acids affects the chromatographic mobility in very different ways, depending on the glycosidic substitution of the flavonoid. This can be clearly observed when comparing the HPLC mobility of the naturally occurring acylated flavonoid glycosides and the corresponding products of alkaline hydrolysis to remove the acyl residue. All the compounds that are glycosylated at the 7 position lead to deacylated compounds with shorter retention times than the naturally occurring acylated derivatives. For those acylated compounds without glycosylation at the 7 position, the corresponding deacylated compounds lead to the similar retention times to those of the naturally occurring acylated glycosides.

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