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Identification of New Flavonoid Glycosides and Flavonoid Profiles To Characterize Rocket Leafy Salads (*Eruca vesicaria* and *Diplotaxis tenuifolia*)

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"Rocket" is a collective name used to term some species within the *Eruca* and *Diplotaxis* genera, whose leaves are characterized by a more or less pungent taste. Different approaches have been carried out to differentiate both genera that have similar leaf morphologies. Following our research in flavonoid profiling of the *Brassicaceae* family using high-performance liquid chromatography/ultraviolet−diode array detection/electrospray ionization mass spectroemtry, we have investigated *Eruca vesicaria* and *Diplotaxis tenuifolia* leaf samples as new ingredients of fresh salads. The MS/MS study allowed the identification of new naturally occurring quercetin mono- and diacyl-tri-O-glucosides and the elucidation of the flavonoid glycosylation and acylation patterns. Important differences between flavonoid profiles of *E. vesicaria* and *D. tenuifolia* were observed. *E. vesicaria* contained kaempferol derivatives as principal compounds whereas *D. tenuifolia* instead accumulated quercetin derivatives. The exhaustive study of the profiling of these species could help further studies concerning the bioavailability of these flavonoids for epidemiological or clinical intervention studies because these species have considerable potential as healthy leafy salads because of the bioactive phytochemicals.

KEYWORDS: ***Eruca vesicaria*; *Diplotaxis tenuifolia*; *Brassicaceae*; crucifers; fresh-cut; HPLC/MS; polyphenols; vegetables**

INTRODUCTION

In the past few years, an important increase in the cultivation of wild plant species has been carried out for the commercial production of traditional Mediterranean green salads. "Rocket" is a collective name used to term some species within *Brassicaceae* whose leaves are characterized by a more or less pungent taste. Although different species are referred under the common name of rocket, the main ones are those belonging to *Eruca* Miller and *Diplotaxis* DC. genera. The main species used for human consumption include *Eruca vesicaria* (L.) Cav. and *Diplotaxis tenuifolia* (L.) DC (*1*).

From the botanical point of view, these plants belong to tribe *Brassiceae* subtribe *Brassicinae*, which includes *Brassica*, *Coincya*, *Erucastrum*, *Hirschfeldia*, *Raphanus*, *Sinapidendron*, *Sinapis*, and *Trachystoma*. The *Brassicinae* subtribe is defined primarily on the basis of elongated (siliquose) dehiscent fruits, the presence of median nectaries, and usually seeded beaks (*2*). It is remarkable that the leaf morphology has not been used to differentiate these species.

Both genera are used as new ingredients for green leafy salads. At optimum commercial maturity (young leaves), both plants are very similar, and this is the cause of misidentification. Different approaches have been carried out to find new tools to identify and differentiate both genera (*1*). In fact, flavonoids have been used as chemotaxonomy markers for the *Diplotaxis* genera (*3*). In the past few years, different studies on metabolite profiling, mainly based on polyphenols, of members of the *Brassicaceae* family have been used to differentiate between crucifer species (*4*−*7*). Thus, the presence of isorhamnetin glucoside allows the differentiation of the *Brassica oleracea* from *Brassica rapa* (*6*). Recently, Bennett et al. have investigated the ontogenic profiling of secondary metabolites of four rocket species including *E. sativa* and *D. tenuifolia* (*4*). They have identified and quantified the major phytochemicals including glucosinolates and also phenolics and flavonoids of different rocket tissues (seeds, roots, leaves, and flowers). They observed that all rocket tissues, except roots, contained significant levels of polyglycosilated flavonoids and separated them into simple and acylated mono-, di-, and triglycosides (*4*). In addition, Weckerle et al. (*8*) identified three quercetin 3,3′,4′-tri-*O*-β-D-glucopyranosides in *E. sativa* leaves as the main flavonoids. These previous reports on leaf flavonoids (*4*, *8*) did not show the complete characterization of the rocket flavonoids. The complex flavonoid pattern found in rocket species has limited

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the interest in the studies of bioavailability and biological effects of phenolics and flavonoids of fresh rocket leaves. Among the biologically active compounds in fresh cut salads, polyphenols are an important group (both qualitative and quantitative) as they are closely related with health-promoting activities such as prevention of cardiovascular diseases and cancer (*9*). Metabolite profiling (or metabolome analysis) represents a tool in studies of plant taxonomy, physiology, and phytochemistry. The use of high-performance liquid chromatography/ultraviolet−diode array detection/electrospray ionization mass spectroemtry (HPLC/UV-DAD/ESI-MS^n) for metabolite profiling of flavonoids has been recently discussed in several papers (*10*, *11*).

The main objective of this work was to characterize two rocket species, *E. vesicaria* (L.) Cav. and *D. tenuifolia* (L.) DC., based on the flavonoid profiling as a tool for qualitative and quantitative analysis of healthy salad phytochemicals.

MATERIALS AND METHODS

Plant Material. *D. tenuifolia* (wild rocket) and *E. vesicaria* (salad rocket) were cultivated in fields located in La Aparecida (Cartagena, Murcia, Spain) and supplied by Agrolito S.L. (Torre Pacheco, Murcia, Spain). At optimum commercial maturity, uniform size plants, free from decay and/or mechanical damage, were selected at random and immediately transported to the laboratory (30 km). Approximately, three replicates of 80 g of each were weighed, frozen at −70 °C, and freeze-dried. The dried samples were ground into a fine powder for further analysis.

The botanical identification was carried out at the Department of Botany (University of Murcia, Spain), and a voucher specimen was deposited in the University Herbarium. Wild rocket and salad rocket corresponded to the botanical names of *D. tenuifolia* (L.) DC. and *E. vesicaria* (L.) Cav. respectively, but the last one was also accepted as *Eruca sativa* Miller.

Phenolics Extraction. The freeze-dried sample (0.5 g) was homogenized with 5 mL of methanol−water (v:v) using an Ultra-Turrax homogenizer. The extract was then centrifuged (10500*g*) for 5 min, and the supernatant was filtered through a 0.45 μm membrane filter (Millex-HV 13MM, Millipore, Bedford, MA).

Isolation of Desacylated Flavonoid Glycosides. In order to study the structures of the desacylated flavonoid glycosides, a previous saponification step of the raw extracts was done before the isolation of the main compounds. A freeze-dried sample (20 g) was macerated overnight at room temperature with water (200 mL). The resulting extract was centrifuged (10500*g*) for 5 min, and the supernatant was collected and filtered. The supernatant was saponificated with 4 N NaOH (200 mL) for 16 h. Finally, the extract was acidified with concentrated HCl until pH 1−2. The acidified extract was mixed with the nonionic polymeric resin Amberlite XAD-2 (enough to fill a column of 3 cm × 50 cm) using a magnetic stirrer for 4 h to allow flavonoid adsorption on the resin particles as described by Llorach et al. (*12*). The resin particles were then poured into the glass column and washed with distilled water (1500 mL). Flavonoids were eluted with methanol (300 mL), and an aliquot was analyzed by HPLC/UV-DAD/ESI-MS^n. The extract was evaporated to dryness under reduced pressure (50 °C) and redissolved in water. The resulting extract was fractionated by the semipreparative HPLC on a Spherisorb ODS-2 column (250 mm × 10 mm, 5 μm particle size) (Tecnokroma, Barcelona, Spain), with different isocratic mixtures of methanol and water. The purity of the isolated compounds was verified by HPLC/UV-DAD/ESI-MS^n. Finally, these compounds were freeze-dried and stored.

Acid Hydrolysis. Total acid hydrolysis was carried out by adding 1 mL of 4 N 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 analyzed by HPLC/UV-DAD/ESI-MS^n.

Mild acid hydrolysis was carried out by adding 5 mL of 2 N HCl to the isolated compounds. Kaempferol-3-sophoroside (<2 mg) from cauliflower was used as standard (*12*). The extracts were distributed in five stoppered test tubes, incubated at 85 °C at different times (5, 10, 15, 30, and 60 min). The solutions were then cooled in ice, filtered, and directly analyzed by HPLC/UV-DAD/ESI-MS^n.

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, LiChrospher100 stationary phase, Merck, Darmstadt, Germany) protected with a LiChroCART guard column (4 mm × 4 mm, RP-18, 5 μm particle size, Merck). The mobile phase consisted of two solvents: water−formic acid (0.1%) (A) and methanol (B). For studying both the free flavonol glycosides and the corresponding acylated derivatives, a linear gradient starting with 20% B was installed to reach 50% B at 30 min. On the other hand, for the analysis of the phenolic acids and the flavonoids aglycones obtained after acid hydrolysis, a linear gradient was used starting with 15% B, reaching 65% B at 50 min and finally 80% B at 52 min. The flow rate was 1 mL min^{-1}, and the injection volume ranged from 10 to 50 μL depending on the compound and the extract assayed. Spectral data from all peaks were accumulated in the range of 240−400 nm. The chromatograms were recorded at 330 nm for glycosides and acylated derivatives and at 330 and 360 nm for hydroxycinnamic acids and flavonoid aglycones, respectively. The HPLC/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 ionization interface and was controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer 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 *m*/*z* 2000 for free glycosides and acylated derivatives and from *m*/*z* 90 up to *m*/*z* 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 ionization mode. MS^n was carried out in the automatic mode on the more abundant fragment ion in MS^{n-1}.

Table 1 shows the most frequent ions that characterize the fragmentation of these flavonoid *O*-glycosides. Other ions were found, but they have not been included due to their low significance.

RESULTS AND DISCUSSION

D. tenuifolia. The HPLC/UV-DAD/ESI-MS^n screening of the hydroalcoholic extract revealed the presence of different flavonoids, mainly trihexoside derivatives of quercetin (UV spectrum), and also some of them acylated with hydroxycinnamic acids (UV spectrum); **1**, **6**, and **11** were the most important of these (**Figure 1A**). Saponification was used to simplify their study, and acid hydrolysis was used to study their aglycones.

The acid hydrolysis showed quercetin as the most abundant aglycone as well as kaempferol and isorhamnetin in much smaller amounts. In this context, the saponification rendered compound **1** as main product that also was the principal compound in the original extract. Other minor compounds (**3** and **5** already observed in the original extract) and hydroxycinnamic acids were detected.

Deacylated Glycosides. The UV study with alkaline and metal reagents (*13*) of the isolated compound **1** showed a blocked hydroxyl at position 3 (UV MeOH: 250sh, 267, 337 nm). The λ of band I (337 nm) as well as its lower absorbance indicated the lack of free hydroxyls in the B ring. In addition, this showed a free hydroxyl at position 7 (+NaOAc: 276, 372), a blocked hydroxyl at position 4′ (+NaOMe: 276, 368↓), and also a free hydroxyl at position 5 (+$AlCl_3$: 252sh, 277, 299sh, 346, 395sh; +$AlCl_3$/HCl: 253sh, 277, 338, 398sh) (*13*).

Table 1. R_t, UV, and MS: $[M-H]^-$, $MS^2[M-H]^-$, $MS^3[(M-H) \rightarrow (M-H\text{-}Glc)]^-$, $MS^4[(M-H) \rightarrow (M-H\text{-}Glc) \rightarrow (M-H\text{-}Glc-Glc)]^-$, and $MS^4[(M-H) \rightarrow (M-H\text{-}Glc) \rightarrow (M-H\text{-}Glc\text{-}Ac1/Ac2Glc)]^-$ Data of Quercetin-3,3′,4′-triglucoside and Hydroxycinnamic Acid Esters Derivatives

compounds[a]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$MS^2[M-H]^-$ (*m/z*) (%)	$MS^3[(M-H \rightarrow (M-H\text{-}Glc)]^-$ (*m/z*) (%)	$MS^4[(M-H) \rightarrow (M-H-H\text{-}Glc) \rightarrow (M-H\text{-}Glc-Glc)]^-$ (*m/z*) (%)
				–G	–G	–G
1 Q-3,3′,4′-TriG	14.1	250sh, 267, 337	787	625 (100)	463 (100)	301 (100)

quercetin-3,4′-diglucoside-3′-(6-acyl-glucosides)

compounds[a]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$MS^2[M-H]^-$ (*m/z*) (%)	$MS^3[(M-H) \rightarrow (M-H\text{-}Glc)]^-$ (*m/z*) (%)	$MS^4[(M-H) \rightarrow (M-H\text{-}Glc) \rightarrow (M-H\text{-}Glc-Glc)]^-$ (*m/z*) (%)					
				–G	–G	–*p*C	–C	–F	–MC	–S	–(AcG)
2 Q-3,4′-diG-3′-(6-MC-G)	18.0		979	817 (100)	655 (100)				463 (57)		301 (100)
4 Q-3,4′-diG-3′-(6-C-G)	19.6		949	787 (100)	625 (100)		463 (52)				301 (100)
6 Q-3,4′-diG-3′-(6-S-G)	21.4	271, 329	993	831 (100)	669 (100)					463 (100)	301 (100)
7 Q-3,4′-diG-3′-(6-F-G)	22.6	271, 327	963	801 (100)	639 (100)			463 (100)			301 (100)
8 Q-3,4′-diG-3′-(6-*p*C-G)	23.4	273, 325	933	771 (100)	609 (100)	463 (12)					301 (100)

quercetin-3-(2-acyl-glucoside)-3′-(6-acyl-glucoside)-4′-glucosides

compounds[a]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$MS^2[M-H]^-$ (*m/z*) (%)	$MS^3[(M-H) \rightarrow (M-H\text{-}Glc)]^-$ (*m/z*) (%)						$MS^4[(M-H) \rightarrow (M-H\text{-}Glc) \rightarrow (M-H\text{-}Glc\text{-}Ac1/Ac2Glc)]^-$ (*m/z*) (%)			
											$MS^4[\rightarrow \rightarrow (...\text{-}Ac1Glc)]^-$		$MS4[\rightarrow \rightarrow (...\text{-}Ac2Glc)]^-$	
				–G	–C	–F	–MC	–S	–(Ac1G)	–(Ac2G)	–Ac2	–Ac2G	–Ac1	–Ac1G
9 Q-3-(2-MC-G)- 3′-(6-S-G)-4′-G	24.2	265, 335	1185	1023 (100)				817 (63)	669 (100)	655 (7)				
10 Q-3-(2-C-G)- 3′-(6-S-G)-4′-G	25.0		1155	993 (100)	831 (38)			787 (57)	669 (100)	625 (62)		301 (100)	463 (70)	301 (100)
11 Q-3-(2-S-G)- 3′-(6-S-G)-4′-G	26.3	275, 329	1199	1037 (100)				831 (5)	669 (100)		463 (100)	301 (73)		
12 Q-3-(2-F-G)- 3′-(6-S-G)-4′-G	26.9	275, 329	1169	1007 (100)		831 (25)			669 (56)	639 (100)	463 (15)	301 (100)		301 (100)
13 Q-3-(2-F-G)- 3′-(6-F-G)-4′-G	28.7	253, 267sh, 337	1139	977 (100)					639 (100)		463 (100)			

[a] Q, quercetin; Glc (G), glucosyl; Ac, acyl; C, caffeoyl; MC, methoxycaffeoyl; *p*C, *p*-coumaroyl; F, feruloyl; and S, sinapoyl.

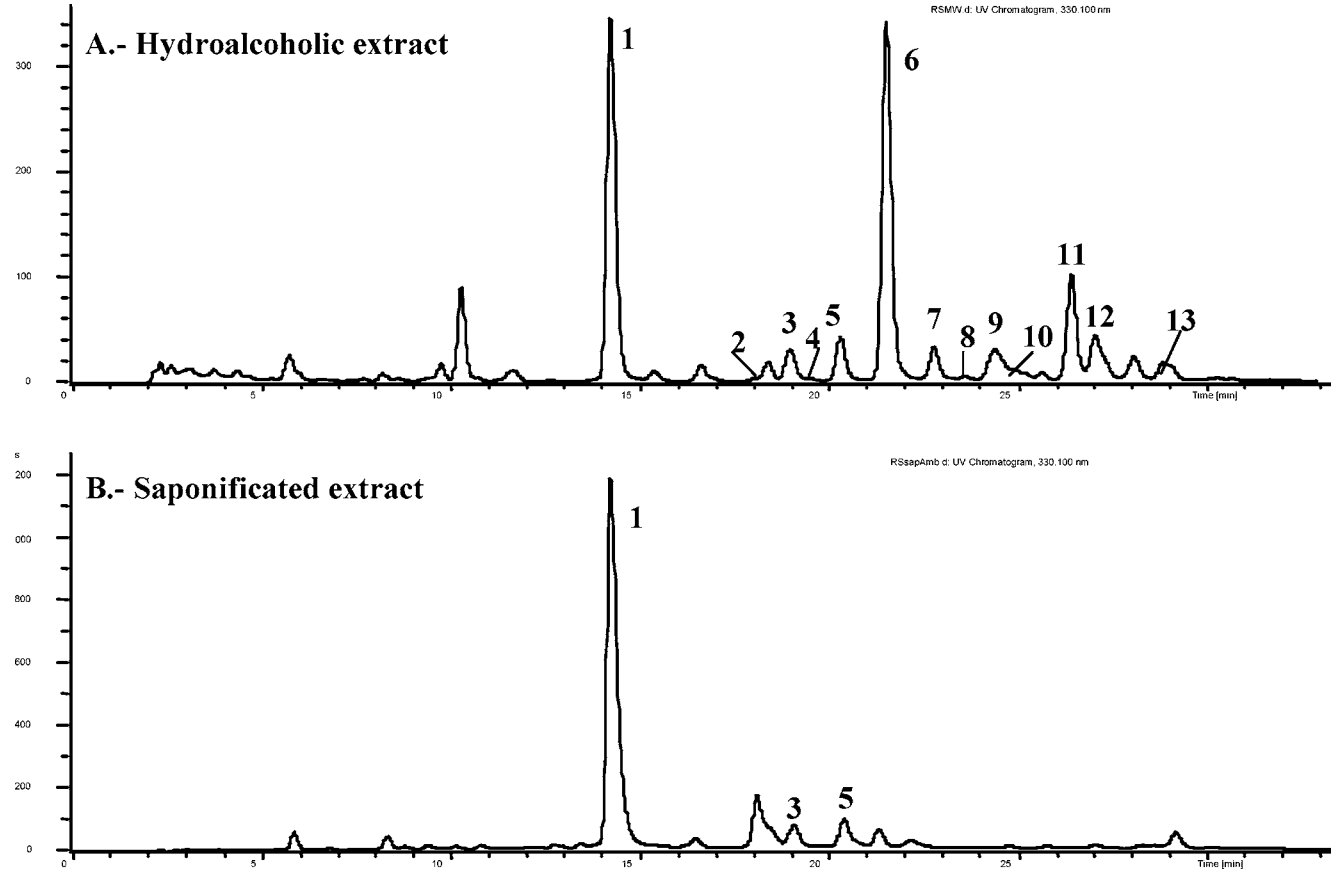


Figure 1. HPLC-DAD chromatogram at 330 nm of the hydroalcoholic and saponificated extracts of *D. tenuifolia*. Compounds: **1**, quercetin-3,3′,4′-triglucoside; **2**, quercetin-3,4′-di-glucoside-3′-(6-methoxycaffeoyl-glucoside); **3**, kaempferol-3,4′-di-glucoside; **4**, quercetin-3,4′-di-glucoside-3′-(6-caffeoyl-glucoside); **5**, isorhamnetin-3,4′-di-glucoside; **6**, quercetin-3,4′-di-glucoside-3′-(6-sinapoyl-glucoside); **7**, quercetin-3,4′-di-glucoside-3′-(6-feruloyl-glucoside); **8**, quercetin-3,4′-di-glucoside-3′-(6-*p*-coumaroyl-glucoside); **9**, quercetin-3-(2-methoxycaffeoyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside; **10**, quercetin-3-(2-caffeoyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside; **11**, quercetin-3-(2-sinapoyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside; **12**, quercetin-3-(2-feruloyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside; and **13**, quercetin-3-(2-feruloyl-glucoside)-3′-(6-feruloyl-glucoside)-4′-glucoside. Compounds were numbered according to HPLC elution order.

On the other hand, the MS study of compound **1** (**Table 1**) gave a deprotonated molecular ion at *m*/*z* 787 corresponding to a quercetin trihexoside, which a similar fragmentation pattern to the flavonoids with glycoside moieties linked to different phenolic hydroxyls (*14*). The following MS^2, MS^3, and MS^4 events showed sequential losses of hexosyl moieties (−162 u) (**Table 1** and **Figure 2**), differing from the fragmentation behavior of the oligosaccharides in which the base peak is the resultant from the loss of the whole glycosylic fraction linked to the phenolic hydroxyls (*14*). Therefore, compound **1** should match with the quercetin-3,3′,4′-tri-*O*-glucoside recently identified in extracts of *E. sativa* (*8*).

The UV and MS studies of the compounds **3** and **5** showed that they were a kaempferol and isorhamnetin di-*O*-hexoside derivatives, respectively, with a hydroxyl at position 3 blocked (UV: λ band I 345 nm).

Acylated Glycosides. Compound **6** (**Figure 1A**) showed a deprotonated molecular ion at *m*/*z* 993 as well as UV−vis spectra characteristic of the hydroxy cinnamoyl derivatives (**Table 1**). The MS^n (*n*, 2−3) fragmentation behavior of this compound (**Table 1**) showed sequential losses of hexosyl residues from the $[M - H]^-$ and $[M - H\text{-}Glc]^-$ ions to give a peak base. Finally, this showed a loss of the sinapoyl (−206) as well as the sinapoyl and hexosyl (−206−162) moieties at $MS^4[(M - H) \rightarrow (M - H\text{-}Glc) \rightarrow (M - H\text{-}Glc - Glc)]^-$ to give the deprotonated aglycon ion (base peak) (**Table 1**). In addition, these were not detected in the HPLC chromatogram of the saponificated extract (**Figure 1B**). Therefore, the MS analysis and the UV spectra suggest that this compound is a sinapoyl derivative of compound **1**. Quercetin-3,4′-di-*O*-glucoside-3′-*O*-(6-sinapoyl-glucoside) was recently identified in *E. sativa* extracts (*8*) suggesting that this has a similar structure to compound **6**.

Compounds **2**, **4**, **7**, and **8** are structurally very close to **6** with the acyl moiety being the unique difference (**Table 1**). Thus, a loss of 192 u in the MS^4 analysis suggests the presence of methoxycaffeic acid as an acyl group (compound **2**), a loss of 162 u corresponds to the loss of caffeic acid (compound **4**), 176 u to ferulic acid (compound **7**), and 146 u to *p*-coumaric acid (compound **8**). Thus, the compounds **2**, **4**, and **6**−**8** have been tentatively identified as quercetin-3,4′-di-*O*-glucoside-3′-*O*-(6-acyl-glucoside), with methoxycaffeic, caffeic, sinapic, ferulic, and *p*-coumaric acids as the corresponding acyl residues, respectively.

Compound **11** was the third most abundant flavonoid (**Figure 1A**). This compound was identified as a disinapoyl derivative of **1** (**Table 1**) and, as for compound **6**, this should match with quercetin-3-*O*-(2-sinapoyl-glucoside)-3′-*O*-(6-sinapoyl-glucoside)-4′-*O*-glucoside recently identified in *E. sativa* extracts (*8*). In addition, **9**, **10**, and **12** also might be identified as dicinnamoyl derivatives of compound **1**, and the same that **11**, derivatives of compound **6** as a result of the acylation of glucose at position 3 with methoxycafeic, cafeic, and ferulic acids, respectively. In this context, compound **13** was also related with compound

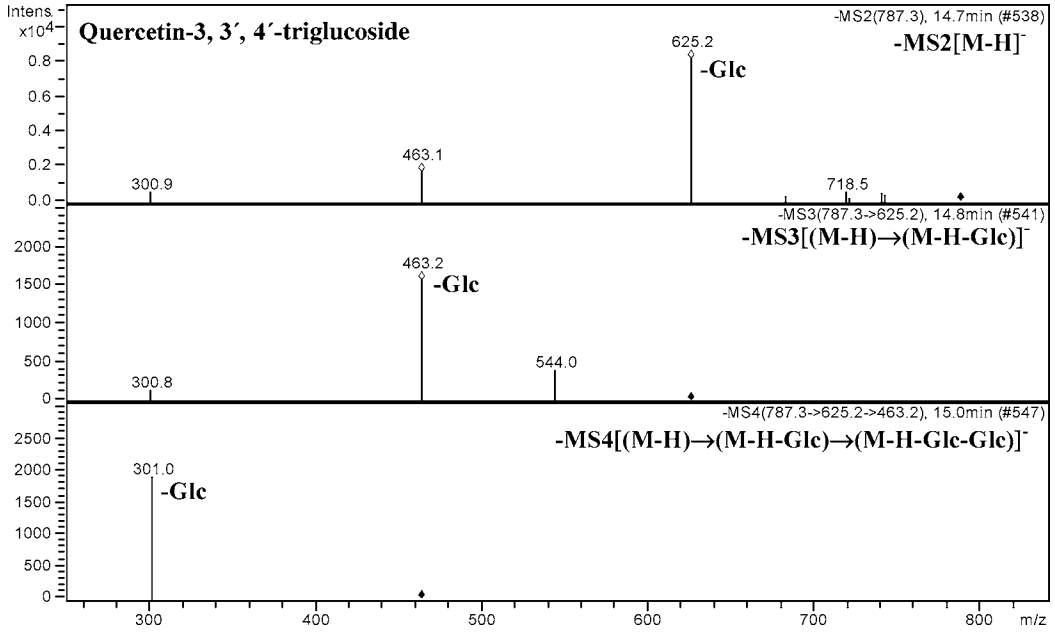


Figure 2. MS^n analysis of quercetin-3,3′,4′-triglucoside (compound **1**).

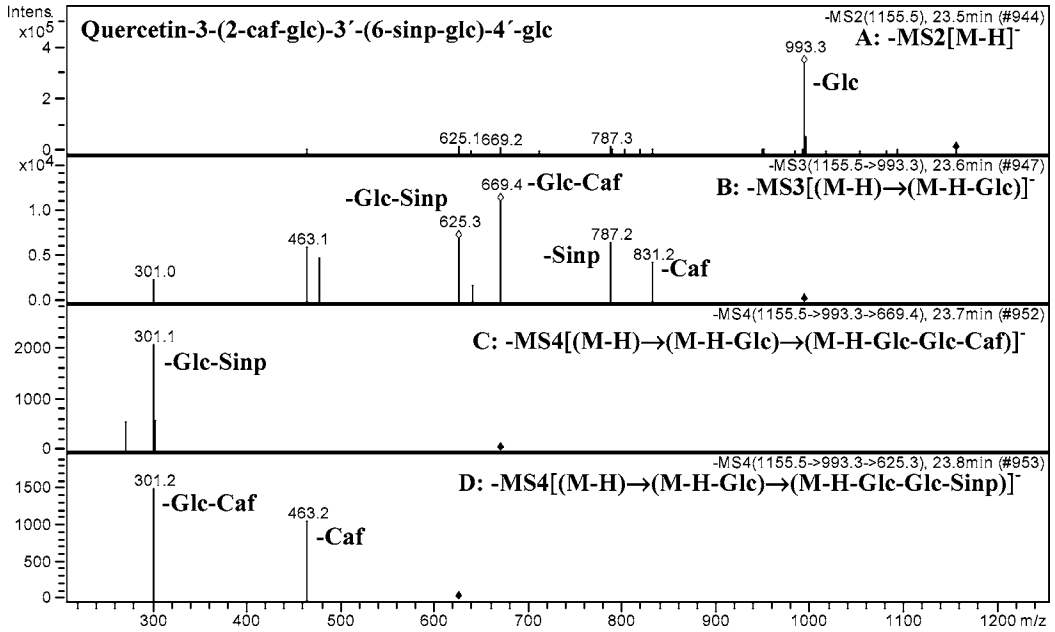


Figure 3. MS^n analysis of quercetin-3-(2-caffeoyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside (compound **10**).

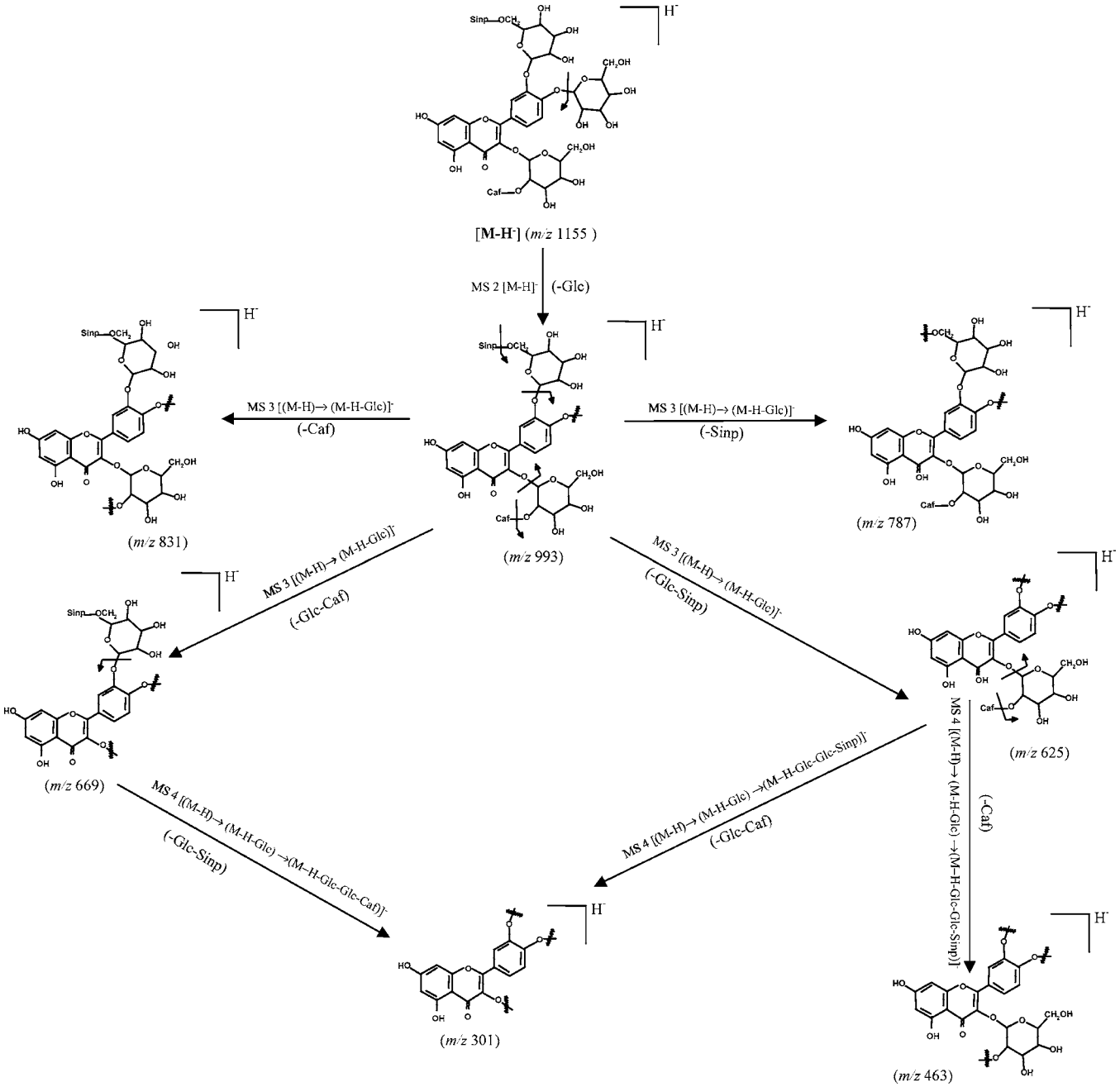
1, being tentatively identified as quercetin-3-*O*-(2-feruloyl-glucoside)-3′-*O*-(6-feruloyl-glucoside)-4′-*O*-glucoside (**Table 1**).

The MS fragmentation of such compounds (**Table 1**) showed a first loss of 162 u from the deprotonated molecular ion that in agreement with the proposed structure it must be a glucose at position 4′ (**Figure 3A** and **Scheme 1**). In the MS^3 event, it was possible to observe a loss of one or both acids, and the loss of glucose with its acyl moiety (**Figure 3B** and **Scheme 1**), where the loss of the acyl-glucosyl radical at position 3 was the base peak in the majority of the studied ions. Finally, the MS^4 events of ions that still have both glucose and acid moieties gave the deprotonated aglycon ion (base peak) (**Figure 3C**,**D**) or the ion coming from the corresponding desacylation (**Figure 3D** and **Scheme 1**).

E. vesicaria. The HPLC/UV-DAD/ESI-MS^n study of *E. vesicaria* showed that the main compound is a kaempferol dihexoside [MS: 609 [M − H]$^-$; MS^2 (609): 447 (46%), 285 (100%)] (**Figure 4**). In addition, the UV spectra and retention time (R_t) (18.8) are similar to compound **3** of *D. tenuifolia*. The UV study of the isolated compound after the addition of alkaline and metal reagents (*13*) showed that the hydroxyl at position 3 is blocked (UV MeOH: 266, 292sh, 345), a hydroxyl free at position 7 (+NaOAc: 275, 373), the blocked hydroxyl at position 4′ (+NaOMe: 275, 297sh, 375↓), as well as the free hydroxyl at position 5 (+$AlCl_3$: 275, 300sh, 344, 395; +$AlCl_3$/HCl: 276, 298sh, 338, 394). After mild acid hydrolysis, it gave a monoglycosylated kaempferol derivative with a free hydroxyl at position 3 [R_t 27.8 min; UV: 251sh, 265, 319sh, 365.; MS: 447 [M − H]$^-$; MS^2 (447): 301 (100%)]. This behavior differs with that of flavonol-3-*O*-diglucosides such as kaempferol-3-sophoroside, which gave only the kaempferol aglycone (*12*). Therefore, this compound has been tentatively identified as kaempferol-3,4′-di-*O*-glucoside.

In the same way, the R_t (20.2 min), UV spectra (253, 265, 345 nm), and the MS study [MS: 639 [M − H]$^-$; MS^2 (639): 477 (50%), 315 (100%)] of compound **5** were similar to those

Scheme 1. ESI-MS^n Fragmentation Pathway of Quercetin-3-(2-caffeoyl-glucoside)-3′-(6-sinapoyl-glucoside)-4′-glucoside (Compound **10**)



of compound **5** of *D. tenuifolia*. In addition, the HPLC-DAD-MS study of the products of mild acid hydrolysis of the original extract rendered a compound with a R_t 30.0, UV spectra (253, 267sh, 367), as well as the MS study [MS: 477 [M − H]$^-$; MS^2 (477): 315 (100%)] that suggested that compound **5** could be tentatively identified as isorhamnetin-3,4′-di-*O*-glucoside.

Following the UV and MS studies for compounds **14**−**16**, they have been assigned as the 3-hexosyl derivatives of quercetin, kaempferol, and isorhamnetin, respectively. After saponification, compound **17** disappeared from the chromatogram showing its acylated nature (**Figure 4A**,**B**). The UV spectra (267, 327 nm) of compound **17**, as well as MS study [MS: 815 [M − H]$^-$; MS^2 (815): 653 (100%), [M − H-Glc]$^-$), 447 (7%, [M − H-Glc-Sinp]$^-$), 285 (3%, [kaempf-H]$^-$); MS^3 (653): 353 (100%), 299 (38%), 285 (50%, [kaempf-H]$^-$)] in the hydroalcoholic extract showed that this compound is a sinapoyl derivative of compound **3**; therefore, it has been tentatively identified as kaempferol-3-*O*-(2-sinapoyl-glucoside)-4′-*O*-glucoside.

***D. tenuifolia* vs *E. vesicaria*.** Important differences between flavonoid profiles of both species were observed (**Figures 1A** and **4A**). The most important one was that the *D. tenuifolia* has quercetin derivatives as principal compounds whereas *E. vesicaria* instead has kaempferol derivatives. These results are in disagreement with those reported by Werckele et al. where leaves of *E. sativa* (Miller) showed quercetin derivatives as main flavonoids (*8*). Compounds identified by Werckele and coworkers are the same as those that we have identified in *D. tenuifolia*, which have not been detected in *E. vesicaria* (*8*).

A recent work by Bennett et al. (*4*) supports the present results as shown that the main flavonoids identified in different *E. sativa* young leaves were kaempferol derivatives while quercetin derivatives were only identified as traces. However, in this paper, both quercetin triglucoside and monosynapoyl triglucoside were

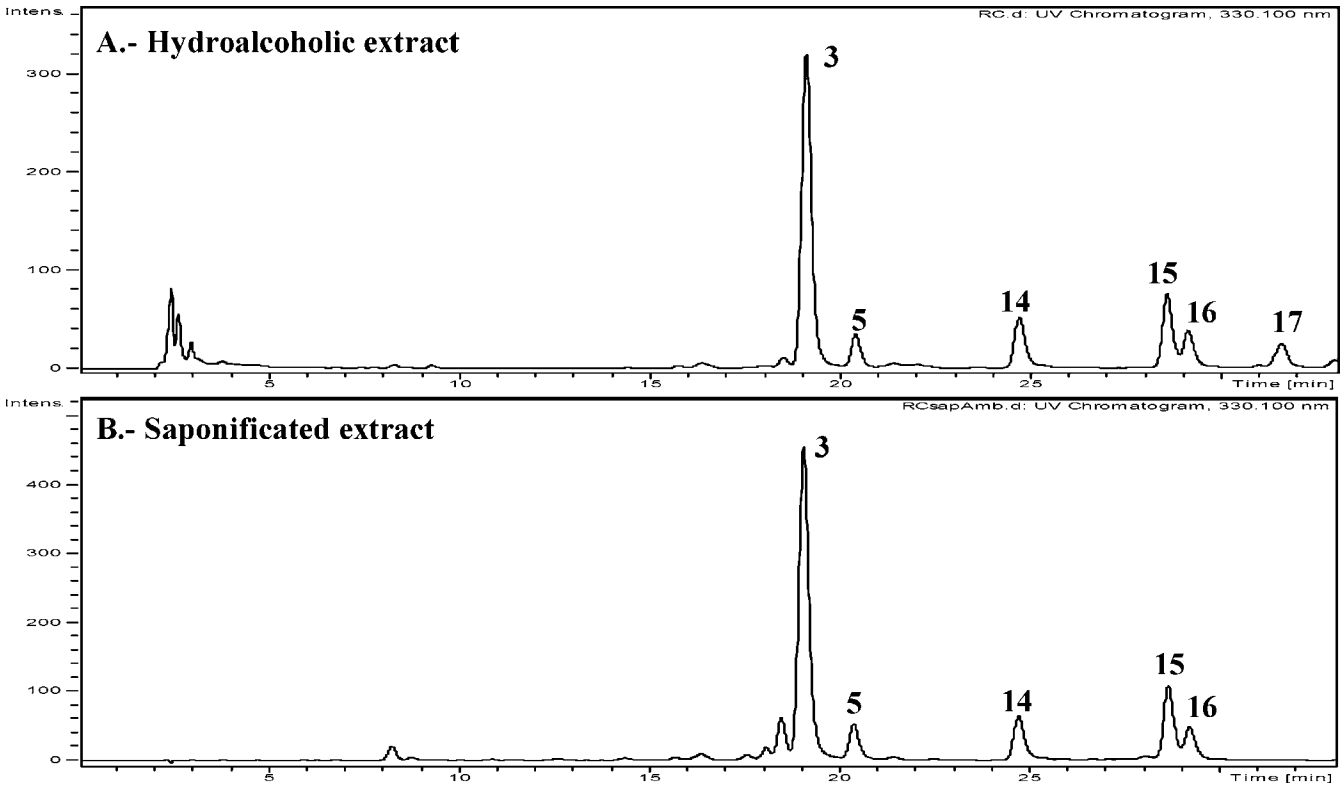


Figure 4. HPLC-DAD chromatogram at 330 nm of the hydroalcoholic and saponificated extracts of *E. vesicaria*. Compounds: **3**, kaempferol-3,4′-di-glucoside; **5**, isorhamnetin-3,4′-di-glucoside; **14**, quercetin-3-glucoside; **15**, kaempferol-3-glucoside; **16**, isorhamnetin-3-glucoside; and **17**, kaempferol-3-(2-sinapoyl-glucoside)-4′-glucoside. Compounds were numbered according to HPLC elution order and numeration of **Figure 1**.

identified as important compounds in the different *E. sativa* seed samples (*4*). Therefore, it is possible to detect quercetin derivatives in leaves in some ecotypes or chemotypes of this plant. The presence of those compounds in seeds could be explained as an answer to specific needs of the seeds (such as germination or defensive responses), and those compounds can disappear along the vegetative cycle of the plant, once they are no longer necessary. However, some flavonoids detected in leaves could be the result of the de novo synthesis.

Some new quercetin triglucosides, both mono- and mainly diacyl derivatives, have been identified in our samples and not in previous metabolite profiling studies in *Brassicaceae* (*4*). Concerning the monoacyl derivatives, both quercetin methoxy-caffeoyl triglucoside and quercetin caffeoyl triglucoside were identified for the first time in *Diplotaxis*. Regarding the diacyl derivatives, dimethoxycaffeic and dicaffeoyl have been identified as new quercetin triglucoside derivatives. A similar acylation pattern was identified in a recent work in *Brassica oleracea* L. var. *costata*; the kaempferol diacyl triglucoside derivatives were the main compounds detected (*15*). To the best of our knowledge, the characterization of these quercetin diacyl tri-*O*-glucosides has not been previously reported in nature, with the exception of compound **11**, recently described by Werckele et al. (*8*).

It is a noteworthy that both *Eruca* and *Diplotaxis* showed a different glycosilation pattern than other closely related species such as *B. oleraceae* (*12*) or *B. rapa* (*6*). The results showed that such a pattern is based on the linkage of glycosyl moieties in different phenolic hydroxyl positions of the flavonoid nucleus (di-*O*-glucosides or tri-*O*-glucosides) in contrast with the *Brassica* species where *O*-di- and *O*-tri-glucosides are the habitual glycosilation pattern (*12*).

Moreover, tetra-*O*-glucosides haven been identified and quantified in young leaf extracts from both *D. tenuifolia* and *D. erucoides* (*4*). In contrast, these compounds have not been identified in the present work in *D. tenuifolia*, and it suggests important differences between ecotypes. However, no structural information on the occurrence of *O*-diglucosides or *O*-triglucosides and on its interglycosidic linkages or the position of linkage in phenolic hydroxyls has previously been reported (*4*). Different tetraglucosides have been identified in *Brassica*, and the interglycosidic linkage was always characterized as 1 → 2 although the sophoroside (glucose 1 → 2 glucose) and sophorostrioside (glucose 1 → 2 glucose 1 → 2 glucose) are more frequently identified in nature (*12*, *16*).

Both *Eruca* and *Diplotaxis* are cosmopolitan genera that are presented in the flora of countries from different continents. Moreover, the bank of genetic resources of these groups showed an important number of possible ecotypes (*1*). For instance, in the Indian subcontinent, particularly in Pakistan, special ecotypes of *E. sativa* are cultivated for seed production (*17*). In addition, *Brassicaceae* has other important secondary metabolites such as glucosinolates, which are involved in many plant functions including natural plant defenses. Recently, Kliebenstien et al. indicated an association between the glucosinolate and the phenylpropanoid pathways, and it appears to be cross-talk between both pathways (*18*). Furthermore, variation in the glucosinolate profile has been found in *Arabidopsis* where after analysis of 39 ecotypes, 14 different glucosinolate profiles were found (*19*). These differences in glucosinolates profile were detected in *D. tenuifolia* (*20*). Therefore, it is possible that the phytochemical differences found between the *Diplotaxis* samples analyzed in this work and those analyzed by Bennett et al. (*4*) are related to different ecotypes or chemotypes. It is also remarkable the high degree of similarities between them. Concerning the *Eruca* profiles, high similarities between our samples and Bennett's samples were found, and in contrast, substantial differences were found regarding those proposed by

Werckele et al. (*8*). These results suggest that a possible incorrect botanical identification could be the explanation for these differences.

In summary, metabolite profiling could be crucial in many aspects such as fundamental research by improving the polyphenols knowledge for the nutritional quality applications of plant food. Moreover, important differences between *Eruca* and *Diplotaxis* genera have been demonstrated, and also, new polyphenols have been tentatively identified for the first time; a study of NMR spectroscopy to confirm these structures was necessary. In this context, further studies concerning the bioavailability of these compounds would be important and mandatory to recommend these species as new source of polyphenols, and additional studies with a larger number of samples of these species are necessary.

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LITERATURE CITED

(1) VVAA. *Rocket: A Mediterranean Crop for the World*; Padulosi, S., Pignone, D., Eds.; International Plant Genetic Resources Institute: Rome, Italy, 1997.

(2) Warwick, S. I.; Francis, A.; La Fleche, J. *Guide to the Wild Germplasm of Brassica and Allied Crops (Tribe Brassiceae, Brassicaceae)*, 2nd ed.; Warwick, S. I., Francis, A., La Fleche, J., Eds.; Ontario, Canada, 2000; available at http://www.brassica.info/resources/crucifer_genetics/brass00.pdf (accessed Jan 8, 2007).

(3) Sánchez-Yélamo, M. D. A chemosystematic survey of flavonoids in the Brassicinae: *Diplotaxis*. *Bot. J. Linn. Soc.* **1994**, *115*, 9−18.

(4) Bennett, R. N.; Rosa, E. A. S.; Mellon, F. A.; Kroon, P. A. Ontogenic profiling of glucosinolates, flavonoids, and other secondary metabolites in *Eruca sativa* (salad rocket), *Diplotaxis erucoides* (wall rocket), *Diplotaxis tenuifolia* (wild rocket), and *Bunias orientalis* (turkish rocket). *J. Agric. Food Chem.* **2006**, *54*, 4005−4015.

(5) Braham, H.; Mighri, Z.; Ben Jannet, H.; Matthew, S.; Abreu, P. M. Antioxidant phenolic glycosides from *Moricandia arvensis*. *J. Nat. Prod.* **2005**, *68*, 517−522.

(6) Romani, A.; Vignolini, P.; Isolani, L.; Ieri, F.; Heimler, D. HPLC-DAD/MS Characterization of flavonoids and hydroxycinnamic derivatives in turnip tops (*Brassica rapa* L. Subsp. *sylvestris* L.). *J. Agric. Food Chem.* **2006**, *54*, 1342−1346.

(7) Zhang, J.; Satterfield, M. B.; Brodbelt, J. S.; Britz, S. J.; Clevidence, B.; Novotny, J. A. Structural characterization and detection of kale flavonoids by electrospray ionization mass spectrometry. *Anal. Chem.* **2003**, *75*, 6401−6407.

(8) Weckerle, B.; Michel, K.; Balazs, B.; Schreier, P.; Toth, G. Quercetin 3,3′,4′;-tri-O-[beta]-glucopyranosides from leaves of *Eruca sativa* (Mill.). *Phytochemistry* **2001**, 547−551.

(9) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727−747.

(10) Vallejo, F. A.; Tomás-Barberán, F. A.; Ferreres, F. Characterisation of flavonols in broccoli (*Brassica oleracea* L. var *italica*) by liquid chromatography-UV diode-array detection-electrospray ionisation mass spectrometry. *J. Chromatogr. A* **2004**, *1054*, 181−193.

(11) Heneidak, S.; Grayer, R. J.; Kite, G. C.; Simmonds, M. S. J. Flavonoid glycosides from Egyptiam species of the tribe Ascleadeae (Apocynaceae, subfamily Asclepiadoideae). *Biochem. Syst. Ecol.* **2006**, 575−584.

(12) Llorach, R.; Gil-Izquierdo, A.; Ferreres, F.; Tomas-Barberan, F. A. HPLC-DAD-MS/MS ESI characterization of unusual highly glycosylated acylated flavonoids from cauliflower (*Brassica oleracea* L. var. *botrytis*) agroindustrial byproducts. *J. Agric. Food Chem.* **2003**, *51*, 3895−3899.

(13) Mabry, T. J. M. K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, 1970.

(14) Ferreres, F.; Llorach, R.; Gil-Izquierdo, A. Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2004**, *39*, 312−321.

(15) Ferreres, F.; Valentao, P.; Llorach, R.; Pinheiro, C.; Cardoso, L.; Pereira, J. A.; Sousa, C.; Seabra, R. M.; Andrade, P. B. Phenolic compounds in external leaves of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC.). *J. Agric. Food Chem.* **2005**, *53*, 2901−2907.

(16) Nielsen, J. K.; Norbaek, R.; Olsen, C. E. Kaempferol tetraglucosides from cabbage leaves. *Phytochemistry* **1998**, *49*, 2171−2176.

(17) Padulosi, S. *Rocket Genetic Resources Network*; Report of the first meeting; International Plant Genetic Resources Institute: Lisbon, Portugal, 1995.

(18) Kliebenstein, D. J.; Rowe, H. C.; Denby, K. J. Secondary metabolites influence Arabidopsis/Botrytis interactions: Variation in host production and pathogen sensitivity. *Plant J.* **2005**, *44*, 25−36.

(19) Kliebenstein, D. J.; Kroymann, J.; Brown, P.; Figuth, A.; Pedersen, D.; Gershenzon, J.; Mitchell-Olds, T. Genetic control of natural variation in Arabidopsis glucosinolate accumulation. *Plant Physiol.* **2001**, *126*, 811−825.

(20) Martínez-Sánchez, A.; Allende, A.; Bennett, R. N.; Ferreres, F.; Gil, M. I. Microbial, nutritional and sensory quality of rocket leaves as affected by different sanitizers. *Postharvest Biol. Technol.* **2006**, *42*, 86−97.

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