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Identification of Flavonoid Diglycosides in Several Genotypes of Asparagus from the Huétor-Tájar Population Variety

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The qualitative and quantitative composition of flavonoids from the Huétor-Tájar population variety of asparagus (commonly known as "*triguero*") was investigated. Flavonoids were analyzed by reversed-phase high-performance liquid chromatography–diode array detection (HPLC–DAD). Liquid chromatography–mass spectrometry (LC–MS) under identical HPLC conditions was used to verify the identities of the flavonoid glycosides from *triguero* asparagus. The quantities of asparagus flavonoids were calculated according to concentration curves constructed with authentic standards. Total flavonoid contents, calculated as the sum of individual compounds, were determined and ranged from 400 to 700 mg/kg fresh weight. The most abundant was rutin, which represented 55–98% of the total flavonoid complement. *Triguero* asparagus were revealed to be an important source of not only quercetin derivatives but also kaempferol and isorhamnetin glycosides. Significant differences ($p < 0.05$) in the content and relative composition of flavonoids were found among the spears of the distinct asparagus genotypes from the Huétor-Tájar population variety.

KEYWORDS: *Triguero* asparagus; flavonoid diglycosides; HPLC–DAD; MS

INTRODUCTION

Flavonoids represent the most common and widely distributed group of plant-food phenolics, and their contents and compositions have been related to the antioxidant properties of different fruits and vegetables (*1*, *2*). The beneficial effects of the consumption of vegetables, such as broccoli (*3*), spinach (*4*), onion, and asparagus (*5*), on human health can, at least partly, be explained by their flavonoid content.

Asparagus is a vegetable that has traditionally been very appreciated for its organoleptic and nutritional characteristics, but this product is also a good source of bioactive compounds that may contribute to enhancing its cultivation and consumption. It has been established that, among the most commonly consumed vegetables, asparagus has the highest antioxidant capacity (*6*, *7*), and this property is associated to a great extent to its total phenolic content (*8*, *9*).

Scarce information is available regarding asparagus spear phenolic characterization, but we have recently investigated the phenolic profile of both white and green asparagus, and the results revealed that, whereas white spears mainly contained hydroxycinnamic acid derivatives, flavonoids were the major phenolics in green asparagus (*10*). In agreement with Maeda et al. (*11*), who reported that rutin represented 60–80% of the total phenolic content of purple and green asparagus extracts, we have found that rutin constituted more than 70% of the total phenolic content of asparagus from commercial hybrids. This flavonoid glycoside has been shown to act as a strong free-radical scavenger and may have a protective role in carcinogenesis and cardiovascular diseases (*2*). The high content of rutin of green asparagus could be directly related to its antioxidant properties. However, other flavonoids that are much more abundant in *triguero* asparagus than in commercial hybrids (*10*) may contribute to that activity and have not been characterized yet.

Wang et al. (*12*) developed a liquid chromatography–mass spectrometry (LC–MS) method for the characterization of the main bioactive compounds, including saponins and flavonoids, in asparagus spears. These authors detected only two flavonoid compounds in asparagus extracts; the major compound was identified as rutin, and the other peak appeared to be a rutin-type flavonoid that should have an extra sugar molecule on its structure.

Several recent studies have been conducted with a focus on the influence of genetics and cultivation area on the phenolic profile of plant foods. Significant differences have been found not only in the total content but also in the flavonoid profile from different varieties of several plant foods, such as strawberries (*13*), grapes (*14*), spinach (*15*), and *Brassica* species (*16*, *17*).

We have previously reported that the flavonoid profile of green asparagus is determined by sample origin and variety (*10*).

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The main objective of the present study was the quantitative determination of the different flavonoid glycosides detected and identified from several genotypes of asparagus from the Huétor-Tájar population variety. Those flavonoids, which are not found in most commercial varieties of green asparagus, were identified and quantified by high-performance liquid chromatography–diode array detection (HPLC–DAD), and LC–MS was used to confirm their structures.

MATERIALS AND METHODS

Plant Material. The samples investigated consisted of spears from 10 native lines of *triguero* asparagus from the Huétor-Tájar population variety and a sample of commercial green asparagus. *Triguero* asparagus are tetraploid subspecies that come from wild asparagus, autochthonous to the Huétor-Tájar area. Asparagus spears were harvested from 10 lines of *triguero* asparagus (HT-1, HT-2, HT-3, HT-4, HT-5, HT-6, HT-7, HT-8, HT-9, and HT-10) from Huétor-Tájar, Granada, Spain, in the spring of 2005 and 2006. These asparagus have been developed and cloned in Las Torres Agricultural Research Center, Alcalá del Río, Sevilla, Spain, during the last year, and they have been classified and selected by their agronomic characteristics. Their chemical characterization, on the basis of flavonoid profiles, may help to establish new criteria for the selection of these *triguero* asparagus.

The spears investigated in this work were harvested from experimental fields of Huétor-Tájar (Granada, Spain). On harvest day, asparagus spears were transported to the laboratory and then weighed, frozen at −20 °C, and freeze-dried. This plant tissue was ground into a fine powder and stored at −20 °C for further analysis.

Phenolic Extraction. Each sample, consisting of 2.5 g of freeze-dried material, was extracted with 100 mL of 80% ethanol (EtOH). The samples were blended in a Sorvall Omnimixer, Model 17106 (Du Pont Co., Newtown, CT), at maximum speed for 1 min, and then filtered through filter paper. Ethanolic extracts were stored at −20 °C until analysis by HPLC. The method was optimized in terms of the extraction of the solvent, sample size, volume, and concentration of ethanol for asparagus spears. All extractions were made in duplicate.

Acid Hydrolysis. The free flavonoid aglycones were released by acidic hydrolysis as follows: 2.5 g of freeze-dried material were extracted with 80 mL of 80% EtOH as described above. A total of 20 mL of 6 M HCl was added, and the solution was incubated for 2 h, with constant mixing, at 90 °C. The extract was filtered through filter paper and made to 100 mL with 80% ethanol. The extracts were stored at −20 °C until analysis.

Qualitative Analysis of Flavonoids by HPLC–DAD. Phenolic compounds were detected and quantified by HPLC using a SYNERGI 4 μHYDRO-RP80A reverse-phase column (25 cm × 4.6 mm i.d., 4 μm; Phenomenex, Macclesfield, Cheshire, U.K.). The gradient profile for the separation of flavonoids was formed using solvent A [10% (v/v) aqueous acetonitrile plus 2 mL/L acetic acid] and solvent B (40% methanol, 40% acetonitrile, and 20% water plus 2 mL/L acetic acid) in the following program: the proportion of B was increased from 10 to 42.5% B for the first 17 min, then to 70% B over the next 6 min, maintained at 70% B for 3.5 min, then to 100% B over the next 5 min, maintained at 100% B for 5 min, and finally returned to the initial conditions. The flow rate was 1 mL/min, and the column temperature was 30 °C. Phenolic compounds were detected using a Jasco-LC-Net II ADC liquid chromatograph system equipped with DAD and a Rheodyne injection valve (20 μL loop). Spectra from all peaks were recorded in the 200–600 nm range, and the chromatograms were acquired at 360 nm.

Isolation of the New Flavonoids Identified in *Triguero* Asparagus. A HPLC method similar to that described above but using a semi-preparative SYNERGI 4 μHYDRO-RP80A reverse-phase column (25 cm × 46 mm i.d., 4 μm; Phenomenex, Macclesfield, Cheshire, U.K.) was developed for the isolation of the new flavonoids. The flow rate was maintained at 10 mL/min, and the injection volume was 400 μL. Elution was monitored by UV at 360 nm, and the flavonoids were manually collected after the UV detector. The two fractions containing each individual compound were then reinjected onto the analytical column, to purify the two isolated flavonoids. Those were concentrated under nitrogen prior to lyophilization.

Table 1. Effect of the Solvent Type and Solvent/Solid Ratio on the Flavonoid Contents Extracted from *Triguero* Asparagus[a]

	mg/kg fresh weight			
	ratio of 1:1	ratio of 1:2	ratio of 1:4	ratio of 1:8
water	214 ± 15	227 ± 9	252 ± 6	250 ± 9
80% ethanol	233 ± 4	545 ± 8	591 ± 6	594 ± 15
80% methanol	443 ± 6	514 ± 17	583 ± 13	594 ± 9

[a] Data are the means of three replicates.

Characterization of Flavonoids by HPLC–DAD–MS. Rutin and the "new flavonoids" detected in asparagus were separated by HPLC, as described above, and identified by their electron impact mass data collected on a quadrupole mass analyzer (ZMD4, Micromass, Waters, Inc., Manchester, U.K.). Electrospray ionization (ESI) mass spectra were obtained at ionization energies of 50 and 100 eV (negative mode) and 50 eV (positive mode), with MS scans from *m*/*z* 100 to 1000. Capillary voltage was 3 kV; desolvation temperature was 200 °C; source temperature was 100 °C; and extractor voltage was 12 V. The flow was maintained at 1 mL min^{-1}.

HPLC–DAD–MS System for Quantitative Analysis. Flavonoid compound quantification was achieved by integration of peak areas, with reference to calibrations made using known amounts of pure compounds.

Results were calculated from the mean of three replicates. Comparisons among samples were done by the analysis of variation (ANOVA) test and the least-square deconvolution (LSD) method at a 95% confidence level.

Validation of the Method of HPLC–DAD–MS for Quantitative Analysis. Calibration curves were established on 8 data points that covered a concentration range of 5–250 μg/mL for each flavonoid glycoside. The linearity response of rutin, kaempferol-3-*O*-rutinoside, and isorhamnetion-3-*O*-rutinoside was determined using standards purchased from Megazyme. Eight concentrations of the mixed standard 80% ethanol solution were injected in duplicate. The calibration curves were constructed by plotting the mean peak area versus the concentration of standards. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

The precision of the method was evaluated from the measurement of intra- and interday variability. For this purpose, the same mixed standard 80% ethanol solution was analyzed 3 times within the same day and then for 3 consecutive days. The assays were realized by triplicate, and the relative standard deviation (RSD) was taken as a measure of precision.

A recovery test was used to evaluate the accuracy of the method. Accurate amounts of the three standards were added to 2.5 g of freeze-dried asparagus (plant material) and then extracted and analyzed as described above. The average percentage recoveries were calculated as the ratio of detected amount versus added amount. The recovery experiment was performed with three replicates at two concentration levels.

RESULTS AND DISCUSSION

Extraction Procedure. Prior to achieving the detailed analysis of the phenolic profile of green asparagus, the influence of different process conditions (raw material, solvent type, solvent/solid ratio, simple or sequential extraction, time of extraction) on the phenolics extraction efficiency was studied. The results (**Tables 1**–**3**) showed the following: (1) The yield of total soluble phenolics was equivalent from fresh asparagus and freeze-dried material. The water content of the spears was 90%, and the values of the distinct samples investigated were not significantly different ($p < 0.05$). (2) The yield of phenolics was also equivalent when using methanol or ethanol aqueous

Table 2. Effect of Sequential Extraction on the Flavonoid Contents Extracted from *Triguero* Asparagus[a]

	mg/kg fresh weight			
	1st extraction	2nd extraction	3rd extraction	4th extraction
1 × 25 mL of 80% EtOH	233 ± 4			
1 × 100 mL of 80% EtOH	591 ± 6			
4 × 25 mL of 80% EtOH	233 ± 4	155 ± 21	71 ± 5	0
4 × 100 mL of 80% EtOH	591 ± 6	9 ± 1	0	0

[a] Data are the means of three replicates.

Table 3. Effect of the Extraction Time on the Flavonoid Contents Extracted from *Triguero* Asparagus[a]

	mg/kg fresh weight			
	1 min	1 h	2 h	16 h
1 × 25 mL of 80% EtOH	233 ± 4	237 ± 6	230 ± 4	235 ± 9
1 × 100 mL of 80% EtOH	591 ± 6	588 ± 11	605 ± 8	593 ± 8

[a] Data are the means of three replicates.

solutions and higher than that reached using water as the extraction solvent. It is noteworthy that the highest results were attained when the alcohol concentration was ≥70%. (3) The solvent/solid ratio greatly influenced the phenolic yield. Preliminary assays were conducted by extracting 25 g of fresh sample and 2.5 g of freeze-dried material with 25–50 and 100 mL of 80% ethanol or water, respectively, and the results revealed that the higher the solvent/solid ratio, the higher the total amount of phenolics solubilized. Extraction volumes higher than 100 mL did not increase the amount of phenolics released, which revealed that the optimal solvent/solid ratio was 1:4 (g of fresh sample/mL of ethanol). (4) Sequential extraction of asparagus samples, using 4 × 25 or 4 × 100 mL aliquots of extraction solvent, did not result in a higher yield of phenolics compared to simple extraction with 100 mL of 80% ethanol. (5) In the same way, increasing the time of extraction did not have a positive effect on extraction efficiency, because extraction for 1, 2, and 24 h with constant mixing did not make a significant difference in the amount of solubilized phenolics compared to that obtained by extracting the samples for 1–2 min.

Characterization of Flavonoid Glycosides from *Triguero* Asparagus. A representative chromatogram of the ethanolic extract from *triguero* asparagus is shown in **Figure 1**. From the HPLC–DAD data, the major peak was identified as rutin, because its retention time and UV spectrum were identical to those of the rutin standard purchased from Sigma. In addition to rutin, there were two significant peaks on the chromatogram of the ethanolic extract from *triguero* asparagus (**Figure 1A**). From the HPLC–DAD data, those peaks were tentatively identified as flavonoid glycosides, because the UV spectra from peak 1 (264, 280sh, and 348) and peak 2 (252, 280, and 356) were similar to that from rutin (255, 279sh, and 355). The chromatogram from the ethanolic extract of commercial green asparagus was also recorded (inset of **Figure 1A**), and it consisted of a very prominent peak of rutin, which was only accompanied by one or two other minor peaks of flavonoids that, in many cases, were only detected in trace amounts.

After acid hydrolysis of the *triguero* and commercial green asparagus ethanolic extracts, three different flavonol aglycones were detected for the former (**Figure 1B**), while a unique peak was found for the latter (inset of **Figure 1B**). The aglycones were identified by a comparison of retention times, DAD information, and co-injection with standards. The results revealed that commercial asparagus only yielded quercetin. Analysis of the hydrolyzate from *triguero* asparagus revealed that quercetin was the most prominent aglycone as expected, because rutin represented more than 50% of the total flavonoid complement in all samples investigated in this study. However, it has been revealed that *triguero* asparagus is also a good source of glycosides from kaempferol and isorhamnetin, flavonols that are not found in most varieties of green asparagus. By a comparison of the UV spectra from the new flavonoid glycosides with those from the aglycones, it can be proposed that peak 1 must be a kaempferol (264, 280sh, and 364) derivative, while peak 2 could derive from quercetin (252, 284sh, and 372) or isorhamnetin (252, 284sh, and 368).

Because UV spectra were not enough to identify the flavonoids, a HPLC–MS method for recording the MS spectra from both aglycones and flavonoid glycosides was developed. The ESI–HPLC–MS analyses allowed the first structure hypotheses to be established. The use of alternating positive/negative ionization modes during recording was preferred to ensure the assignment of the molecular weights.

Figure 2A shows the negative-ion MS spectrum of rutin (*m*/*z* 609). It can be observed that, apart from the molecular ion, the main product ions were at *m*/*z* 301, 300, 271, 255, and 151. As reported previously (*18*), deprotonated flavonoid-*O*-glycosides, such as rutin (quercetin-3-*O*-rhamnoglucoside), provide both a radical aglycone anion $(Y_0 - H)^{-\bullet}$ at *m*/*z* 301 and an aglycone product ion (Y_0^-) at *m*/*z* 300.

The $[M - H]^-$ product ion spectra of rutin also reveals an abundant $[Y_0 - H - CO - H]^-$ ion at *m*/*z* 271 and a $[Y_0 - H - CO_2 - H]^-$ ion at *m*/*z* 255. These ions provide structural information for isomeric differentiation and determination of the glycosylation position (*19*), because their presence is indicative of 3-O-glycosylation, while 7-O-glycosylated flavonoids would provide an abundant $[Y_0 - CO]^-$ ion at *m*/*z* 273, which was not detected in this case.

The relative abundances of the Y_0^- and $[Y_0 - H]^{-\bullet}$ ions have been proposed to be related to the flavonoid glycosylation position. According to Ferreres et al. (*20*), the Y_0^- ion was the base peak for the flavonoid *O*-diglycosides, whereas it was represented about 30% relative abundance for the flavonoid di-*O*-glycosides. In agreement with these findings, **Figure 2A** shows that rutin, with the two sugars linked in position 3, yielded Y_0^- as the main ion in the negative mode.

The positive-ion spectrum of flavonoid glycosides provides additional and complementary information about structural characteristics, mainly on those aspects related to the position of the sugars. As observed in **Figure 2B**, the ESI spectra of the $[M - H]^+$ ion of rutin, at *m*/*z* 611, showed two main product ions corresponding to two successive losses of sugar residues. The first loss corresponded to a rhamnose residue (146 units), yielding the Y_1^+ ion at *m*/*z* 465, and this was followed by the elimination of glucose (162 units), giving the Y_0^+ ion at *m*/*z* 303.

On the basis of the hypothesis illustrated above by the characterization of rutin with the application of ESI–MS techniques in positive- and negative-ion modes, it has been possible to determine the tentative structures of the two new flavonoid diglycosides detected in *triguero* asparagus. The MS analysis of compound 1 showed a molecular ion at *m*/*z* 593 (**Figure 3**). Its $[M - H]^-$ product ion spectrum gave rise to the Y_0^- [M – H – 308] at *m*/*z* 285 as the base peak and also revealed an abundant $[Y_0 - H - CO - H]$ ion at *m*/*z* 255. This fragmentation pattern indicates that compound 1 is a kaempferol

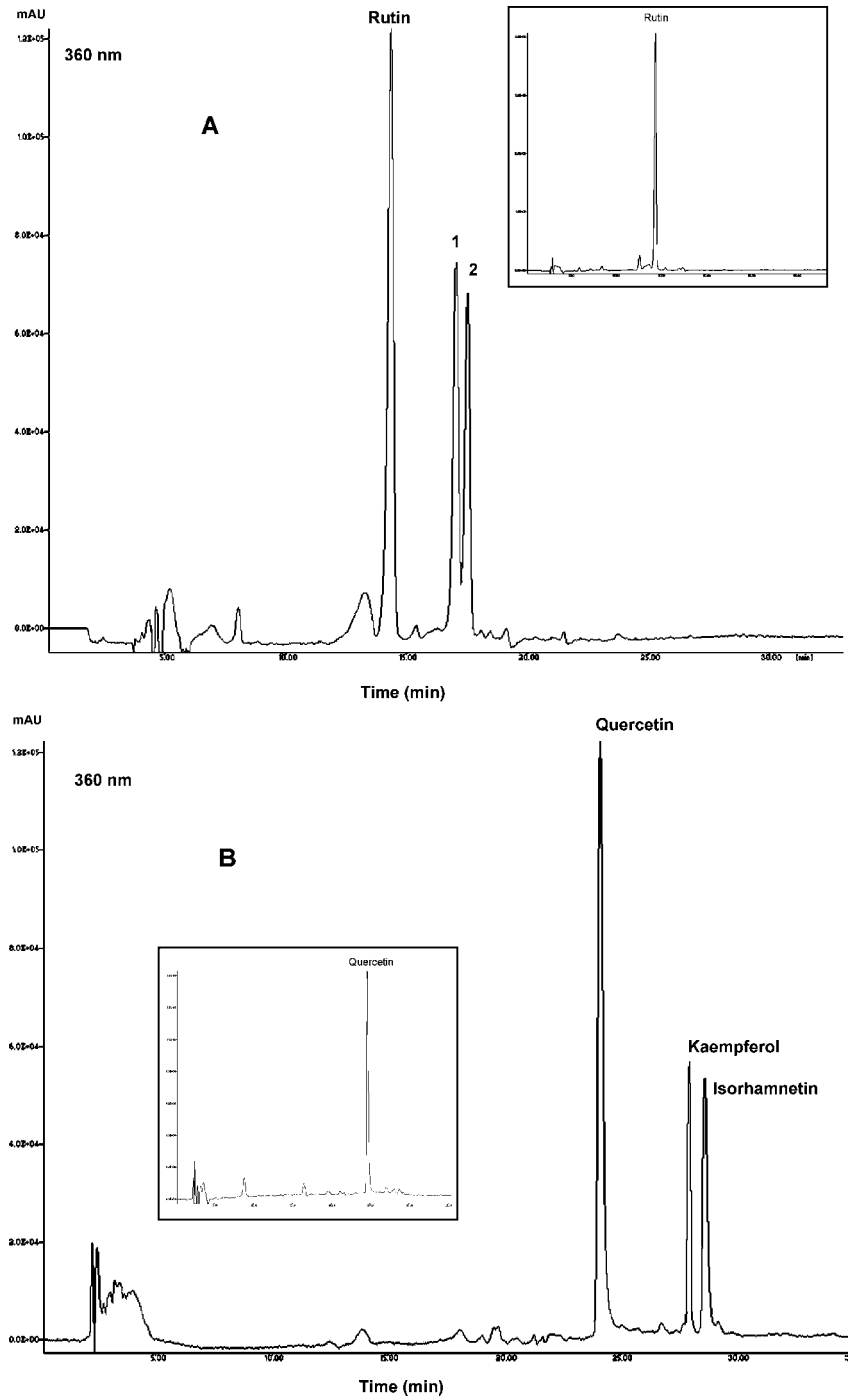

Figure 1. Chromatographic profiles acquired by HPLC–DAD (360 nm) of the 80% ethanolic extract of *triguero* asparagus (A) and its acid hydrolyzate (B). (Insets) Chromatograpahic profiles acquired by HPLC–DAD (360 nm) of the 80% ethanolic extract of commercial asparagus (A) and its acid hydrolyzate (B).

glycosylated with two sugar residues, consisting of a hexose, likely glucose (162 units), and a deoxyhexose, likely rhamnose (146 units). According to Ferreres et al. (*20*), the flavonoid diglycosides with sugar moieties linked to different phenolic positions of the flavonoid nucleus provided a Y_1^- [M – H – 162] ion, which is formed by a loss of a glucosyl from the [M $-$ H]$^-$ as the base peak, and the Y_0^- represented around 30% relative abundance, whereas the flavonoid diglycosides with two sugar moieties linked to the same phenolic position yielded the aglycone ion product (Y_0^-) as the base peak. As described above for rutin, an aglycone product ion is the base peak for compound 1, because it can be observed in its negative-ion spectrum (**Figure 3A**). It has also been established that the glycosylation position significantly influences the fragmentation behavior of flavonoid *O*-glycosides and has been shown to affect the relative abundances of radical aglycone ions, which are most pronounced for flavonol 3-*O*-glycosides. The presence of an abundant [Y_0 – H]$^{-\bullet}$ ion at *m*/*z* 284 and a significant [Y_0 – H – CO – H] ion at *m*/*z* 255 supports the fact that the rhamnose and glucose residues are located at the 3-O positions. In addition to these findings, the fact that the characteristic [Y_0 – CO]$^-$ ion of the 7-*O*-glycosides was not detected is consistent with the two sugar residues being located at the 3-O position. Detailed analysis of the positive-ion MS spectrum allowed for the confirmation of the nature and position of the sugars linked to this kaempferol derivative. The ESI spectrum of the [M $-$ H]$^+$ ion at *m*/*z* 595

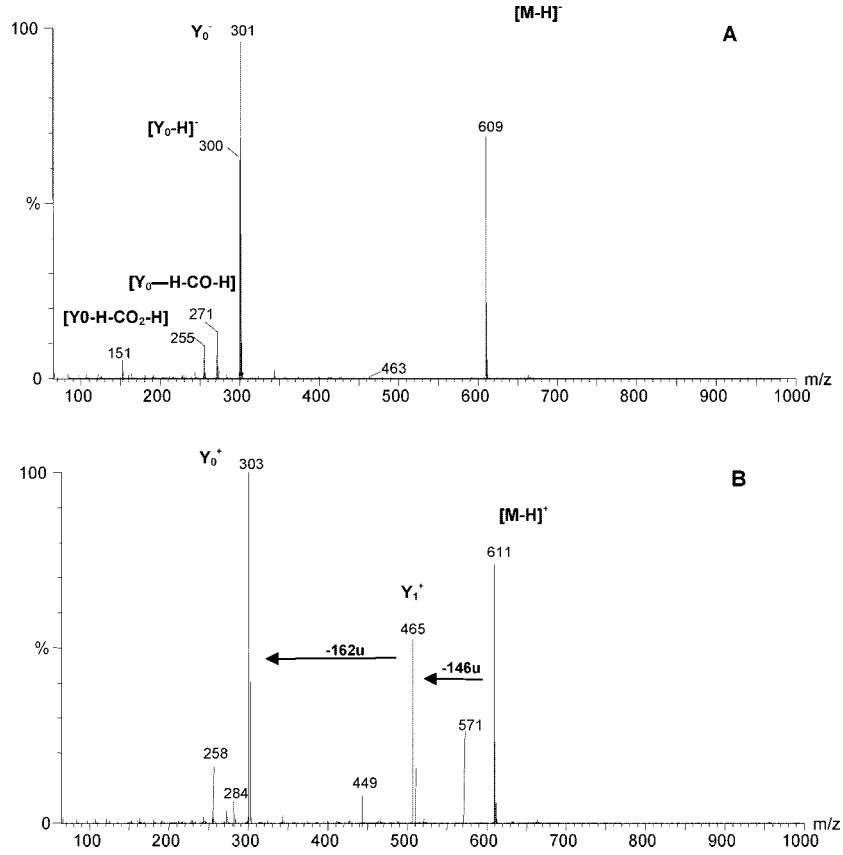

Figure 2. ESI spectra of rutin in negative (A) and positive (B) modes.

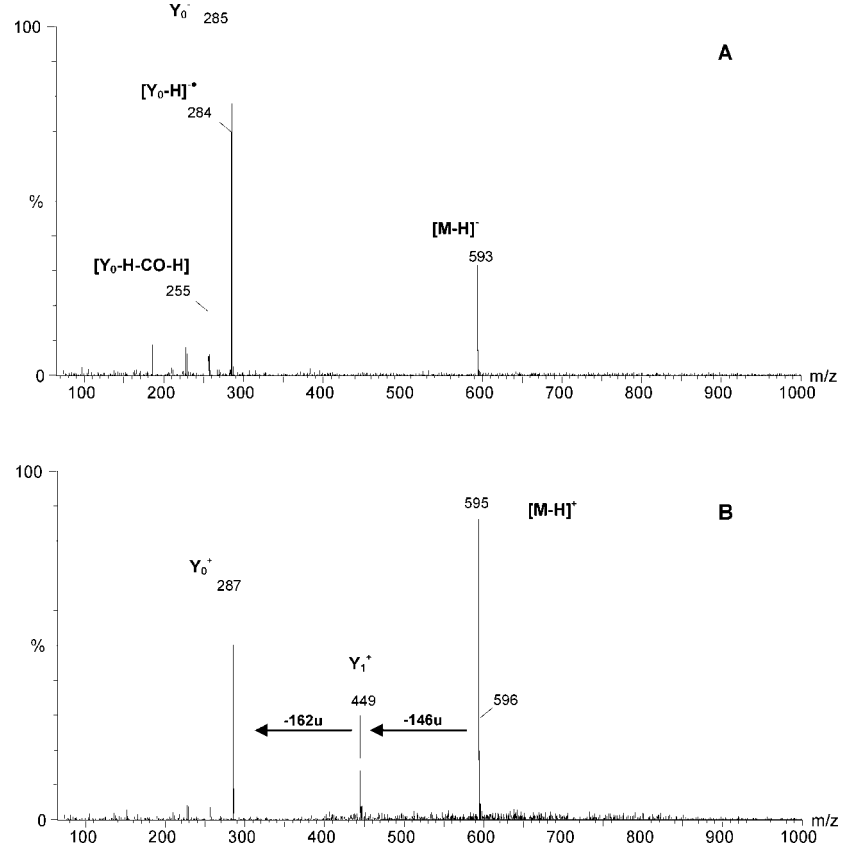

Figure 3. ESI spectra of kaempferol diglycoside in negative (A) and positive (B) mode.

from compound 1 (**Figure 3B**) showed two main product ions, indicating two losses of sugar residues. The first loss corresponded to a rhamnose (146 units), giving Y_1^+ at *m*/*z* 449, and then the loss of glucose (162 units) yielded the Y_0^+ ion at *m*/*z* 287, which was assigned as protonated kaempferol. These results are in agreement with the fragmentation behavior of a

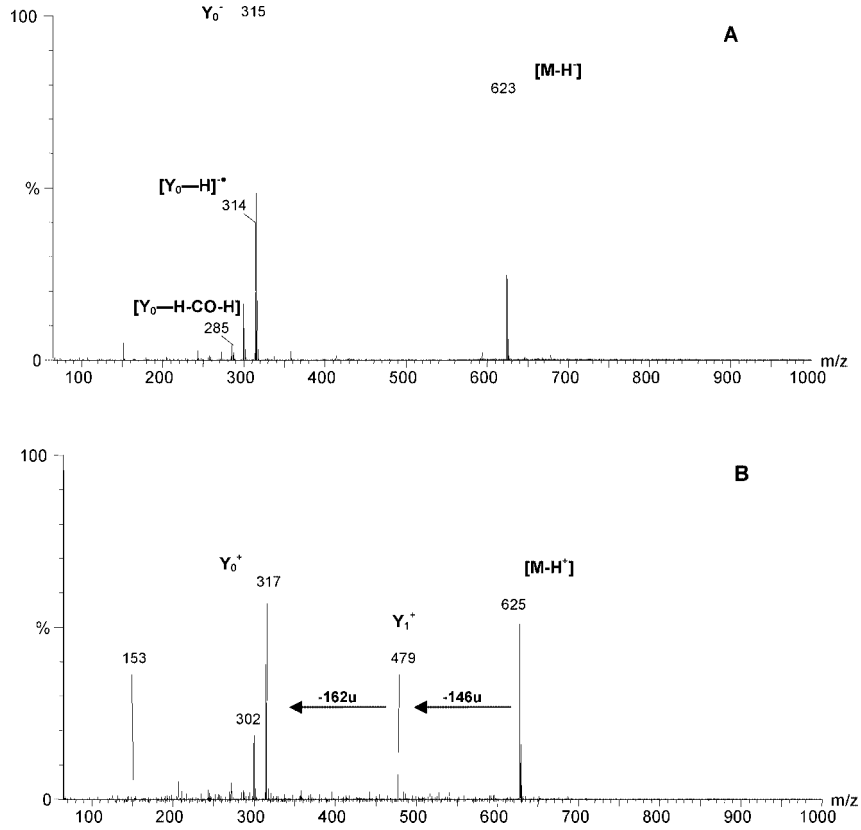

Figure 4. ESI spectra of isorhamnetin diglycoside in negative (A) and positive (B) mode.

3-*O*-rutinoside flavonol, which, in this case, would be the kaempferol-3-*O*-rutinoside. This compound, known as nicotiflorin, has not been previously detected in asparagus, but its presence has been reported in other plant food, such as quince fruit (*21*).

The mass spectra of compound 2 revealed that this was also a flavonoid diglycoside, whose fragmentation pattern was similar to that of rutin and compound 1. **Figure 4** shows the negative and positive spectra of compound 2. After ensuring the assignment of its molecular weight (623), as well as that of the corresponding aglycone (315), by analyzing the fragments issued from the $[MH]^-$ ion at *m*/*z* 623 (**Figure 4A**), the nature and position of the two sugar residues present in this compound were determined from the information generated by the fragmentation of the $[M - H]^+$ ion at *m*/*z* 625 (**Figure 4B**). Following the premises established for the characterization of compound 1, the other flavonoid detected in *triguero* asparagus has been tentatively assigned as isorhamnetin-3-*O*-rutinoside. This compound, which is also present in significant quantities in the *triguero* cultivars investigated in this work, had not been previously described in asparagus, because it is not present in the green spears from commercial hybrids. To our knowledge, almonds are the unique plant food that contain isorhamnetin-3-*O*-rutinoside as a predominant flavonoid (*22*).

The identities of kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside were confirmed by injection of authentic standards purchased from Extrasynthese. Authentic compounds were injected alone and with their corresponding flavonoids isolated from *triguero* asparagus extracts. Retention times and UV and MS profiles were the same for pure kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside as for those of peaks 1 and 2.

Validation of the Method for Quantitative Analysis of Flavonoids from *Triguero* Asparagus. To ensure the accurate assessment of the contents of the three flavonoid glycosides found in *triguero* asparagus, the HPLC–DAD–MS method was validated prior to its application for the quantitative analysis of different asparagus cultivars. The calibration curves of rutin, nicotiflorin, and narcissin showed good linear regression within test ranges, as observed in **Table 4**. The limit of detection, defined as the lowest sample concentration that can be detected (signal-to-noise-ratio = 3), was 4.61 μg/mL for rutin, 1.89 μg/mL for nicotiflorin, and 2.90 μg/mL for narcissin, and the limit of quantification, defined as the lowesr sample concentration that can be quantitatively determined with suitable precision and accuracy (signal-to-noise ratio = 10), was 15.39 μg/mL for rutin, 6.31 μg/mL for nicotiflorin, and μg/mL for narcissin. As shown in **Table 5**, the developed analytical method provided good precision and stability, because the overall intra- and interday variations were less than 4.1% for all flavonoids. To test the recovery of the method, one asparagus sample was added to known quantities of each of the reference flavonoids. The samples were analyzed before and after the additions in triplicate. Results showed that 105% of rutin, 93% of kaempferol-3-*O*-rutinoside, and 95% of isorhamnetin-3-*O*-rutinoside were recovered (**Table 5**).

Table 4. Calibration Curves, LODs, and LOQs of the Three Flavonoid Diglycosides[a]

flavonoid diglycoside	calibration curve	r^2	test range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
rutin	$y = 25\,074x + 126\,136$	0.9978	25–250	4.61	15.39
k-3-*O*-rutinoside	$y = 27\,162x - 55\,839$	0.9996	5–250	1.89	6.31
i-3-*O*-rutinoside	$y = 29\,329x + 32\,576$	0.9991	5–250	2.90	9.67

[a] Data are the means of three replicates.

Analytical characteristics of the calibration graphs as well as the precision and accuracy of the method were satisfactory and comparable to those reported by other authors that have

Table 5. Precisions and Recoveries of the Three Flavonoid Diglycosides[a]

flavonoid diglycoside	precision		recovery ($n = 3$)	
	intraday RSD (%)	interday RSD (%)	recovery (%)	RSD (%)
rutin	0.45	0.46	104.93	2.80
k-3-*O*-rutinoside	0.42	1.33	92.97	4.09
i-3-*O*-rutinoside	0.43	0.54	95.94	1.67

[a] Data are the means of three replicates.

Table 6. *Triguero* Asparagus Flavonoids Identified by HPLC–DAD and HPLC–MS[a]

asparagus line	mg/kg fresh weight						
	rutin	(%)	k-3-*O*-rutinoside	(%)	i-3-*O*-rutinoside	(%)	total flavonoids
HT-1	336 ± 15	(83)	5 ± 0	(1)	66 ± 0	(16)	407 ± 13
HT-2	476 ± 30	(86)	13 ± 1	(2)	65 ± 1	(12)	553 ± 44
HT-3	332 ± 16	(69)	39 ± 4	(8)	111 ± 18	(23)	481 ± 37
HT-4	401 ± 13	(78)	6 ± 0	(1)	108 ± 3	(21)	515 ± 16
HT-5	382 ± 28	(55)	108 ± 2	(16)	203 ± 16	(29)	692 ± 47
HT-6	368 ± 15	(67)	35 ± 1	(6)	147 ± 2	(27)	549 ± 14
HT-7	498 ± 12	(72)	34 ± 1	(5)	162 ± 4	(23)	694 ± 18
HT-8	548 ± 8	(97)	5 ± 0	(1)	9 ± 4	(2)	562 ± 5
HT-9	230 ± 5	(55)	31 ± 1	(7)	157 ± 3	(38)	418 ± 9
HT-10	411 ± 4	(75)	42 ± 2	(8)	97 ± 4	(18)	549 ± 6

[a] Data are the means of three replicates. Data in parentheses represent the relative percent.

developed HPLC–DAD–MS analytical methods for the determination of flavonoid compounds from several plant materials (*23–25*). It can be concluded that the recommended method is reliable and accurate for the qualitative and quantitative determination of flavonoid diglycosides from asparagus.

Quantification of Flavonoids from 10 Varieties of *Triguero* Asparagus. The main flavonoid glycosides found in all of the asparagus varieties were rutin, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside, but significant differences were found in the total quantities and the relative compositions of the distinct asparagus varieties (**Table 6**). The flavonoid content of the 10 varieties investigated varied between 400 and 700 mg/kg fresh weight, which was within the range of the values reported for green asparagus from commercial varieties (*12, 26*). Rutin has been reported as the main phenolic compound in green asparagus, and it represents more than 80% of the total phenolic complement of commercial hybrids (*10, 11*). Therefore, this has been described as the main flavonoid related to the antioxidant activity of ethanolic extracts from different varieties of green asparagus (*11, 26, 27*), and although the presence of other related flavonoids accompanying rutin has recently been reported (*12*), they have not been characterized yet. As observed in **Table 6**, there are several varieties of *triguero* asparagus, such as HT-1, HT-2, and HT-8, whose flavonoid profiles are similar to that found in commercial hybrids (**Figure 1A**). However, there is another group of *triguero* varieties, such as HT-5, HT-6, and HT-9, in which kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside represent nearly 50% of the total flavonoid content. Fuleki (*27*) has reported that rutin is the only flavonoid present in green asparagus spears, and this author suggested that other minor peaks detected in the chromatogram from asparagus methanolic extracts were impurities, because most of them were even detected in commercial rutin used as a standard. From the results of the present paper, it can be proposed that some of those peaks did not correspond to impurities but to the kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside, which are present in significantly greater quantities in *triguero* asparagus than other green varieties. These findings may explain why these compounds had not been previously described in green asparagus, because of the fact they are not found or are present in very low quantities in most commercial varieties.

A recent study about the characterization of antioxidant components of some Italian edible wild greens (*8*) showed that wild asparagus species, such as *Asparagus acutifolius*, have a considerable antioxidant capacity that seems to be related to their flavonoid content. The detailed analysis of their flavonoid profiles revealed that, apart from rutin and other quercetin glycosides, those asparagus contain significant amounts of kaempferol and isorhamnetin derivatives. Because the samples were analyzed after acidic hydrolysis, just the aglycones were determined and flavonoid content was quantified as quercetin equivalents, because that was the most abundant aglycone. These data are in agreement with our results and support the fact that *triguero* asparagus may come from wild species distinct from *Asparagus officinalis*, which gives them a characteristic phytochemical profile that can be used for differentiating and revalorizating native asparagus cultivars. It has been established that the bioactive properties of flavonoid compounds are dependent upon their structure and that minor differences in the number and position of –OH and sugar residues linked to the flavonol skeleton may lead to great differences in the bioactive properties of the individual compounds (*28*). Thus, deeper studies on the isolation and structural characterization of flavonoids and other bioactive compounds from *triguero* asparagus will establish relationships between individual components and specific beneficial actions associated with asparagus, mainly derived from its antioxidant capacity.

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