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Flavonoid Profile of Green Asparagus Genotypes

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The determination of flavonoid profiles from different genotypes of *triguero* asparagus and their comparison to those from green asparagus commercial hybrids was the main goal of this study. The samples consisted of 32 commercial hybrids and 65 genotypes from the Huétor-Tájar population variety (*triguero*). The analysis of individual flavonoids by HPLC-DAD-MS has allowed the determination of eight naturally occurring flavonol derivatives in several genotypes of *triguero* asparagus. Those compounds included mono-, di-, and triglycosides of three flavonols, that is, quercetin, isorhamnetin, and kaempferol. The detailed analysis of the flavonoid profiles revealed significant differences among the distinct genotypes. These have been classified in three distinct groups as the result of a *k*-means clustering analysis, two of them containing both commercial hybrids and *triguero* asparagus and another cluster constituted by 21 genotypes of *triguero* asparagus, which contain several key flavonol derivatives able to differentiate them. Hence, the triglycosides tentatively identified as quercetin-3-rhamnosyl-rutinoside, isorhamnetin-3-rhamnosyl-rutinoside, and isorhamnetin-3-*O*-glucoside have been detected only in the genotypes grouped in the above-mentioned cluster. On the other hand, the compound tentatively identified as isorhamnetin-3-glucosyl-rutinoside was present in most genotypes of triguero *asparagus*, whereas it has not been detected in any of the commercial hybrids.

KEYWORDS: Asparagus; genotypes; cluster analysis; flavonoid glycosides; HPLC-DAD-MS

INTRODUCTION

Asparagus known as *triguero* are tetraploid subspecies from Huétor-Tájar, Granada, which proceed from wild species that have traditionally been collected and consumed throughout southern Spain. These green-purple asparagus are very appreciated for their organoleptic and nutritional properties, but their cultivation is being replaced by commercial hybrids, which yield more homogeneous production and spears of greater caliber.

Because health-promoting characteristics in food are increasingly demanded and included in the purchase decision by the discriminating consumer, the investigation of the bioactive compounds responsible of the beneficial effects associated with a specific product is of great interest for its revalorization.

Experimental evidence has demonstrated that each plant species is characterized by a limited number of phytochemicals; and within the same species, the nature of those compounds can vary from organ to organ but is constant enough toward several other factors (*1, 2*). Therefore, the characterization of fruits and vegetables can be made from their phytochemical profile, and it may be used, for example, to differentiate varieties of a plant food (*3–5*).

We have previously reported that there is high correlation between antioxidant capacity and total phenol content in *triguero* asparagus (*6*), which suggests that phenols could be mainly responsible for that activity as happens for other plant-derived products (*7–10*). It has also been established that flavonoids are the most abundant phenolics in green asparagus and that their profile is significantly different from that found in green spears from commercial hybrids (*11, 12*). The composition of flavonoids in plants is influenced by both genetic factors and environmental conditions. The former seem to be the most determinant factor because significant differences were found between *triguero* native spears and commercial hybrids both cultivated in Huétor-Tájar (*12*). The determination of the flavonoid profiles from different genotypes of *triguero* asparagus and their comparison to those from green asparagus hybrids developed in recent years by major international asparagus

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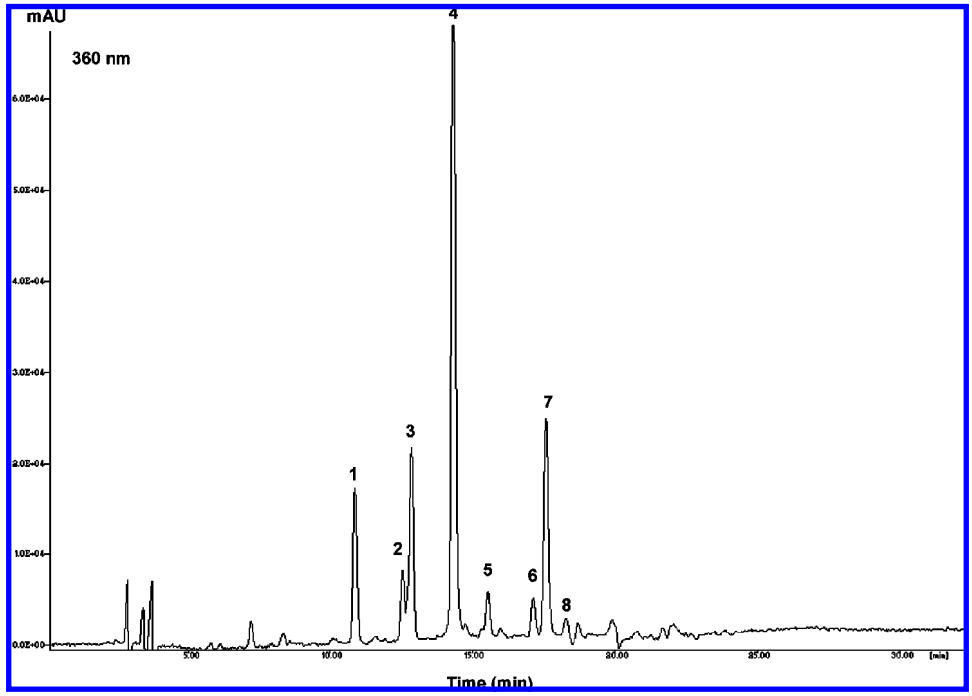

Figure 1. Chromatograpahic profile acquired by HPLC-DAD (360 nm) of a *triguero* asparagus ethanolic extract.

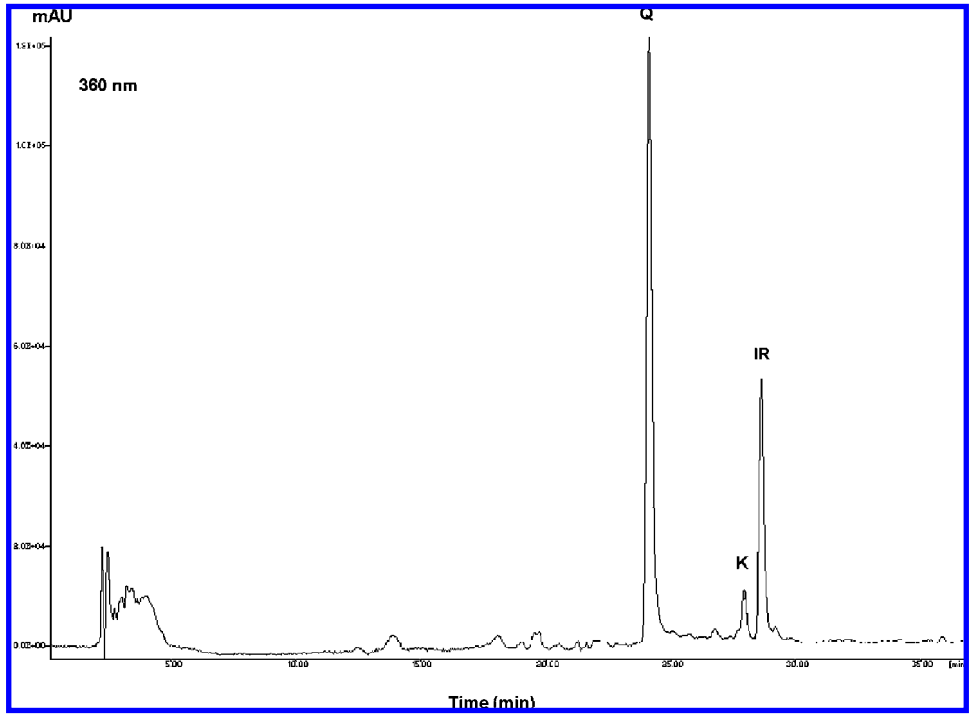

Figure 2. HPLC-DAD chromatogram at 370 nm of the hydrolysate extract of *triguero* asparagus.

programs was the main goal of this study. The chemical characterization of those native spears may allow new criteria to be established for selection and contribute to the promotion of the cultivation and consumption of a very high quality product that is still poorly known.

MATERIALS AND METHODS

Plant Material. The samples investigated consisted of spears from 32 green asparagus hybrids developed in recent years by major international asparagus programs and 65 different native lines of *triguero* asparagus from the Huétor-Tájar population variety. The first were cultivated in Las Torres Agricultural Research Center, Alcalá del Río, Sevilla, Spain, and the *triguero* asparagus samples were collected from Huétor-Tájar, Granada, Spain.

The asparagus samples were collected over a 2 year period and from experimental fields under controlled conditions. The spears were harvested at the same point of the harvest period (April−May 2005 and 2006), cut to the same length, and kept under refrigerated conditions ($T^a = 4$ °C) from the field to the laboratory to minimize the influence of environmental and storage conditions on the flavonoid profile.

Prior to being analyzed, the spears were washed with sodium hypochlorite solution (50 ppm of active Cl_2) and cut to a distance of 20 cm from the tip. Asparagus samples were 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.

Table 1. Peak Numbers, Retention Times (t_R), Assigned Structures, UV, and Main Ion Species Observed during HPLC-DAD-MS Analysis of the Flavonoids from Green Asparagus

peak	t_R (min)	structure	UV (nm)	$[M - H]^-$ (*m*/*z*)	$[M - H]^+$ (*m*/*z*)	ions (ESI^+) (*m*/*z*)
1	10.9	Q-triglycoside	255, 267sh, 352	755	757	611, 465, 303
2	12.6	Q-triglycoside	256, 267sh, 354	771	773	627, 481, 303
3	12.9	IR-triglycoside	254, 268sh, 355	769	771	625, 479, 317
4	14.4	Q-3-*O*-rhamnoglucoside (rutin)	255, 267sh, 355	609	611	465, 303
5	15.5	IR-triglycoside	256, 268sh, 347	785	787	625, 463, 317
6	17.1	K-3-*O*-rhamnoglucoside (nicotiflorin)	264, 296sh, 348	593	595	449, 287
7	17.6	IR-3-*O*-rhamnoglucoside (narcissin)	252, 268sh, 356	623	625	479, 317
8	18.6	IR-3-*O*-glucoside	254, 268sh, 354	477	479	317

Table 2. Flavonoid Content in 32 Commercial Hybrids of Green Asparagus (Milligrams per Kilogram of Fresh Weight)[a]

	Q-triglyc	Q-triglyc	IR-triglyc	rutin	IR-triglyc	nicotiflorin	narcissin	IR-3-*O*-gluc	sum
Apollo	nd	104	nd	649	nd	tr	11	nd	763
Aragon 1978	nd	26	nd	233	nd	tr	tr	nd	259
Atlas	nd	49	nd	438	nd	7	tr	nd	494
Backlim	nd	31	nd	376	nd	tr	11	nd	418
Dulce Verde	nd	21	nd	260	nd	nd	31	nd	312
Ercole	nd	84	nd	484	nd	nd	tr	nd	568
Fileas	nd	54	nd	206	nd	tr	tr	nd	260
G Welph	nd	33	nd	328	nd	7	tr	nd	368
Grande	nd	8	nd	448	nd	8	tr	nd	465
G. Millenium	nd	55	nd	337	nd	tr	tr	nd	392
Italo	nd	79	nd	248	nd	tr	tr	nd	328
Jersey Deluxe	nd	76	nd	219	nd	tr	nd	nd	296
Jersey Giant	nd	118	nd	265	nd	8	tr	nd	391
Jersey King	nd	113	nd	345	nd	8	tr	nd	466
Jersey Night	nd	100	nd	338	nd	13	tr	nd	451
Jersey Supreme	nd	149	nd	399	nd	tr	tr	nd	547
JWC-1	nd	37	nd	544	nd	tr	tr	nd	581
NJ1016	nd	60	nd	324	nd	6	tr	nd	390
NJ953	nd	76	nd	337	nd	12	tr	nd	425
NJ956	nd	108	nd	421	nd	tr	tr	nd	529
NJ977	nd	60	nd	214	nd	tr	tr	nd	274
Pacific Purple	nd	10	nd	277	nd	tr	tr	nd	288
Purple Passion	nd	0	nd	477	nd	tr	tr	nd	477
Plaverd	nd	28	nd	407	nd	tr	12	nd	447
Rally	nd	55	nd	441	nd	7	12	nd	515
Ramada	nd	35	nd	342	nd	7	tr	nd	385
Rambo	nd	37	nd	331	nd	tr	tr	nd	369
Rapsody	nd	53	nd	706	nd	11	tr	nd	770
Ravel	nd	25	nd	273	nd	tr	tr	nd	297
Solar	nd	20	nd	542	nd	8	12	nd	582
UC115	nd	53	nd	299	nd	7	tr	nd	359
UC157	nd	44	nd	274	nd	tr	tr	nd	318

[a] Data are the mean of three replicates. Standard deviation was <5%. nd, not detected; tr, traces.

Chemicals and Reagents. Authentic standards of quercetin (Q), kaempferol (K), isorhamnetin (IR), and rutin (quercetin 3-*O*-rutinoside) were purchased from Sigma-Aldrich Quimica (Madrid, Spain); kaempferol-3-*O*-rutinoside (nicotiflorin), isorhamnetin 3-*O*-rutinoside (narcissin), and isorhamnetin 3-*O*-glucoside were purchased from Extrasynthese (Genay, France).

All solvents were of HPLC grade purity (Romyl, Teknokroma, Barcelona, Spain). All sample solutions were prepared using Milli-Q water.

Flavonoids Extraction. Phenolic compounds, mainly flavonoids, were extracted as described in Fuentes-Alventosa et al. (*12*). 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 (DuPont 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. 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 was extracted with 80 mL of 80% EtOH as described above. Twenty milliliters 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 up to 100 mL with 80% ethanol. The extracts were stored at −20 °C until analysis.

HPLC-DAD Analysis. Analyses of flavonoids were carried out using a Jasco-LC-Net II ADC liquid chromatograph system equipped with a diode array detector (DAD). Flavonoid compounds were separated by using a SYNERGI 4 μHYDRO-RP80A reverse phase column (25 cm × 4.6 mm i.d., 4 μm particle size; 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, 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 set at 30 °C. Spectra from all peaks were recorded in the 200−600 nm range, and the chromatograms were acquired at 360 nm for flavonoid glycosides and at 370 nm for their aglycones.

Table 3. Flavonoid Content in Spears from 65 Genotypes of *Triguero* Asparagus (Milligrams per Kilogram of Fresh Weight)

	Q-triglyc	Q-triglyc	IR-triglyc	rutin	IR-triglyc	nicotiflorin	narcisin	IR-3-*O*-gluc	sum
HT-1	nd	nd	nd	335	9	tr	65	nd	409
HT-2	nd	nd	nd	476	19	12	65	nd	572
HT-3	nd	53	nd	331	24	38	116	nd	562
HT-4	nd	25	nd	401	35	6	108	3	578
HT-5	nd	nd	nd	382	11	108	202	nd	703
HT-6	nd	47	nd	378	33	31	203	1	693
HT-7	nd	44	nd	497	21	34	162	nd	758
HT-8	nd	28	nd	542	nd	tr	16	nd	586
HT-9	nd	16	nd	233	nd	32	159	nd	440
HT-10	41	59	nd	408	23	43	94	2	670
HT-11	nd	nd	nd	702	11	tr	tr	nd	713
HT-12	nd	16	nd	478	5	nd	tr	nd	499
HT-13	nd	19	nd	437	nd	nd	tr	nd	456
HT-14	nd	42	nd	406	16	tr	tr	nd	464
HT-15	nd	11	nd	561	3	nd	tr	nd	575
HT-16	nd	33	nd	275	nd	nd	tr	nd	308
HT-17	nd	nd	nd	583	13	nd	tr	nd	596
HT-18	nd	42	nd	220	nd	nd	tr	nd	262
HT-19	nd	17	nd	610	72	12	tr	nd	711
HT-20	nd	76	nd	716	11	tr	tr	nd	803
HT-21	nd	nd	nd	443	nd	tr	tr	nd	443
HT-22	nd	63	nd	538	10	tr	tr	nd	611
HT-23	nd	18	nd	267	nd	tr	tr	nd	285
HT-24	nd	nd	nd	490	39	tr	13	nd	542
HT-25	65	20	24	195	18	9	16	nd	347
HT-26	nd	50	nd	410	13	tr	tr	nd	473
HT-27	nd	3	nd	73	18	10	34	nd	138
HT-28	37	32	50	366	60	tr	65	9	619
HT-29	nd	nd	nd	418	nd	tr	tr	nd	418
HT-30	nd	35	nd	384	10	nd	tr	nd	429
HT-31	nd	54	nd	478	12	tr	tr	nd	544
HT-32	nd	nd	nd	344	nd	tr	16	nd	360
HT-33	nd	57	nd	606	13	tr	tr	nd	676
HT-34	nd	3	nd	495	nd	nd	tr	nd	498
HT-35	nd	18	nd	485	nd	tr	tr	nd	503
HT-36	nd	nd	nd	462	18	tr	13	nd	493
HT-37	nd	35	nd	493	11	tr	tr	nd	539
HT-38	75	25	86	119	13	7	63	nd	388
HT-39	nd	36	nd	535	nd	6	tr	nd	577
HT-40	nd	nd	nd	717	14	tr	tr	nd	731
HT-41	nd	56	nd	390	nd	tr	nd	nd	446
HT-42	45	26	22	276	63	9	33	3	477
HT-43	nd	39	nd	458	68	18	32	nd	615
HT-44	nd	29	nd	416	42	8	26	nd	521
HT-45	43	nd	63	155	nd	14	29	nd	304
HT-46	nd	44	nd	518	nd	nd	nd	nd	562
HT-47	152	245	nd	202	20	13	116	3	751
HT-48	11	69	9	343	17	tr	14	nd	463
HT-49	nd	31	nd	328	nd	nd	nd	nd	359
HT-50	nd	23	nd	423	nd	tr	nd	nd	446
HT-51	nd	34	nd	432	8	tr	nd	nd	474
HT-52	nd	70	28	617	nd	tr	nd	nd	715
HT-53	nd	nd	nd	394	nd	tr	nd	nd	394
HT-54	80	75	35	100	21	tr	22	2	335
HT-55	nd	31	nd	377	15	tr	tr	nd	423
HT-56	38	21	24	272	39	6	30	nd	430
HT-57	36	34	nd	360	43	12	40	nd	525
HT-58	nd	96	nd	606	13	nd	tr	nd	715
HT-59	36	7	1	247	6	10	tr	nd	307
HT-60	63	82	95	371	60	9	120	4	804
HT-61	nd	86	nd	517	14	nd	nd	nd	617
HT-62	nd	55	nd	424	8	tr	tr	nd	487
HT-63	40	37	nd	172	5	8	42	5	309
HT-64	79	30	nd	310	54	tr	39	nd	512
HT-65	66	28	nd	303	47	tr	36	nd	480

Isolation of the New Flavonoids Identified in Green Asparagus. A HPLC method similar to that described above, but using a semipreparative SYNERGI 4 HYDRO-RP80A reverse phase column (25 cm × 46 mm i.d., 4 μm; Phenomenex), 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 four fractions containing each individual compound were then re-injected onto the analytical column to purify the four isolated flavonoids. Those were concentrated under nitrogen prior to lyophilization.

Characterization of Flavonoids by HPLC-DAD-MS. Flavonoid glycosides detected in green asparagus were separated by HPLC as described above and identified by their electron impact mass data

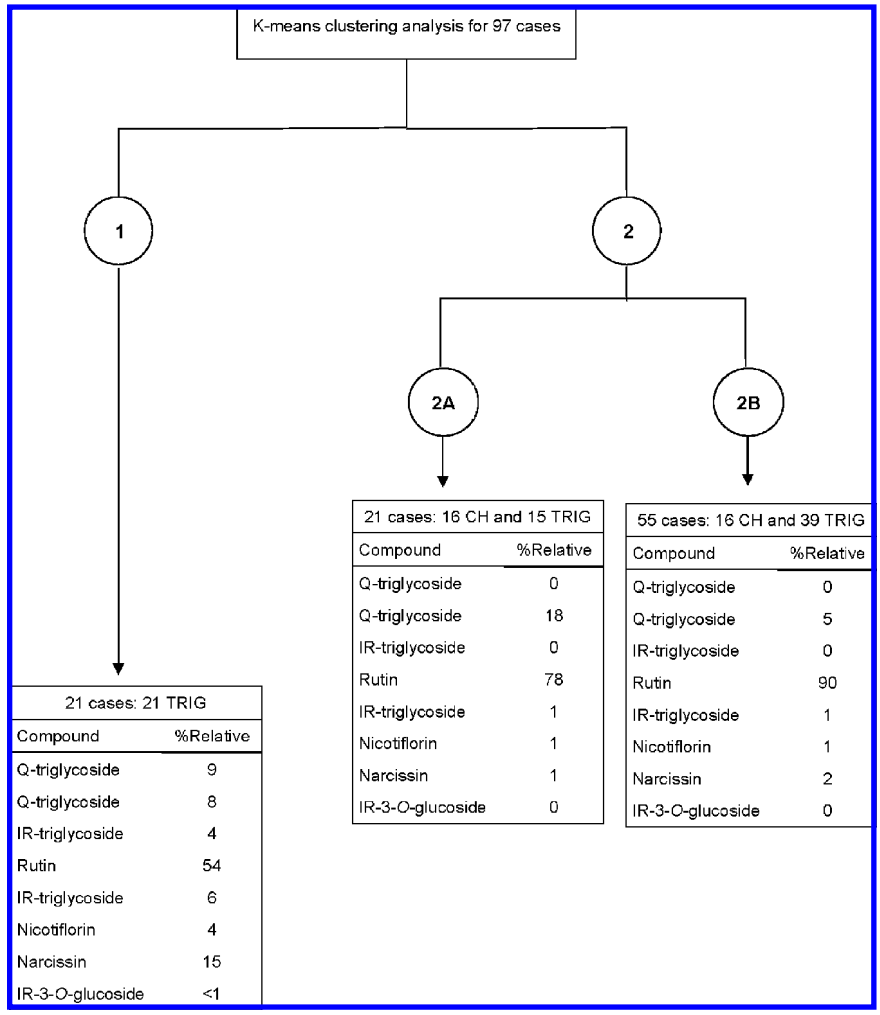

Figure 3. Classification of genotypes of green asparagus in three clusters obtained by the application of a *k*-means clustering analysis.

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}.

Identification and Quantification of Individual Flavonoids. Quantitative evaluation of flavonoid content was carried out as described by Fuentes-Alventosa et al. (*12*). Identification of individual flavonoid glycosides was carried out using their retention times and both spectroscopic and mass spectrometric data. Quantification of individual flavonoid monoglycosides and flavonoid diglycosides was directly performed by HPLC-DAD using an eight-point regression curve in the range of 0−250 μg on the basis of standards. When standards were not available, as in the case of the new flavonid triglycosides described in the present work, quantification was based on an average value for that class of compound, because responses were essentially similar within classes. Results were calculated from the mean of three replicates. Comparisons among samples were done by the ANOVA test and the LSD method at 95% confidence level.

RESULTS AND DISCUSSION

Determination of Flavonoids from Green Asparagus. The separation of flavonol glycosides in a *triguero* asparagus ethanolic extract is presented in **Figure 1**. As can be seen, the analytical method allowed the determination of eight flavonol glycosides. From HPLC-DAD data, all flavonoids are glycosylated derivatives of three flavonols, that is, quercetin (252, 267sh, 372), kaempferol (264, 296sh, 364), and isorhamnetin (252, 268sh, 368). No free aglycones were detected in the ethanolic extracts from triguero asparagus, but acid hydrolysis of the samples confirmed that *triguero* asparagus flavonoids are derivatives of three different aglycones, quercetin (Q) being the major flavonol, followed by isorhamnetin (IR) and kaempferol (K) (**Figure 2**). The identities of these aglycones were confirmed by the comparison of the HPLC-DAD-MS data of the hydrolysate to those of commercial standards.

Peaks 4, 6, 7, and 8 were respectively identified as rutin (quercetin-3-*O*-rutinoside), nicotiflorin (kaempferol-3-*O*-rutinoside), narcissin (isorhamnetin-3-*O*-rutinoside), and isorhamnetin-3-*O*-glucoside, on the basis of their spectral characteristics and comparison to standards. The above three flavonol diglycosides had been previously described in *triguero* asparagus (*12*), and the isorhamnetin-3-*O*-glucoside is the first flavonol monoglycoside detected in green asparagus. This compound has been described in other plant tissues, such as grapes (*13*), calendula (*14*), and turnip tops (*15*).

The four flavonol triglycosides, which had not been previously described in asparagus, were tentatively identified by means of a combination of the retention times (t_R), UV, and mass spectra obtained by HPLC-DAD-MS. Peaks 1 and 2 were tentatively identified as quercetin derivatives and peaks 3 and 5 as isorhamnetin derivatives.

As the UV spectra from all of the flavonol glycosides are very similar, they were not very useful for identification. MS fragmentation patterns of these compounds were used to obtain more information about their molecular masses and structural

characteristics. **Table 1** shows the values obtained for each of the detected compounds (numbered from 1 to 8 following their retention times).

The MS negative ion mode spectrum of compound **1** showed a deprotonated molecular ion at *m/z* 755 and an ion at *m/z* 301 corresponding to the deprotonated aglycone, which showed that this compound is a triglycosylated quercetin. It is well established that most flavonol derivatives from plant tissues are 3-*O*-glycosylated and/or 7-*O*-glycosylated flavonoids and that they are usually linked to glucose and rhamnose residues (*16–18*). The fact that the deprotonated ion from the aglycone was the base peak is indicative that the sugars are linked only at the 3 position (*16*). The positive ion spectrum of flavonoid glycosides provided additional information about the sugars linked to the flavonol structures. The ESI spectra of the $[M + H]^+$ ion of compound **1** at *m/z* 757 showed two main product ions corresponding to three successive losses of sugar residues. The first loss corresponded to a rhamnose residue (146 u), yielding the major ion at *m/z* 611. Ion *m/z* 611 then decomposed into another prominent ion at *m/z* 465, resulting from the loss of a second unit of rhamnose (146 u). Finally, the loss of a glucose residue (162 u) generated the major ion at *m/z* 303, corresponding to the protonated aglycone (quercetin). This fragmentation pattern could be compatible with a quercetin-3-rhamnosyl-rutinoside.

The MH^- spectrum of compound **2** showed a deprotonated molecular ion at *m/z* 771 and an ion at *m/z* 301 corresponding to the deprotonated aglycone, which showed that this is another quercetin triglycoside. Data from the positive ion spectrum suggested that this flavonoid derivative contained a rhamnose (146 u), the loss of which gave rise to the ion at *m/z* 627, and two residues of glucose (−324 u), the loss of which yielded a characteristic fragment ion of the protonated aglycone (quercetin) at *m/z* 303. These results suggest that compound **2** is a quercetin-3-glucosyl-rutinoside.

The MH^- spectrum of compound **3** gave a deprotonated molecular ion at *m/z* 769 and an ion at *m/z* 315 corresponding to the deprotonated aglycone (isorhamnetin). Complementary information from the positive ion spectrum revealed that the fragmentation pattern to this flavonoid derivative was similar to that from compound **1**, but the aglycone was isorhamnetin instead of quercetin. Compound **3** was tentatively identified as isorhamnetin-3-rhamnosyl-rutinoside.

The MH^- spectrum of compound **5** showed a deprotonated molecular ion at *m/z* 785 and an ion at *m/z* 315 corresponding to a deprotanated aglycone. On the other hand, according to the MH^+ data, this flavonoid followed the same fragmentation pattern explained above for compound **2**, but the aglycone is isorhamnetin instead of quercetin. It can be proposed that compound **5** is an isorhamnetin triglycoside with two glucoses and a rhamnose residue linked to the flavonol. This compound could be isorhamnetin 3-glucosyl-rutinoside.

On the other hand, it has been reported that the chromatographic behavior of flavonoid compounds under reversed-phase HPLC shows that, in the same conditions, a higher degree of glycosylation leads to a shorter retention time (*19*). Our results are in consonance with these findings, because peaks 1 (t = 10.9 min) and 2 (t_R = 12.6 min), which have been tentatively identified as quercetin triglycosides, elute earlier than rutin (t_R = 14.4 min). A similar behavior has been observed for the isorhamnetin derivatives. Therefore, peaks 3 (t_R = 12.9 min) and 5 (t_R = 15.5 min), tentatively identified as isorhamnetin-triglycosides, elute earlier than peak 7 (t_R = 17.6 min), which has been identified as isorhamnetin-rutinoside. Finally, the unique flavonoid monoglycoside detected in asparagus samples, identified as isorhamnetin-glucoside, was the last compound eluted from the column (t_R = 18.6 min).

The structures of the four new flavonol triglycosides described in this study were tentatively assigned on the basis of t_R, UV, and MS data, but further data are needed for a complete structural identification. Thus, the above four flavonoid triglycosides have been isolated and purified, and their analysis by RMN techniques will make it possible to establish the precise position of the sugars within the flavonoid molecule.

Quantitative Analysis of Flavonoids from Several Asparagus Cultivars. Flavonoid contents of the 32 commercial hybrids of green asparagus are shown in **Table 2**. The flavonoid content of these varieties varied between 259 and 763 mg/kg of fresh weight. Rutin was the main flavonoid glycoside, and its value was ≥70% of the total flavonoid complement in all of the samples investigated. Compound **2**, which has been tentatively identified as quercetin 3-*O*-glucosyl-rutinoside, was quantified in 31 of the 32 genotypes studied, but its content varied between 2 and 30% among the different hybrids of green asparagus. Significant quantities of nicotiflorin and narcissin were detected in most of the 32 genotypes of commercial hybrids, but these flavonoid diglycosides represented only 1−3% of the total flavonoid content, with the exception of the variety called Dulce Verde, which contained 10% of narcissin.

The detailed composition of flavonoids from 65 genotypes of *triguero* asparagus is shown in **Table 3**. Total flavonoid average content was 519 mg/kg of fresh weight, and rutin represented 78% of that quantity, which is comparable to the values calculated from commercial hybrids. However, significant differences were found among the flavonoid compositions from the 65 genotypes of *triguero* asparagus.

The diverse genotypes of asparagus investigated in the present work have been classified in three distinct groups as the result of a *k*-means clustering analysis. This statistical test produces exactly *k* different clusters of greatest possible distinction. The total content of flavonoids and the relative percent of each of the eight individual flavonoids identified in green asparagus have been used as factors of classification. The distribution of the 97 genotypes of green asparagus in three clusters and the average composition of each group are shown in **Figure 3**.

In the first dimension, samples were divided in two clusters (1 and 2), with very different means. It is noteworthy that all 21 genotypes within the first group came from *triguero* asparagus, whereas the second group included both commercial hybrids and *triguero* genotypes. In the second dimension, the second group was divided in two clusters (2A and 2B) constituted by mixtures of commercial hybrids and *triguero* genotypes. Despite all of the samples within these two clusters containing a high percent of rutin, which represented up to 70% in all cases, significant differences were found between the two groups.

Cluster 1 included only genotypes from *triguero* asparagus, the flavonoid composition of which is significantly different from those samples within the other two clusters. These genotypes contained the greatest variety of flavonoid compounds. Rutin content was about 50% of the total flavonoids, and this was accompanied by up to seven more flavonoids, including monoglycosides (trace amounts), diglycosides, and triglycosides. The average composition for this group consisted of 54% rutin and significant quantities of other seven flavonoids, each of which represented 1−15% of total flavonoid content.

Cluster 2A comprised 16 commercial hybrids and 5 genotypes of *triguero* asparagus. These genotypes had an average com-

position consisting of 78% of rutin, which was accompanied by a significant quantity of compound **2**, identified as quercetin-3-glucosyl-rutinoside, and minor quantities of other flavonoid di- and triglycosides. Cluster 2B was the greatest group, containing a mixture of 55 samples, 16 from commercial hybrids and 39 from *triguero* asparagus. The genotypes within this group contained almost solely rutin, which represented about 90% of the total flavonoids, accompanied by only small quantities of two or three more flavonoid glycosides.

From these results, it can be concluded that the flavonoid composition of *triguero* asparagus population is similar to that of commercial hybrids. This can be explained by the fact that several American hybrids of green asparagus have been included in the cultivation areas of *triguero* asparagus during recent years, which can influence and vary the original characteristics of the native varieties. However, the analysis of each of the 65 different genotypes of *triguero* asparagus revealed that several of them (the 21 genotypes classified within cluster 1) possessed a flavonoid composition very distinct and statistically different from the majority of the samples, grouped in the two other clusters. That subgroup of *triguero* asparagus contained several key flavonol derivatives able to differentiate those 21 genotypes from the rest, including both commercial hybrids and *triguero* asparagus. Therefore, compounds **1**, **3**, and **8**, which have been tentatively identified as quercetin-3-rhamnosyl-rutinoside, isorhamnetin-3-rhamnosyl-rutinoside, and isorhamnetin-3-*O*-glucoside, respectively, have been detected only in the genotypes grouped in cluster 1. On the other hand, compound **5**, tentatively identified as isorhamnetin-3-glucosyl-rutinoside, is present in a greater number of genotypes of *triguero* asparagus but has not been detected in any of the commercial hybrids investigated in this study. This flavonol triglycoside is one of the minor flavonoid components, as can be observed in **Table 3** and **Figure 3**. However, the fact that it is not present in any of the commercial hybrids (**Table 2**) suggests that this isorhamnetin triglycoside is key for distinguishing the *triguero* asparagus population from other green asparagus cultivars.

It is well established that the flavonoid profile of vegetables is influenced by genetic and environmental factors (*20–24*), and we have previously reported that the first determine to a greater extension both the phenolic composition and the antioxidant activity of green asparagus (*6*). On the other hand, it has been reported that the antioxidant activity of flavonol glycosides is greatly modified by the position of the sugar group attached to the basic diphenylpropane structure (*25*). Thus, Yamamoto et al. (*26*) demonstrated that Q40G, which has no catechol group, is greatly inferior to catechol-containing Q3G in preventing lipid peroxidation in human low-density lipoprotein (LDL). From the results of the present work it can be concluded that there are several flavonol glycosides, specific to *triguero* asparagus, which could be an alternative factor determining the bioactive properties of this product. Further investigations are required to determine if the characteristic flavonoid composition of *triguero* asparagus, reported in this work, is related to specific functional properties that may distinguish this product from other green asparagus.

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