J. Braz. Chem. Soc., Vol. 19, No. 3, 483-490, 2008.
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0103 - 5053 $6.00+0.00

Article

Study of *C*- and *O*-glycosylflavones in Sugarcane Extracts using Liquid Chromatography - Exact Mass Measurement Mass Spectrometry

Renata Colombo,[a] Janete H. Yariwake*,[a] and Michael McCullagh[b]

[a]*Instituto de Química de São Carlos, Universidade de São Paulo, CP 780, 13560-970 São Carlos-SP, Brazil*

[b]*Waters Corporation, Floats Rd., Wythenshave, Manchester, M23, 9LZ, UK*

Os flavonóides presentes nos extratos de cana-de-açúcar *(Saccharum officinarum*) foram analisados por cromatografia líquida acoplada à espectrometria de massas (LC-MS), e o estudo da rota de fragmentação dos flavonóides selecionados foi realizado utilizando a espectrometria de massas com aceleração ortogonal por tempo de vôo e ionização eletrospray (ESI-oa-ToF MS). Sete flavonas ***C***- e ***O***-glicosiladas foram identificadas nos extratos: schaftosídeo, isoschaftosídeo, luteolina-8-***C***-(ramnosilglicosídeo), vitexina, orientina, tricina-7-***O***-neohesperidosídeo e tricina-7-***O***-glicosídeo. Destas, cinco foram identificadas sem comparação direta com seus respectivos padrões. O método descrito também permitiu a diferenciação das 6-***C*** e 8-***C*** flavonas isoméricas, schaftosídeo e isoschaftosídeo. A combinação dos dados de fragmentação e a medida de massa exata mostraram ser complementares às técnicas de HPLC-UV-MS previamente utilizadas para discriminação de isômeros nos estudos da cana-de-açúcar.

The flavonoids present in sugarcane *(Saccharum officinarum*) extracts were analyzed by liquid chromatography – mass spectrometry (LC-MS), and a study of the fragmentation patterns of selected flavonoids was conducted using orthogonal acceleration time-of-flight electrospray ionization mass spectrometry (ESI-oa-ToF MS). Seven ***C***- and ***O***-glycosylflavones were identified in the extracts, namely, schaftoside, isoschaftoside, luteolin-8-***C***-(rhamnosylglucoside), vitexin, orientin, tricin-7-***O***-neohesperidoside and tricin-7-***O***-glucoside. Of these, five were identified in the absence of direct comparison with their respective standards. The described method also permitted the differentiation of the 6-***C*** and 8-***C*** isomeric flavones, schaftoside and isoschaftoside. The combination of fragmentation data and exact mass measurement showed to be complimentary to the HPLC-UV-MS techniques previously utilized for isomers discrimination in sugarcane studies.

Keywords: *Saccharum officinarum*, flavonoids, orthogonal acceleration time-of-flight electrospray ionization mass spectrometry (ESI-oa-ToF MS)

Introduction

The flavonoids are one of the largest and most widespread class of plant compounds and possess diverse pharmacological and biological properties. By virtue of these attributes, many flavonoid-containing plant species may be utilized as phytomedicines or as functional foods.[1,2] There is thus considerable interest in the systematic characterisation of flavonoids in crop plants, the derivative products of which could be of enhanced commercial value.

LC coupled with MS has proven to be a powerful technique for the on-line identification of target compounds in complex mixtures, especially crude plant extracts.[3] Moreover, the recent development of new commercial mass spectrometric systems makes possible the rapid and accurate characterization of structural features of flavonoids through the analysis of either pure compounds or of actual extract samples.[4-6] The exact mass measurements that are

*e-mail: janete@iqsc.usp.br

attainable with orthogonal acceleration time-of-flight (oa-ToF) mass spectrometers offer improved precision with respect to elemental composition, hence providing increased confidence in the structural identification of unknown compounds in real samples. We have recently demonstrated that the combination of mass measurement and in-source collision-induced dissociation (CID) is a valuable tool for the unequivocal identification of isomeric *C*-glycosylflavonoids in *Passiflora* extracts.[7]

Sugarcane (*Saccharum officinarum* L.; Gramineae) is the main source of industrial sugar in tropical countries and is also one of the most important crop plants in Brazil. Various HPLC-UV-MS techniques (including APCI-MS/MS and UV detection using post-column addition of shift reagents) have been employed previously in the investigation of sugarcane components, resulting in the identification of 15 flavone glycosides and aglycones.[8,9] Whilst relevant information could be obtained using these techniques, the unambiguous characterisation of the structures of isomers proved to be a difficult task, particularly in the absence of direct comparison with reference standards. Thus, the aim of the present study was to carry out a systematic investigation of sugarcane flavonoids using HPLC combined with oa-ToF/electrospray ionisation (ESI)/MS exact mass measurements in order to evaluate the potential value of sugarcane derivatives, such as juice, leaves and bagasse, as functional foods.

Materials and Methods

Plant material

Leaves of *S. officinarum* L. were obtained from a commercial plantation in Araraquara, SP, Brazil, whilst bagasse was supplied by the São Martinho sugar Mill in Pradópolis, SP, Brazil. All plant material was oven dried at *ca.* 40ºC to constant weight prior to extraction. Samples of sugarcane juice, obtained by crushing cleaned and peeled sugarcane stems, were purchased from various suppliers in São Carlos, SP, Brazil. Juice samples were transferred to plastic bottles and frozen at *ca.* –10ºC for storage. Immediately prior to sample preparation, the sample bottles were thawed and homogenized.

Chemicals and standard solutions

HPLC-grade acetonitrile was obtained from Riedel-de Häen (Seelze, Germany), analytical grade methanol and dimethyl sulphoxide (DMSO) were supplied by J. T. Baker (Phillipsburg, NJ, USA), formic acid and ammonium acetate were purchased from Merck (Darmstadt, Germany). HPLC-quality water was prepared using a Millipore Milli-Q system (Millipore Corp., New Bedford, MA, USA). Standard solutions (containing 250 mg L^{-1}) of reference flavonoids were prepared by dissolving appropriate amounts of vitexin and orientin (Carl Roth, Karlsruhe, Germany) in methanol and of diosmin (Sigma, St Louis, MO, USA) in DMSO.

Sample preparation

Flavonoids were extracted from dried leaves or bagasse by ultrasonic maceration of 1 g of material for 1.5 min at room temperature. Leaf samples were extracted with methanol : water (1:1; 20 mL); the resulting extract was filtered, reduced in volume to 2 mL on a rotary evaporator and then purified by solid-phase extraction (SPE) using Oasis HLB cartridges (3cc, 60 mg; 30 µm particle size; Waters, Milford, MA, USA) pre-conditioned with 1 mL of methanol and 1 mL of water. The interfering compounds were eluted with 3 mL of water, whilst the flavonoids were obtained by elution with 3 mL of methanol. Samples of bagasse were extracted with 35 mL of methanol; the extract was filtered, diluted with 2 mL of water, reduced in volume to 2 mL on a rotary evaporator and purified by SPE as described above. Sugarcane juice samples (10 mL) were sonicated with 10 mL of methanol for 1.5 min at room temperature, the extract was filtered, mixed with 2.0 mL of water, reduced in volume to 2 mL on a rotary evaporator and purified by SPE as described above. Purified flavonoid extracts were filtered through 0.5 µm Fluorpore membranes (Millipore) prior to injection into the HPLC system. All samples were prepared and analyzed in triplicate.

HPLC analyses

HPLC analyses were carried out on a Waters Alliance 2795 liquid chromatographic system equipped with an HT Sample Management autosampler and a model 996 photodiode array detector. Separations were performed at 40^{0}C on a Waters_SymmetryShield RP18 column (250 x 4.6 i.d; 5µm) protected by a SymmetryShield C_{18} guard column (20 x 3.9 mm i.d.; 5µm). The mobile phase consisted of 0.2% formic acid in water (solvent **A**) and acetonitrile (solvent **B**). The elution program for leaf and juice extracts was: 0-8 min - linear gradient from 10 to 13% **B**; 8-25 min - linear gradient from 13 to 20% **B**; 25-40 min - linear gradient from 20 to 40% **B**; and 40-45 min - linear gradient from 40-60% **B**. For bagasse extracts the elution program was: 0-3 min - isocratic at 18% **B**; and 3-40 min - linear gradient from 18-70% **B**. The flow rate was 1.2 mL min^{-1} and the sample injection volume was 10

μL. UV spectra were acquired between 200 and 400 nm. Data were processed using Mass Lynx version 3.5 software (Micromass, Manchester, UK).

ESI-oa-ToF MS analyses

Experiments were carried out using a Micromass model LCT oa-ToF mass spectrometer equipped with a LockSpray dual ESI source. The instrument was operated in the positive ion mode with both reference and sample sprays maintained at + 3 kV. The source and desolvation temperatures were set at 120 and 250ºC, respectively, and the nitrogen desolvation and nebuliser gas flow rates were 711 L h^{-1} and 4 L h^{-1}, respectively. Four different sample cone voltages (30, 50, 65 and 95 V) were employed. Prior to analyses, the instrument was calibrated over the mass range 100-800 Da using a solution containing PEG 200, 400, 600 and 1000 (Sigma) dissolved in acetonitrile: 10 mmol L^{-1} ammonium acetate (1:1, v/v). For exact mass measurements, a solution containing 0.25 ng mL^{-1} of leucine enkephalin ($[M+H]^+$ = 556.2771) in acetonitrile: water (1:1) was infused through the reference spray at a rate of 10 μL min^{-1} with the aid of an Harvard Apparatus (South Natick, MA, USA) model 22 syringe pump. The lock mass reference was sampled every 5 s.

Results and Discussion

Sugarcane extracts contained a complex mixture of *O*- and *C*-glycosylated flavonoids (Figure 1). The HPLC-ToF/MS total ion current (TIC) profiles of the juice and leaves extracts (Figures 1a and 1b, respectively) were comparable with those obtained by HPLC-UV[8] and HPLC-APCI/MS.[9] However, since the only compound identified in the TIC of the bagasse extract (Figure 1c) was the component associated with peak **6**, which was also found in juice and leaves extracts, detailed ESI-oa-ToF MS analysis were performed on juice and leaf extracts alone.

ESI-oa-ToF and in-source collision-induced dissociation (CID) analyses were performed at a low cone voltage (30 V) in order to measure accurately the molecular masses of the flavonoids, and at increasingly higher cone voltages (50, 65 and 95 V) in order to obtain characteristic fragmentation patterns that would be of value in the identification of individual peaks in the sugarcane extracts. Moreover, the high specificity of the exact mass measurement permitted the efficient assignment of the fragments using an elemental composition calculator that served to generate the probable elemental formula for each of the observed fragments. Analysis of commercial reference flavonoid standards revealed that the measurement error obtained between the calculated and the experimental mass was smaller than 8 mDa (< 17 ppm), indicating a good coincidence of theoretical and experimental data (Table 1). Hence an exact mass measurement could be employed in the identification of unknown flavonoids present in crude extracts even when the respective authentic standards were not available for analysis.

The MS data obtained for the studied sugarcane extracts are discussed with respect to the differentiation of the various groups of flavonoids detected; the nomenclature proposed by Domon and Costello[10] has been adopted in the discussion of fragmentation data. MS spectra are given in the *Supplementary Information* Section.

8-C-glycosylflavones

Components associated with peaks **4** and **5** in the sugarcane extracts were identified as vitexin (*m/z* 433.1152, $[M+H]^+$) and orientin (*m/z* 449.1085, $[M+H]^+$), respectively. The exact mass measurement data for protonated **4** and **5** and the characteristic fragments were fully consistent with those obtained by analysis of the respective reference standards. The compound associated with peak **3** was identified as luteolin-8-*C*-(rhamnosylglucoside) (*m/z* 595.1677, $[M+H]^+$), and its fragmentation pattern subsequent to the loss of the rhamnose moiety (i.e. *m/z* 449.1042; *m/z* 329.0652; *m/z* 299.0570) was consistent with that of orientin. The exact mass data for compounds **3** – **5** are presented in Table 2 and the proposed fragmentation pathways are given in Figure 2 .

7-O-glycosylflavones

Although no 7-*O*-glycosylflavones or their respective aglycones were available as reference standards in this study, the relevant correlations could be deduced by analysis of the standard 7-*O*-neohesperoside flavone, diosmin (*m/z* 609.1815; calcd. 609.1819, $[M+H]^+$). At a cone voltage of 50 V the flavone lost one sugar moiety giving rise to the fragment $[M+H\text{-}Rha]^+$ (*m/z* 463.1232). At the higher cone voltage of 65 V, the glucose moiety was also lost yielding the aglycone diosmetin $[A+H]^+$ (*m/z* 301.0718) (Table 1).

Compounds associated with peaks **6** and **7** were identified on the basis that their aglycone tricin differed from diosmetin only by the presence of an additional methoxy group. Thus, compound **6** was identified as tricin-7-*O*-neohesperidoside from its exact mass (*m/z* 639.1928, $[M+H]^+$; empirical formula $C_{29}H_{34}O_{16}$) by taking into consideration the additional mass of 30.0260 (calcd.) for the extra methoxy group on the $[M+H]^+$ ion and on the fragment ions observed with standard diosmin.

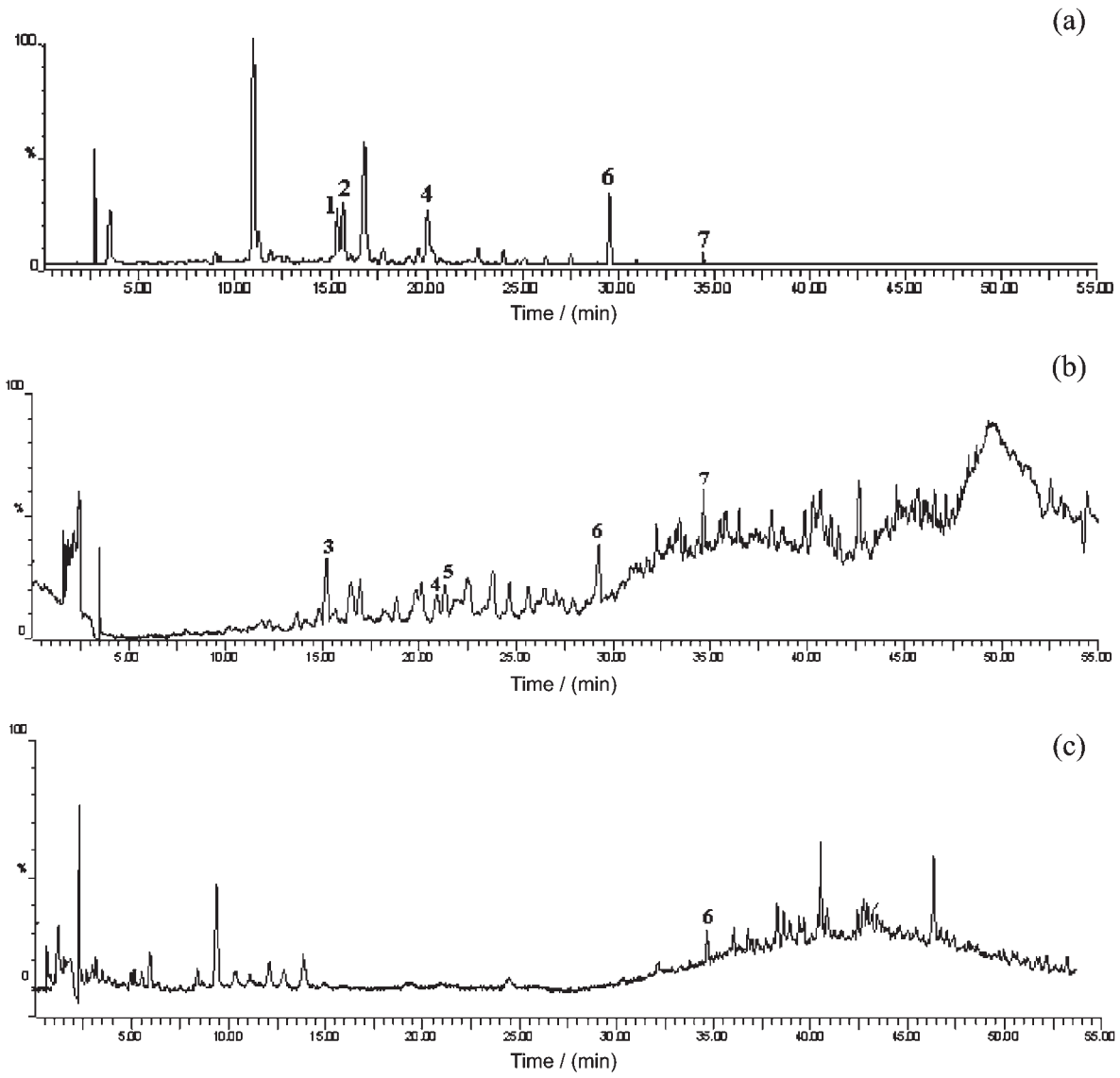


Figure 1. LC/ToF/MS total ion chromatogram of sugarcane extracts: (a) juice, (b) leaves (c) bagasse. Peak identification: **1**, schaftoside; **2**, isoschaftoside; **3**, luteolin-8-*C*-(rhamnosylglucoside); **4**, vitexin; **5**, orientin; **6**, tricin-7-*O*-neohesperidoside; **7**, tricin-7-*O*-glucoside.

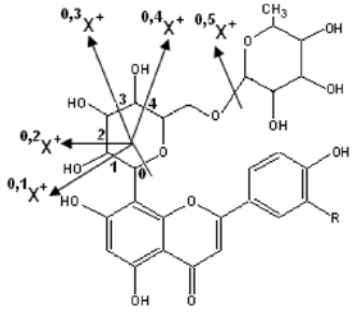
At cone voltages of 50 and 65 V, the major fragments of **6** were at *m/z* 493.1369 and 331.0837 corresponding, respectively, to ions $[M+H-146]^+$ and $[M+H-146-162]^+$. The losses of 146 and 162 Da are characteristic of rhamnose and glucose sugar moieties, respectively, and the ion at *m/z* 331.0837 is characteristic of the aglycone tricin. At a cone voltage of 95 V, the most abundant fragment ion, at *m/z* 301.0754, was produced by loss of a methoxy group, and the structure of tricin could thus be confirmed through direct comparison with the $[A+H]^+$ ion of standard diosmin (*m/z* 301.0718). Similar considerations lead to the identification of compound **7** as tricin-7-*O*-glucoside (*m/z* 493.1369, $[M+H]^+$; empirical formula $C_{23}H_{24}O_{12}$), which exhibited an ion at *m/z* 301.0713 (comparable with the $[A+H]^+$ of diosmin) as the most abundant fragment when a cone voltage of 95 V was applied. At cone voltages of 50 and 65 V, the major fragment ion of **7** was at *m/z* 331.0809, corresponding to $[M+H-162]^+$. The ESI-oa-ToF MS spectrum of standard diosmin in comparison with those of the sugarcane flavonoids **6** and **7**, and the proposed fragmentation pathways are presented in Figure 3.

6,8-di-C-glycosylflavones

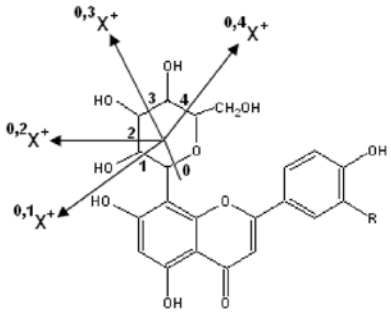
Components associated with peaks **1** and **2** in the sugarcane extracts were identified as the isomeric flavones schaftoside and isoschaftoside. These structures were proposed, in the absence of direct comparison with reference

Table 1. Comparison of experimental and calculated exact mass data of protonated flavonoid (reference standards) at different cone voltages

Compound or fragment	calculated	experimental	error (mDa)	error (ppm)
Diosmin ($C_{28}H_{32}O_{15}$) $[M+H]^+$ (30 V)	609.1819	609.1815	-0.4	-0.7
base peak (*m/z*) at 50 V				
$C_{22}H_{23}O_{11}$	463.1240	463.1232	-0.8	-2.0
base peak (*m/z*) at 65 V				
$C_{16}H_{13}O_6$	301.0712	301.0718	0.6	2.0
base peak (*m/z*) at 95 V				
$C_{16}H_{13}O_6$	301.0712	301.0711	- 0.1	- 0.3
Orientin ($C_{21}H_{20}O_{11}$) $[M+H]^+$ (30 V)	449.1084	449.1009	-7.5	-16.7
base peak (*m/z*) at 50 V				
$C_{17}H_{13}O_7$	329.0661	329.0662	0.1	0.3
base peak (*m/z*) at 65 V				
$C_{17}H_{13}O_7$	329.0661	329.0662	0.1	0.3
base peak (*m/z*) at 95 V				
$C_{16}H_{11}O_6$	299.0556	299.0542	-1.4	-4.7
Vitexin ($C_{21}H_{20}O_{10}$) $[M+H]^+$ (30 V)	433.1135	433.1122	-1.3	-3.0
base peak (*m/z*) at 50 V				
$C_{17}H_{13}O_6$	313.0712	313.0731	1.9	6.1
base peak (*m/z*) at 65 V				
$C_{17}H_{13}O_6$	313.0712	313.0731	1.9	6.1
base peak (*m/z*) at 95 V				
$C_{16}H_{11}O_5$	283.0606	283.0637	3.1	10.9

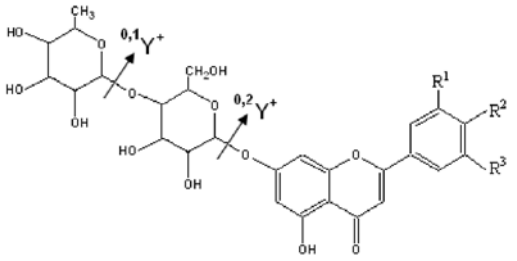


luteolin-8-*C*-(rhamnosylglucoside), **3**

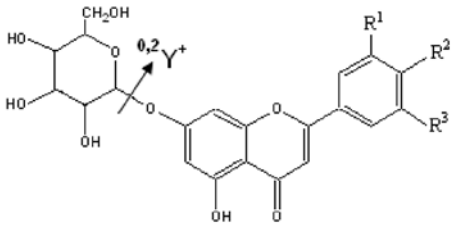


vitexin, **4** (R = H) orientin, **5** (R = OH)

Figure 2. Structure and proposed fragmentation pathway of the 8-*C*-glycosides: **3**, luteolin-8-*C*-(rhamnosylglucoside) **4**, vitexin (R=H) and **5**, orientin (R=OH). Nomenclature of the fragments according to Domon and Costello.[10]



diosmin (standard): R^1=OH; R^2= OCH_3; R^3=H
tricin-7-*O*-neohesperidoside, **6** (R^1= OCH_3; R^2= OH; R^3= OCH_3)



tricin-7-*O*-glucoside, **7** (R^1= OCH_3; R^2= OH; R^3= OCH_3)

Figure 3. Structure and proposed fragmentation pathway of 7-*O*-glycosyl-flavones: diosmin (commercial standard); **6**, tricin-7-*O*-neohesperidoside; **7**, tricin-7-*O*-glucoside. Nomenclature of the fragments according to Domon and Costello.[10]

Table 2. Exact mass data (cone voltage: 65 V) of the protonated 8-*C*-glycosylflavones, luteolin-8-*C*-(rhamnosylglucoside) (**3**), vitexin (**4**) and orientin (**5**) and of the most intense fragments

Compound	*m/z*	Ions (relative abundance %)[a]
Luteolin-8-*C*-(rhamnosylglucoside) (**3**)	595.1677	$[M+H]^+$ (16)
	449.1042	$[M+H-146]^+$: $^{0,5}X^+$ (100)
	431.1003	$[M+H-146-18]^+$: $-H_2O$ (29)
	413.0872	$[M+H-146-36]^+$: $-2\ H_2O$ (28)
	395.1009	$[M+H-146-54]^+$: $-3\ H_2O$ (12)
	383.0836	$[M+H-146-30-36]^+$: $^{2,3}X^+-2H_2O$(10)
	353.0916	$[M+H-146-96]^+$: $^{0,4}X^+-2\ H_2O$ (22)
	330.0728	$[M+H-146-120]^+$+H: $^{0,2}X^+$+H (15)
	329.0652	$[M+H-146-120]^+$: $^{0,2}X^+$ (77)
	300.0631	$^{0,2}X^+$- CHO (8)
	299.0570	$[M+H-146-150]^+$: $^{0,1}X^+$ (16)
Vitexin (**4**)	433.1152	$[M+H]^+$ (18)
	415.1029	$[M+H-18]^+$: $-\ H_2O$ (16)
	397.0925	$[M+H-36]^+$: $-2\ H_2O$ (26)
	379.0810	$[M+H-54]^+$: $-3\ H_2O$ (10)
	367.0857	$[M+H-30-36]^+$: $^{2,3}X^+-2\ H_2O$ (10)
	337.0913	$[M+H-96]^+$: $^{0,4}X^+-2\ H_2O$ (12)
	314.0740	$[M+H-120]^+$+H: $^{0,2}X^+$+H (18)
	313.0711	$[M+H-120]^+$: $^{0,2}X^+$ (100)
	284.0877	$^{0,2}X^+$- CHO (14)
	283.0617	$[M+H-150]^+$: $^{0,1}X^+$ (28)
Orientin (**5**)	449.1085	$[M+H]^+$ (26)
	431.1012	$[M+H-18]^+$: $-H_2O$ (16)
	413.0879	$[M+H-36]^+$: $-2\ H_2O$ (24)
	395.1023	$[M+H-54]^+$: $-3\ H_2O$ (9)
	383.0855	$[M+H-30-36]^+$: $^{2,3}X^+-2\ H_2O$ (8)
	353.0919	$[M+H-96]^+$: $^{0,4}X^+-2\ H_2O$ (10)
	330.0721	$[M+H-120]^+$+H: $^{0,2}X^+$+H (16)
	329.0638	$[M+H-120]^+$: $^{0,2}X^+$ (100)
	300.0614	$^{0,2}X^+$- CHO (24)
	299.0519	$[M+H-150]^+$: $^{0,1}X^+$ (10)

[a] Nomenclature according to Domon and Costello.[10]

Table 3. Exact mass data (cone voltage 95 V) of the isomers 6,8-*C*-glycosides schaftoside (**1**) and isoschaftoside (**2**)

Compound	*m/z*	Ions (relative abundance %)[a]
Schaftoside (**1**)	565.1533	$[M+H]^+$ (59)
	547.1439	$[M+H-18]^+$: $-\ H_2O$ (18)
	475.1248	$[M+H-90]^+$: $^{0,6}Z^+$ (10)
	445.0888	$[M+H-120]^+$: $^{0,2}Z^+$ (25)
	409.0928	$[M+H-156]^+$: $^{0,5}Z^+ + 2\ H_2O$ (11)
	379.0808	$[M+H-186]^+$: $^{0,1}Z^+$+2 H_2O (27)
	355.0859	$[M+H-210]^+$: $^{0,6}Z^+ + {}^{0,2}Z^+$ (06)
	337.0712	$[M+H-228]^+$: $^{0,6}Z^+ + {}^{0,2}Z^+ + H_2O$ (39)
	325.0713	$[M+H-240]^+$: $^{0,6}Z^+$ + $^{0,1}Z^+$ or $^{0,5}Z^+$ + $^{0,2}Z^+$ (100)
	295.0595	$[M+H-270]^+$: $^{0,5}Z^+ + {}^{0,1}Z^+$ (27)
Isoschaftoside (**2**)	565.1570	$[M+H]^+$ (74)
	547.1438	$[M+H-18]^+$: $-\ H_2O$ (14)
	475.1246	$[M+H-90]^+$: $^{0,6}Z^+$ (31)
	445.1006	$[M+H-120]^+$: $^{0,2}Z^+$ (17)
	409.0920	$[M+H-156]^+$: $^{0,5}Z^+ + 2\ H_2O$ (23)
	379.0818	$[M+H-186]^+$: $^{0,1}Z^+$+2 H_2O (38)
	355.0857	$[M+H-210]^+$: $^{0,6}Z^+ + {}^{0,2}Z^+$ (14)
	337.0685	$[M+H-228]^+$: $^{0,6}Z^+ + {}^{0,2}Z^+ + H_2O$ (60)
	325.0695	$[M+H-240]^+$: $^{0,6}Z^+$ +$^{0,1}Z^+$ or $^{0,5}Z^+$ + $^{0,2}Z^+$ (100)
	295.0590	$[M+H-270]^+$: $^{0,5}Z^+ + {}^{0,1}Z^+$ (49)

[a] Nomenclature adapted from Domon and Costello:[10] $^{0\ 0,1}Z^+$ to $^{0,4}Z^+$, glucose fragmentation; $^{0,5}Z^+$ to $^{0,8}\ Z^+$ arabinose fragmentation.

standards, on the basis of the excellent agreement previously demonstrated between the experimental and theoretical (calculated) data for other reference standards and samples. Flavones **1** and **2** both presented the empirical formula $C_{26}H_{28}O_{14}$ and exhibited, respectively, $[M+H]^+$ ions at *m/z* 565.1533 and 565.1570 (calcd. 565.1557). At a cone voltage of 50 V, the characteristic fragmentation pathway of *C*-glycosylated flavonoids resulted in the formation of ions at $[M+H-90]^+$, corresponding to the loss of an arabinose unit ($^{0,6}Z^+$, *m/z* 475.1248 and 475.1246, respectively, for **1** and **2**), and at $[M+H-120]^+$, corresponding to the loss of a glucose unit ($^{0,2}Z^+$, *m/z* 445.0888 and 445.1006, respectively, for **1** and **2**). At the higher cone voltage of 95 V, further fragmentations of the sugar moieties were observed generating, for **1** and **2**, respectively, $[M+H-186]^+$ ions at *m/z* 379.0808 and 379.0818, $[M+H-210]^+$ ions at *m/z* 355.0859 and 355.0857, $[M+H-240]^+$ ions (forming the base peaks) at *m/z* 325.0713 and 325.0695, and $[M+H-270]^+$ ions at *m/z* 295.0595 and 295.0591 (Table 3 and Figure 4).

A number of studies have explored the potential of MS methods in the determination of the type of sugar substituted at the 6-*C* and 8-*C* positions in 6,8-di-*C*-glycosylflavones. MS/MS in the negative ion mode using an ion trap instrument,[11] and CID using ion trap,[12] triple quadrupole[13] or magnetic sector[14] spectrometers all revealed differences in the fragmentation patterns between schaftoside and isoschaftoside sufficient to allow their differentiation and a positive ion method using FAB-CID has also been described.[15] According to such reports,[11] preferential fragmentation is of the sugar moiety at the 6-*C* rather than the 8-*C* position. In the present study it was observed that the unequivocal distinction of schaftoside and isoschaftoside was possible through comparison of the differences in the relative abundance of fragments at *m/z* 475 ($^{0,6}Z^+$) and 445 ($^{0,2}Z^+$). Considering the preferential fragmentation at the *C*-6 position, the ion at *m/z* 475.1246 corresponding to the arabinose unit presented, at a cone voltage of 95 V, a relative abundance of >30% in the spectrum of isoschaftoside compared with 10% in schaftoside. Under the same conditions, the fragment ion at *m/z* 445.0888 corresponding to the glucose unit exhibited a relative abundance of 25% in the spectrum of schaftoside and 17% in isoschaftoside.

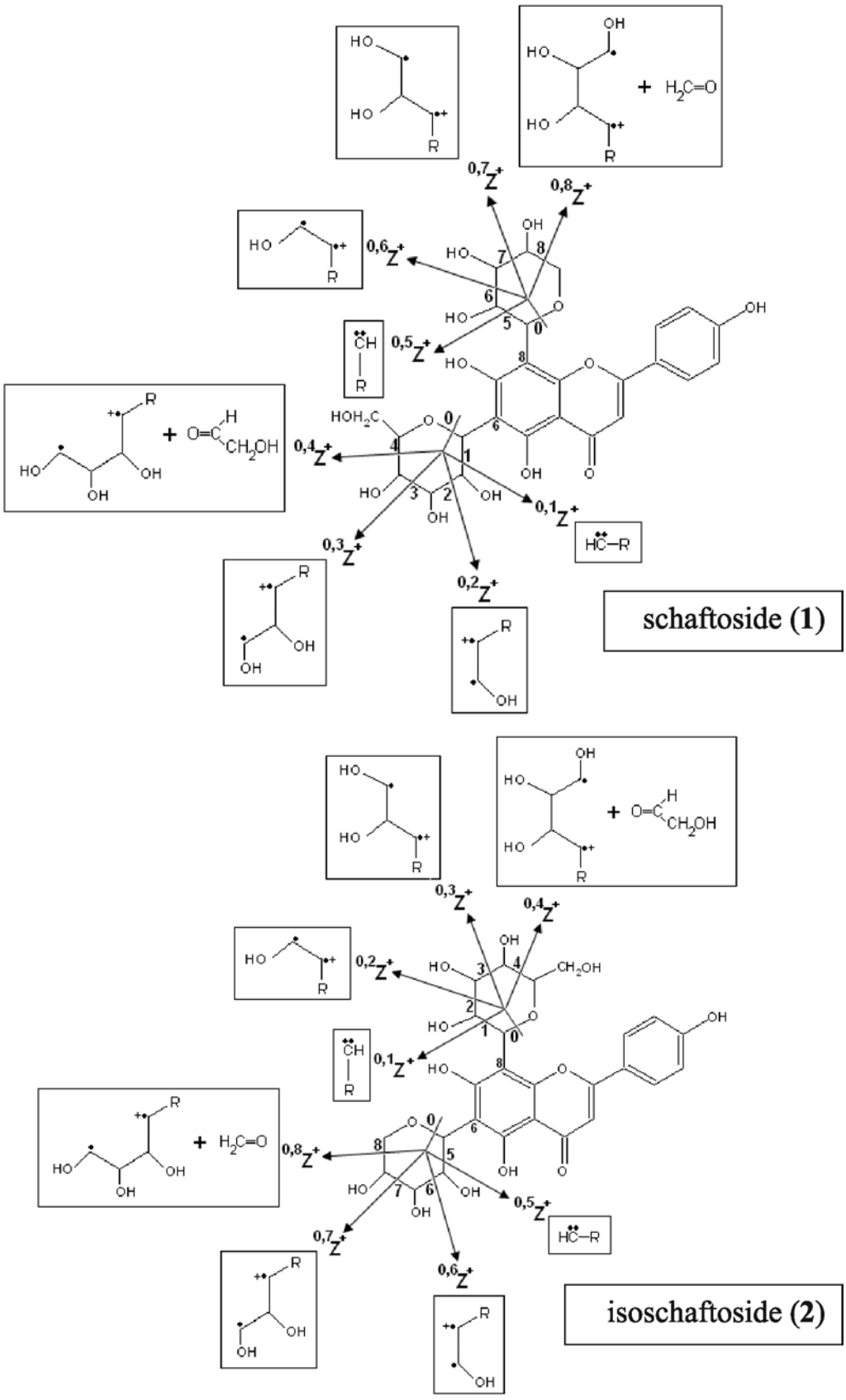


Figure 4. Structure and proposed fragmentation pathway of the 6,8-*C*-glycosides: **1**, schaftoside **2**, isoschaftoside. Nomenclature of the fragments adapted from Domon and Costello:[10] $^{0,1}Z^+$ to $^{0,4}Z^+$ glucose fragmentation; $^{0,5}Z^+$ to $^{0,8}Z^+$ arabinose fragmentation.

Conclusions

It has been demonstrated that LC combined with ESI-oa-ToF MS exact mass measurement provides an accurate tool for the structural analysis of glycosylated flavonoids. The data obtained using commercial reference standards allowed conclusions to be drawn concerning the structures of components in extracts of *S. officinarum*. Using this strategy, five of the seven *C*- and *O*-glycosylflavones studied (schaftoside, isoschaftoside, luteolin-8-*C*-(rhamnosylglucoside), vitexin, orientin, tricin-7-*O*-neohesperidoside and tricin-7-*O*-glucoside) could be identified without direct comparison with their respective reference standards. Moreover, the data obtained from the analysis of the isomer pair schaftoside/isoschaftoside enabled a relationship between the relative abundance of the fragments $^{0,6}Z^+$ and $^{0,2}Z^+$ and the structure of the sugar moieties linked in 6-*C* and 8-*C* positions to be established. The combination of fragmentation data and exact mass measurement is clearly complimentary to the HPLC-UV-MS techniques previously utilized in sugarcane studies[8,9] in that it permits the precise discrimination of isomers.

Acknowledgments

The authors would like to thank Waters Corporation, UK, for the provision of facilities that enabled this study to be carried out. The financial support of FAPESP (fellowship to R. C. [00/11645-2] and research grant to J. H. Y. [02/00493-2]), financial support of CNPq (research fellowship to J. H. Y.) and the Post-Graduate Dean's Office of the Universidade de São Paulo (travel fellowship to R. C.), are gratefully acknowledged.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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Received: May 08, 2007
Web Release Date: February 29, 2008

FAPESP helped in meeting the publication costs of this article.

J. Braz. Chem. Soc., Vol. 13, No. 3, S1-S3, 2008.
Printed in Brazil - ©2008 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Study of *C*- and *O*-glycosylflavones in Sugarcane Extracts using Liquid Chromatography - Exact Mass Measurement Mass Spectrometry

Renata Colombo,[a] Janete H. Yariwake*,[a] and Michael McCullagh[b]

[a]Instituto de Química de São Carlos, Universidade de São Paulo, CP 780, 13560-970 São Carlos-SP, Brazil

[b]Waters Corporation, Floats Rd., Wythenshave, Manchester, M23, 9LZ, UK

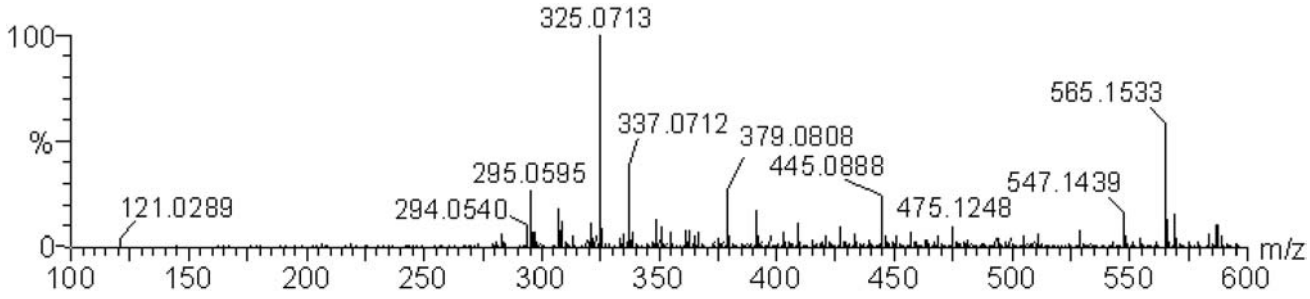


Figure S1. ESI-oa-ToF MS spectra (95V) of **1**, schaftoside (see also Table 3).

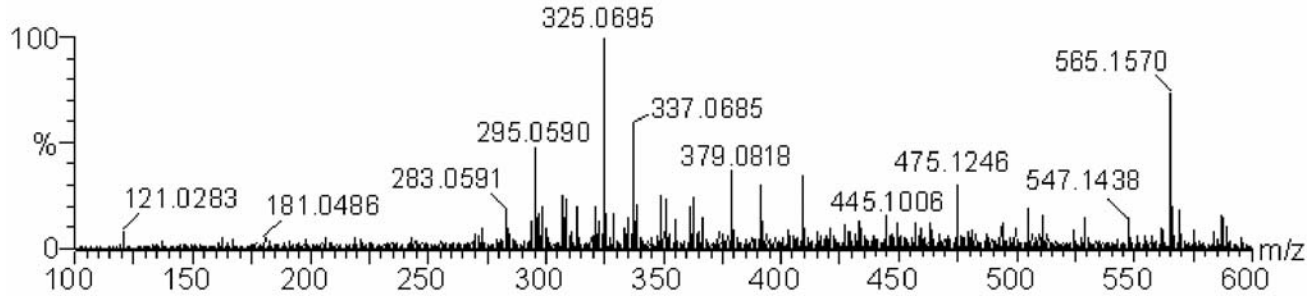


Figure S2. ESI-oa-ToF MS spectra (95V) of **2**, isoschaftoside (see also Table 3).

*e-mail: janete@iqsc.usp.br

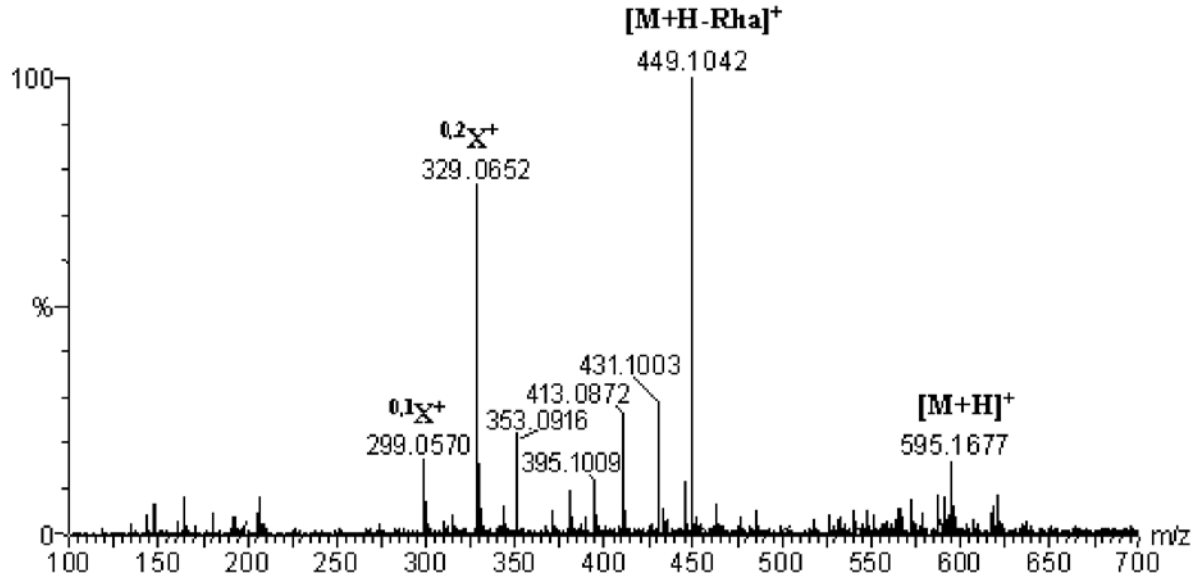


Figure S3. ESI-oa -ToF MS spectra (65 V) of **3**, luteolin-8-*C*-(rhamnosylglucoside).

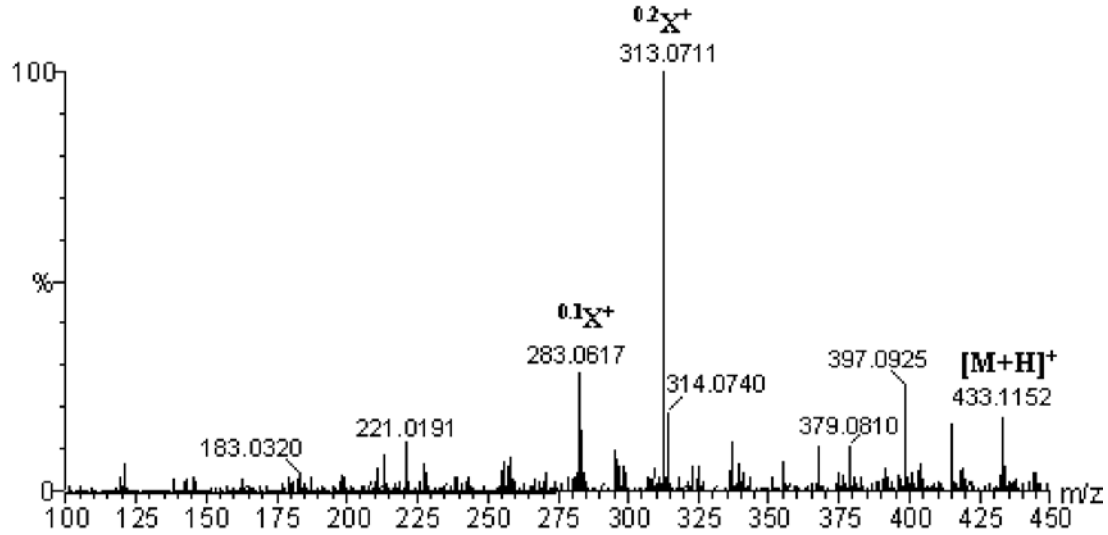


Figure S4. ESI-oa -ToF MS spectra (65 V) of **4**, vitexin (R=H).

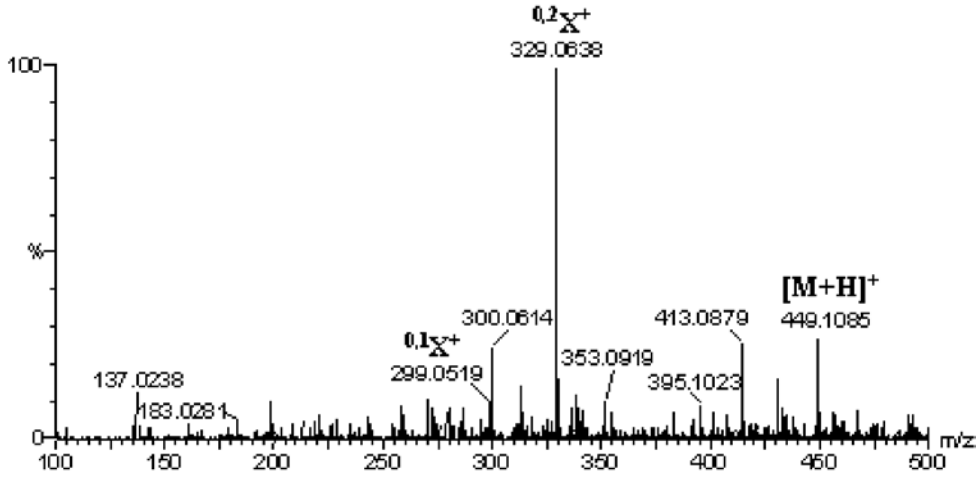
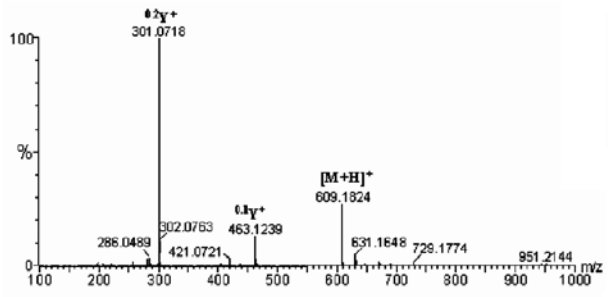
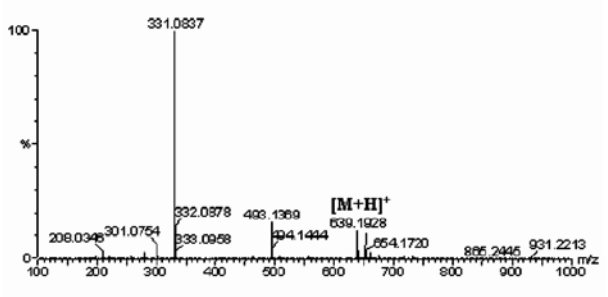


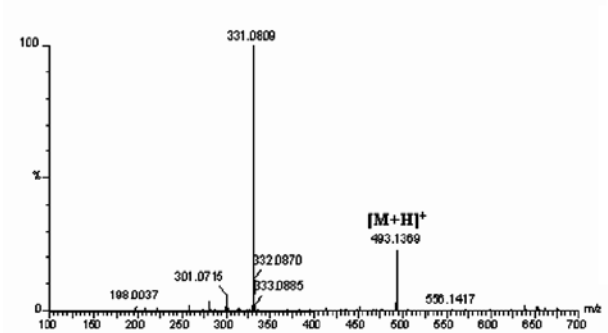
Figure S5. ESI-oa -ToF MS spectra (65 V) of **5**, orientin (R=OH).



Compound *m/z*	ions[a] (relative abundance %)
Diosmin	
609.1824	$[M+H]^+$ (28)
463.1239	$[M+H-146]^+$: $^{0,1}Y^+$ (12)
301.0718	$[M+H-162]^+$: $^{0,2}Y^+$ (100)



(6)	
639.1928	$[M+H]^+$ (13)
493.1369	$[M+H-146]^+$: $^{0,1}Y^+$ (17)
331.0837	$[M+H-162]^+$: $^{0,2}Y^+$ (100)
301.0754	$[M+H-162-30]^+$: $^{0,2}Y^+$-OCH_3 (7)



(7)	
493.1369	$[M+H]^+$: (25)
331.0809	$[M+H-146]^+$: $^{0,1}Y^+$ (100)
301.0715	$[M+H-162-30]^+$: $^{0,2}Y^+$-OCH_3 (6)

Figure S6. ESI-oa -ToF MS spectra (65 V) of 7-*O*-glycosides: **diosmin** (commercial standard); **6**, tricin-7-*O*-neohesperidoside; **7**, tricin-7-*O*-glucoside. [a]Nomenclature of the fragments according to Domon and Costello. [10]