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On-line identification of sugarcane (*Saccharum officinarum* L.) methoxyflavones by liquid chromatography–UV detection using post-column derivatization and liquid chromatography–mass spectrometry

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Abstract

Sugarcane (*Saccharum officinarum* L., Gramineae) bagasse and leaves were investigated for their flavonoid content and transgenic sugarcane ("Bowman-Birk" and "Kunitz") was compared with non-modified ("control") plants. Analyses were carried out by high-performance liquid chromatography coupled to diode array UV detection (LC/UV), also using post-column addition of shift reagents, and tandem MS (atmospheric pressure chemical ionization–MS/MS and collision-induced dissociation–MS). On-line UV and MS data demonstrated the presence of methoxyflavone glycosides and aglycones in a total of seven compounds. Three naturally occurring flavones glycosides and two unusual *erythro*- and *threo*-diastereoisomeric flavolignan 7-*O*-glucosides were identified together with their aglycones.
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Keywords: *Saccharum officinarum*; LC/UV; LC/MS; Flavonoids; Transgenic plants

1. Introduction

Flavonoids are polyphenols which occur in higher plants, including medicinal and edible plants (vegetables, fruits, etc.) and also in some foods and beverages such as tea, red wine and juices. In recent years, an increasing number of publications have reported on the chemistry of flavonoids especially due to their biological properties (antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic, vasodilatory, anti-mutagenic and neoplastic) and their ability in the diet to protect against or inhibit the development of cancer [1,2].

The recent progress in LC-hyphenated techniques such as high-performance liquid chromatography–UV–photodiode array detection (LC/UV-DAD) and LC–mass spectrometry (LC/MS) has led to instrumental developments and different modes of operations of such techniques, providing powerful analytical tools for the efficient detection and rapid characterization of natural products in complex biological matrices such as plant extracts. Furthermore, LC-hyphenated techniques are playing increasingly important roles in support of phytochemical investigations, such as targeting the isolation of new active compounds, for the dereplication of known plant constituents and for metabolite profiling studies [3,4].

Sugarcane (*Saccharum officinarum* L.) is one of the most important crops in Brazil and the flavonoid content is under investigation because these compounds may justify the potential utilization of sugarcane derivatives as nutraceutical foods. Products such as the sugarcane juice and syrup, besides some sweets produced from sugarcane juice, are traditionally consumed by the populations of several tropical

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countries, including Brazil and Central America. The major flavonoids are the flavone *O*- and *C*-glycosides, most of them being derivatives of apigenin, luteolin or tricin [5,6]. Other cultivated *Saccharum* species also have high amounts of flavone glycosides [7].

Due to the economical importance of sugarcane, several studies are in progress to search for new and more productive varieties or in order to achieve disease resistance. Among these studies, one approach in pest management is the genetic modification of sugarcane in order to introduce soybean trypsin inhibitors ("Kunitz") and proteinase inhibitors ("Bowman-Birk") [8]. The resistance of sugarcane genetically modified with soybean genes against the major insect pest of sugarcane in Brazil and other South American countries (sugarcane borer, *Diatraea saccharalis*) is being evaluated in order to introduce transgenic sugarcane into future commercial cultivation [9].

Therefore, in the present study, the flavonoids of sugarcane (bagasse and leaves extracts) were separated by high-performance liquid chromatography and in order to obtain additional structural information, shift reagents were added using a post-column derivatization system (LC/UV). The identification was performed by tandem MS techniques (atmospheric pressure chemical ionization, APCI–MS/MS and collision-induced dissociation, CID–MS). These LC-hyphenated techniques were also utilized for the comparative analysis of the flavonoids from non-modified and transgenic sugarcane ("Bowman-Birk" and "Kunitz").

2. Experimental

2.1. Plant material

The sugarcane material analyzed in this study was the species *S. officinarum* L. (Gramineae). The leaves were obtained from a commercial plantation in Araraquara, SP, Brazil and the bagasse was supplied by the sugar mill "São Martinho", Pradópolis, SP, Brazil.

The leaves of transgenic sugarcane plants were provided by Prof. Dr. Márcio de Castro Silva Filho (ESALQ-Universidade de São Paulo, Piracicaba, SP, Brazil) and were modified with two soybean proteinase inhibitor genes ("Bowman-Birk" and "Kunitz") [8]. 'Control' plants (genetically non-modified plants) were grown in greenhouses under the same conditions of transgenic plants, during 10 months; thereafter, the leaves of control and transgenic sugarcane plants were harvested at the same time.

All the plant material was dried before extraction at ∼40 °C until constant weight.

2.2. Chemicals

HPLC-grade acetonitrile (MeCN) was obtained from Romil (Cambridge, UK). Deionized water was prepared using a Reinstwasser-System Clear Cartridge System (SG, Hamburg, Germany). Both the solvents were passed through Millipore filters (water: 0.45 μm HA; MeCN: 0.50 μm FH; Bedford, MA, USA). Analysis-grade formic acid was purchased from Fluka (Buchs, Switzerland). Sodium hydroxide (NaOH), aluminium chloride ($AlCl_3$) and sodium acetate (NaOAc) were purchased from Sigma–Aldrich Chemie (Steinheim, Germany).

2.3. Extraction and "clean-up" of plant material

Extraction of sugarcane flavonoids was made by ultrasonic maceration of 1 g of dried plant material for 1.5 min at room temperature. A methanol/water 1:1 solution (20 mL) was used for sugarcane leaves, the extract was filtered, evaporated in rotatory evaporator down to 2 mL and the resulting aqueous extract was purified. Methanol (35 mL) was used for bagasse, the extract was filtered, added to 2 mL water, evaporated in rotatory evaporator to 2 mL and the resulting aqueous extracts were purified.

The purification of extracts was made by solid-phase extraction using 3 cc (60 mg), 30 μm particle size Oasis HLB cartridges (Waters, Milford, MA, USA), pre-conditioned with 1 mL of methanol and 1 mL of water. The interfering compounds were eluted in 3 mL of water and the flavonoid fractions were obtained by elution in 3 mL of methanol. Afterwards, the extracts of leaves and bagasse were filtered through a 0.5 μm Fluorpore membrane (Millipore, New Bedford, MA, USA) prior to injection (10 μL) into the HPLC system.

2.4. LC/UV–APCI–MS analyses

Analyses were performed using a Hewlett-Packard 1100 (Waldbronn, Germany) photodiode array detector (DAD) liquid chromatography system with a Waters Symmetry C_{18} column (25 mm × 4.6 mm i.d.; 5 μm) eluted with a linear gradient of acetonitrile (solvent B): water 0.2% formic acid (solvent A). Conditions utilized for analysis of leaf extracts: 0–8 min, 10–13% B; 8–25 min, 13–20% B; 25–40 min, 20–40% B; 40–45 min, 40–60% B. Analysis of bagasse extract was done as follows: 0–3 min, 18% B; 3–40 min, 18–70% B at a flow rate of 1.2 mL/min. A 10 μL aliquot of the extract was injected. The UV spectra (DAD) were recorded between 200 and 400 nm. LC/MS detection was performed directly after UV-DAD measurements. Analyses were performed using a Finnigan MAT (San Jose, CA, USA) ion trap mass spectrometer equipped with a Finnigan APCI interface operated under the following conditions: positive ion mode; capillary voltage, 11 V; capillary temperature, 150 °C; source voltage, 5 kV; sheath gas, nitrogen at 60 psi. Analyses were made in the full scan mode (150–900 u).

2.5. CID–MS analyses

Analyses were made using a Finnigan MAT TSQ-700 triple stage quadrupole instrument operating under the following conditions: positive ion mode; CI reagent gas, methane; heating, 50–100 °C in 1 min.

2.6. UV shift reagents

The classical shift reagents were prepared according to the literature [10]. The reagents used in the post-column derivatization system were as follows: strong base, aqueous sodium hydroxide (0.01 M); aqueous aluminium chloride (0.3 M; with this reagent, the reaction coil was heated to 60 °C); aqueous sodium acetate (0.5 M).

2.7. LC/UV analysis with post-column addition of shift reagents

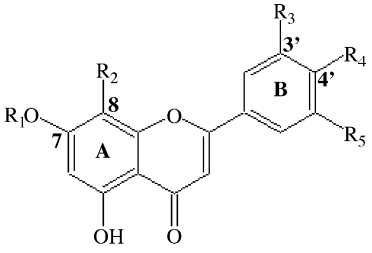
The solvent delivery system comprised two M-6000 pumps, a M-720 gradient controller and a U6K injector (Waters). The photodiode array detector HP-1040A (Hewlett-Packard) coupled with an HP-85 personal computer (Hewlett-Packard) was used for recording chromatograms and UV–vis spectra. For post-column derivatization, an Eldex Model A-30-5-2 (Eldex Labs, Menlo Park, CA, USA) pump and a reaction coil were employed. Shift reagents were added to the eluent at a flow rate of 0.3 mL/min.

3. Results and discussion

3.1. General aspects

A total of seven flavones (Fig. 1) were identified by the combination of LC/UV and LC/MS data. Firstly, several HPLC conditions were tested, but in order to perform further quantitative analysis, different gradient conditions were required for the separation of the peaks of the extracts of leaves and bagasse (cultivated sugarcane, Fig. 2). The extracts of leaves of transgenic and control plants (Fig. 3) were analyzed under the same conditions as cultivated sugarcane leaves. The chromatographic profiles of all the extracts were very complex, with several flavonoid peaks. Photodiode array detection allowed the on-line recording of UV spectra and a rapid attribution of peaks which corresponded to flavonoids in the chromatograms. The assignment of flavone peaks was unambiguous since these exhibit characteristic UV spectra, with two absorption bands [10] (Table 1): an example is shown in Figs. 2 and 3 in which chromatograms and UV spectra are found. Most of the peaks not identified in these chromatograms are of simple phenolic compounds, which were not the focus of our study.

Sugarcane cultivated in ‘real life’ conditions (Fig. 2) showed the presence of different flavones from those found in plants cultivated in a greenhouse (transgenic and control plants, Fig. 3). However, transgenic and non-modified (control) plants showed a similar chromatographic profile, but with differences in peak area. A quantitative study of the flavonoid content in cultivated sugarcane and also in the transgenic varieties is under progress at our laboratory.



	Compound	R_1	R_2	R_3	R_4	R_5
1	diosmetin-8-*C*-glycoside-arabinoside	H	Glc-Ara	OH	OCH_3	H
2	diosmetin-8-*C*-glycoside	H	Glc	OH	OCH_3	H
3	tricin-7-*O*-rhamnosilgalactoronide	Rha-(Galur)	H	OCH_3	OH	OCH_3
4a/4b	tricin-4’-*O*-(*erythro* or *threo* guaiacylglyceryl) ether-7-*O*-glucopyranoside	Glc	H	OCH_3	guaiacylglyceryl *erythro* or *threo*	OCH_3
5a/5b	tricin-4’-*O*-(*erythro* or *threo* guaiacylglyceryl) ether	H	H	OCH_3	guaiacylglyceryl *erythro* or *threo*	OCH_3

Ara = arabinose; Galur = galacturonic acid; Glc = glucose; Rha = rhamnose.

Fig. 1. Structure of the seven methoxyflavones identified in sugarcane extracts.

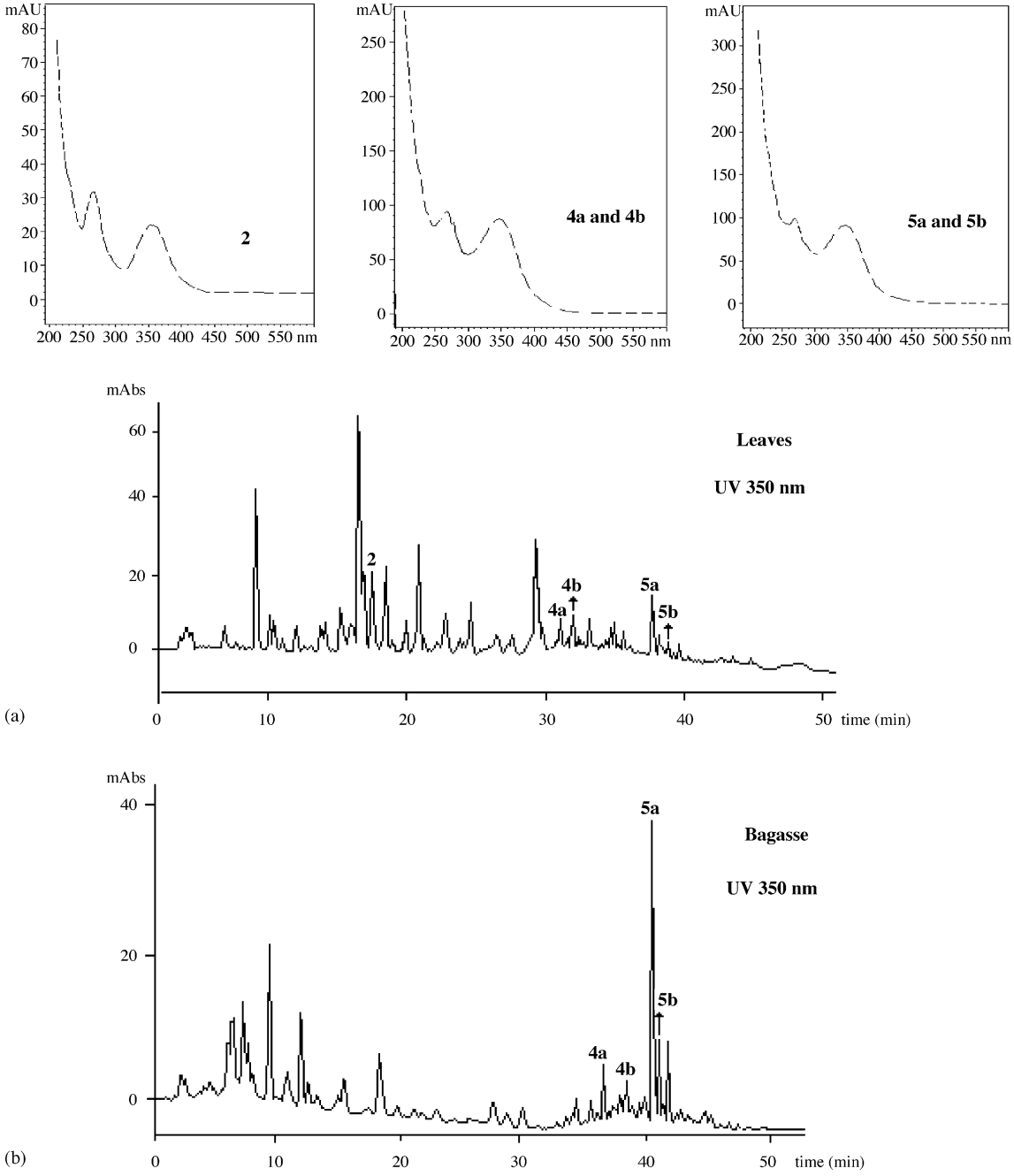


Fig. 2. HPLC-UV chromatogram of cultivated (commercial variety) sugarcane extracts. *Chromatographic conditions:* (a) Leaves, column Symmetry C_{18} (25 mm × 4.6 mm i.d.; 5 μm); linear gradient of acetonitrile (solvent B), water 0.2% formic acid (solvent A), 0–8 min: 10–13% B; 8–25 min: 13–20% B; 25–40 min: 20–40% B; 40–45 min: 40–60% B, at a flow rate of 1.2 mL/min. (b) Bagasse, column Symmetry C_{18} (25 mm × 4.6 mm i.d.; 5 μm); linear gradient of acetonitrile (solvent B), water 0.2% formic acid (solvent A), 0–3 min: 18% B; 3–40 min: 18–70% B, at a flow rate of 1.2 mL/min.

3.2. LC/MS APCI analysis and LC/UV analysis with post-column addition of shift reagents

In order to obtain more detailed structural information and also the molecular weights of the flavone glycosides, APCI–LC/MS analyses of the extracts were carried out. Since APCI is a soft ionization technique, it was possible to obtain the pseudomolecular ions $[M+H]^+$, an important structural information. Similar mass spectra to those measured under CID–MS were obtained which present a main peak corresponding to a pseudomolecular ion $[M+H]^+$, with losses of 120 u leading to $[A+H+42]^+$ peak, a fragmentation characteristic of a *C*-glycoside aglycone [11–13]. The mass difference between $[M+H]^+$ and the protonated aglycone ($[A+H]^+$) ions gives important information on the nature of the sugar or acid moiety.

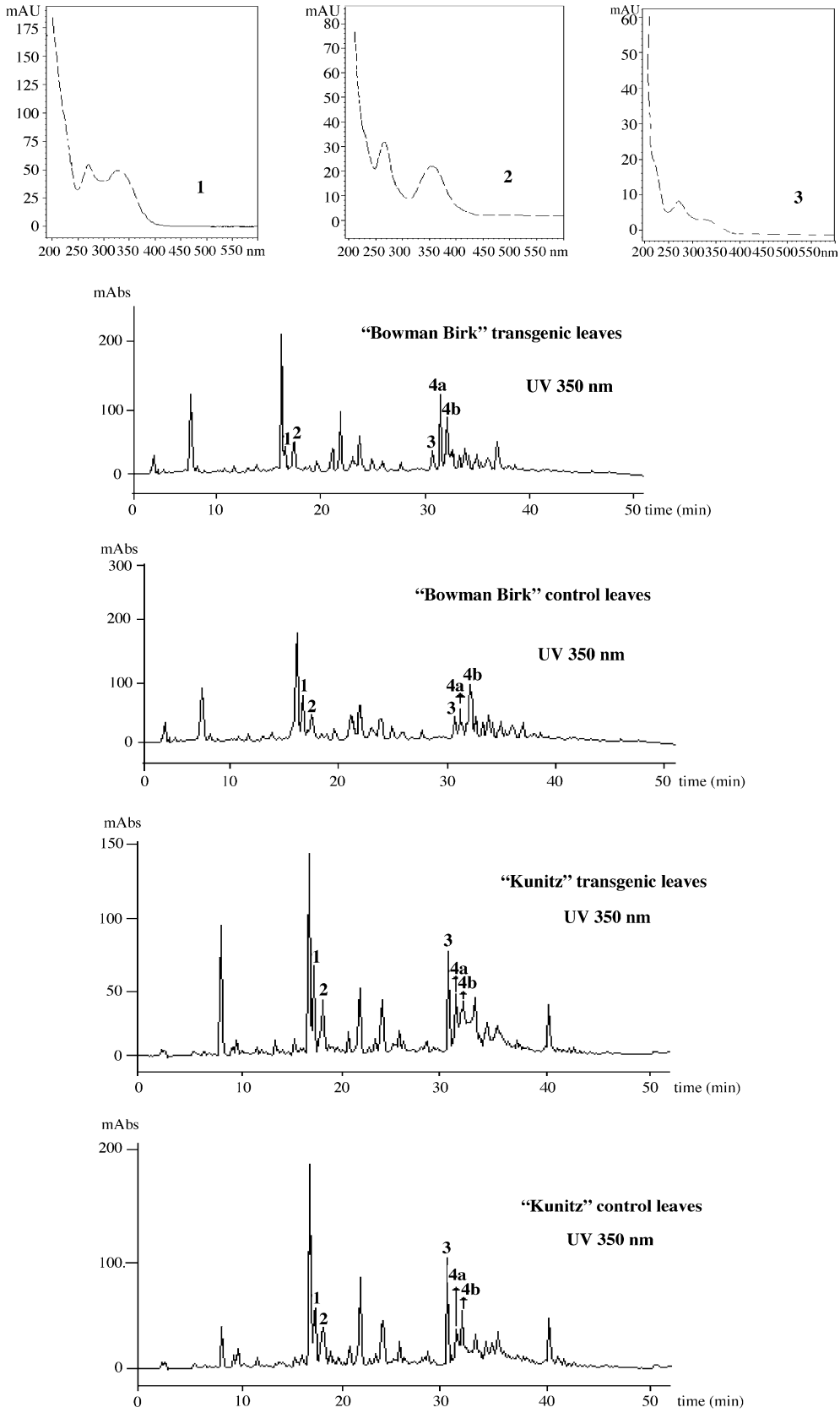


Fig. 3. HPLC-UV chromatogram and UV spectra of extracts of leaves of transgenic sugarcane and control plants (Bowman-Birk and Kunitz). *Chromatographic conditions:* same as Fig. 2a.

Table 1
On-line UV data of sugarcane flavonoids identified in this study

	Flavonoids	UV spectra (nm)		Shifted UV spectra NaOAc (nm)		Shifted UV spectra $AlCl_3$ (nm)	
		Band I	Band II	Band I	Band II	Band I	Band II
1	Diosmetin-8-*C*-glycoside-arabinoside	321	270	401, 320 sh	280	357	270
2	Diosmetin-8-*C*-glicoside	350	260	400, 310 sh	272	390	260
3	Tricin-7-*O*-rhamnosilgalactoronide	328	270	382, 330 sh	270	378	270
4a, **4b**	Tricin-4′-*O*-(*threo* or *erythro* guaiacylglyceryl) ether-7-*O*-glucopyranoside	340	270	400	270	382	270
5a, **5b**	Tricin-4′-*O*-(*threo* or *erythro* guaiacylglyceryl) ether	348	260	400	270	385	270

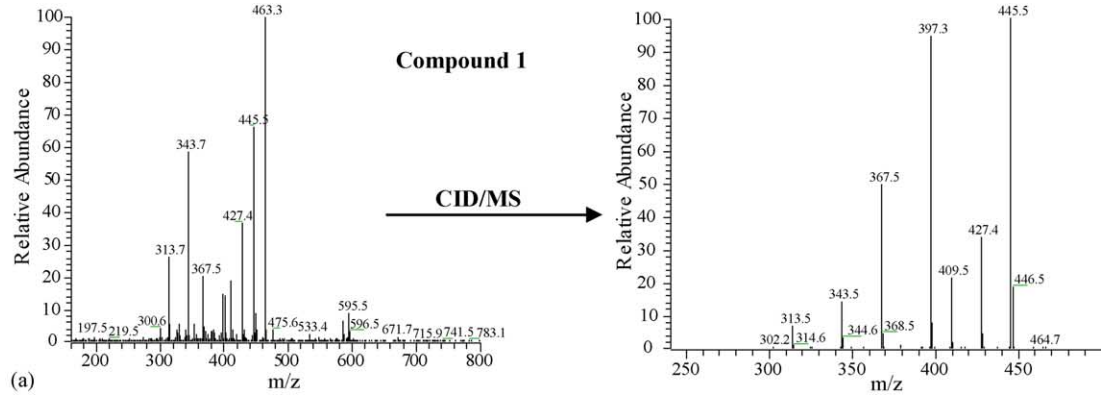
sh: Shoulder.

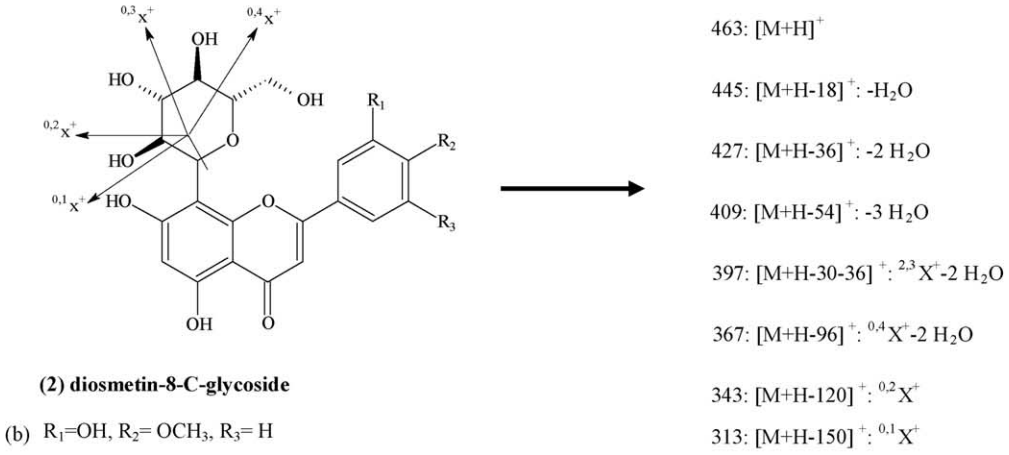
The LC/UV data (Table 1) in combination with shift reagents added post-column allows the determination of the methoxylation pattern and the position of the sugar or other component linked on the aglycone. A strong base reacts with all the phenolic groups except those in the *peri* position to the keto function. Al^{3+} forms a complex with *ortho*-dihydroxyl groups and keto groups having a hydroxyl in the *peri* position. NaOAc deprotonates the more acidic phenolic groups (3, 7 and 4′-OH groups) affecting mainly the UV band I; the shift on band II shows the presence of a free-OH group at position 7, and this 7-OH group influences the shift and the presence of a shoulder on band I, which indicates a free-OH group at position 3′ or/and 4′. Analyses and, therefore, measurements of UV spectra in HPLC with post-column addition of reagents are normally performed in methanol [14,15]. However, the conditions described here involved HPLC with an acidic acetonitrile–water system. Therefore, the shift reagents were tested on different reference flavones under the same chromatographic conditions. For band I, all of the compounds showed a bathochromic shift with $AlCl_3$ in the range of 36–50 nm (Table 1), confirming the flavone skeleton (ref. [10]: 35–55 nm) and the absence of an *ortho*-dihydroxyl group (3′ and 4′).

As shown for **1** (Fig. 4a) the peak in the APCI spectrum corresponding to the pseudomolecular ion $[M + H]^+$ was observed at *m*/*z* 595 while CID–MS fragmentation at *m*/*z* 463 was attributed to $[M + H - 132]^+$, with the loss of the 132 u fragment corresponding to a pentose moiety (possibly arabinose, which is the pentose most frequently found in flavonoids). For compound **2** the pseudomolecular ion $[M + H]^+$ was observed at *m*/*z* 463 and the same fragments obtained for compound **1** were observed in the CID–MS. According to the fragmentation route proposed in Fig. 4b, the fragment at *m*/*z* 343 was attributed to $[A + H + 42]^+$, representing the aglycone diosmetin plus a part of the glucose moiety. For both compounds (**1** and **2**) the UV analysis with post-column addition of shift reagents showed a bathochromic shift of band I with addition of NaOAc, indicating a free-OH group at position 3′, but the absence of *ortho*-dihydroxyl and the presence of a shoulder on band I demonstrate a substitution at 4′. For band II, the shift with NaOAc of 10 and 12 nm, respectively for compounds **1** and **2**, indicated a non-substituted 7-OH group, so the sugar moiety was attributed to the 8-position (Fig. 5a and b).

For compound **3** (Fig. 4c), two peaks were found at *m*/*z* 653 and 639, corresponding to the pseudomolecular ion, $[M + H]^+$. The difference of 14 u is expected for the methyl ester and free acid form, respectively, of the galacturonic acid moiety [5]. The ions observed at *m*/*z* 331, $[M + H - 308]^+$ corresponded to tricin aglycone (M^+, 330) and the loss of 308 u was due to the rhamnosylgalacturonide moiety (rhamnose and galacturonic acid). The appearance of a shoulder on band I showed that the position 3′ is blocked and showed a free-OH group at position 4′ [16], which confirmed the structure of the aglycone tricin. The identification of tricin-7-*O*-rhamnosylgalacturonide was confirmed also by the absence of a bathochromic shift on band II with addition of NaOAc, showing that position 7 is blocked by the rhamnosylgalacturonide moiety (Fig. 5c and d).

APCI–LC/MS and CID–MS analyses of isomeric compounds **4a** and **4b** gave identical results (Fig. 4d and e) comprising a protonated molecule at *m*/*z* 689, $[M + H]^+$ and major fragments *m*/*z* 527, $[M + H - 162]^+$ *m*/*z* 331, $[M + H - 358]^+$, attributed respectively to the loss of a hexose group and to the aglycone tricin. The same molecular ion and fragments were already detected in the unusual isomeric flavonolignan glycosides found in the leaves of *Hyparrhenia hirta* Stapf (Poaceae) [14]. For the isomeric compounds **5a** and **5b**, their MS spectra were also identical (Fig. 4f). The peak at *m*/*z* 527 corresponded to the $[M + H]^+$ pseudomolecular ion, while $[A + H]^+$ at *m*/*z* 331 was attributed to the tricin aglycone (M^+, 330). The UV analysis with shift reagents of the pair **4a**/**4b** confirmed the 4′ position to be blocked by the guaiacylglyceryl group, due to the absence of a shoulder on band I with addition of NaOAc. On band II the absence of shift with NaOAc was characteristic for a substituted 7 position, probably due the sugar moiety (Fig. 5e and f). In the pair **5a**/**5b**, the addition of NaOAc presented a shift of 10 nm on band II showing a free-OH group at 7-position. The distinction between the *erythro*- and *threo*-forms of diastereoisomeric pairs **4a**/**4b** and **5a**/**5b**, however, would require additional data to those obtained by LC/MS or LC/UV.





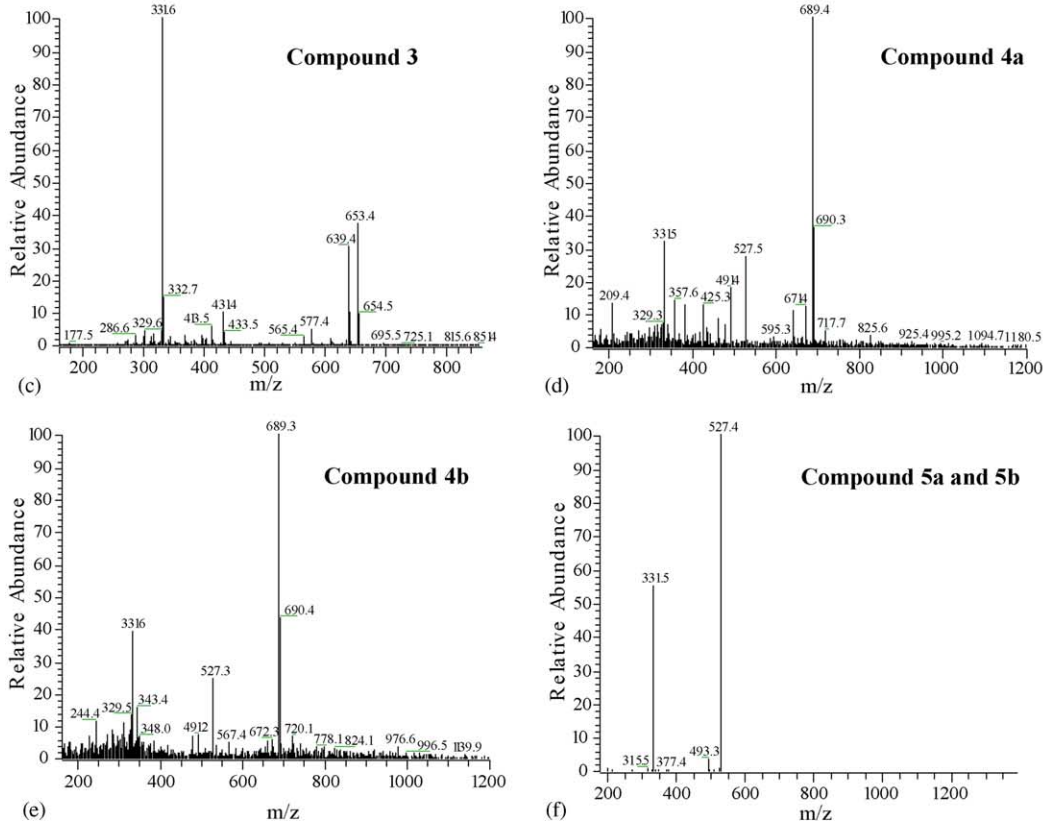


Fig. 4. APCI–MS and CID–MS spectra of compounds **1**, **3**, **4a**, **4b**, **5a** and **5b**. Structure and fragmentation pathways of **2**.

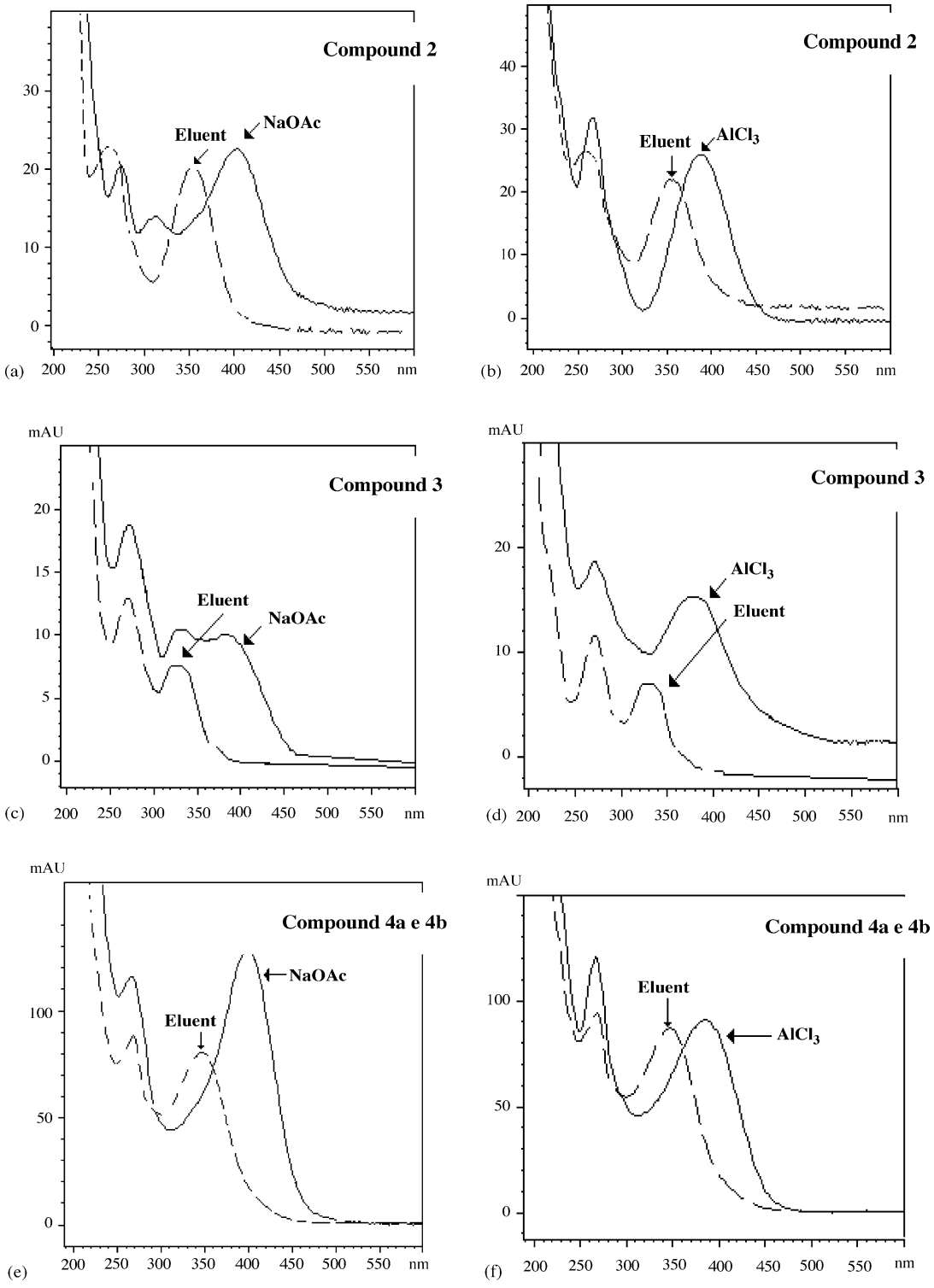


Fig. 5. UV spectra of compounds **2**, **3**, **4a**/**4b** obtained after the post-column addition of different shift reagents.

4. Conclusions

The combination of data obtained by LC/UV with shift reagents and LC/techniques allowed us to the on-line structure elucidation of seven methoxyflavones of *S. officinarum* in complex extracts and using reduced amounts of plant material. LC/UV allowed to decide whether the sugar moiety was attached to the aglycone and how many hydroxyls group is substituted. Besides, the present study also showed that the LC/MS fragmentation pathway proposed in literature for hydroxylated *C*-glycosides [12] is applied also to methoxylated *C*-glycosides. Only one of the methoxyflavones identified in

the present work (**3**, tricin-7-*O*-rhamnosilgalactoronide) has been previously reported in former studies of sugarcane [5], while the pairs of diastereoisomeric flavolignans (**4a**/**4b)** and their glycosides (**5a**/**5b)** have been previously reported in another plant species [14]. The other compounds have never been reported in further studies of sugarcane.

The structural data herein reported about the sugarcane compounds and the strategy of application of LC-hyphenated techniques analysis [17] is being applied in studies in progress focusing the metabolite profiling [18] of transgenic sugarcane in order to assessing the chemical equivalence of genetically modified plants and cultivated (commonly believed to be "safe") sugarcane.

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