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Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass spectrometry for the differentiation of *C*-glycosidic flavonoid isomers

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Abstract

LC–MS–MS is becoming a very important tool for the on-line identification of natural products in crude plant extracts. For an efficient use of this technique in the dereplication of natural products, a careful study of the parameters used to generate informative MS–MS spectra is needed. In this paper, the collision-induced dissociation (CID) MS–MS spectra of ubiquitous *C*-glycosidic flavonoids have been systematically studied using hybrid quadrupole time-of-flight and ion-trap (IT) mass analysers under various CID energy conditions. Efficient differentiation of flavonoid *C*-glycoside isomers was possible, based on the comparison of CID-MS–MS spectra of particular *C*-glycoside unit fragments. Striking differences between 6-*C* and 8-*C* flavonoid glycosides were especially observed in the product ion spectra of their $^{0,2}X^{+}$ fragments ($[M+H-120]^{+}$). Some guidelines for the on-line characterisation of *C*-glycosidic flavonoids by LC–MS–MS or LC–multiple-stage MS are given. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Mass spectrometry; Collision-induced dissociation; Flavonoids; Glycosides

1. Introduction

The introduction of LC–MS in crude plant extract analysis has represented an important step for the on-line identification of natural products [1,2]. The recent development of atmospheric pressure ionisation (API) sources has rendered this technique more sensitive and easy to handle and its use has been spread in many phytochemical laboratories for the screening of crude plant extracts and for the dereplication of known natural products [3]. Electrospray ionisation (ESI) [4] or atmospheric pressure chemical ionisation (APCI) [5] are soft ionisation techniques which generate mainly protonated molecules for relatively small plant metabolites such as flavonoids. Molecular mass information alone, however, is not sufficient for the on-line structure determination of natural products and fragment information generated by collision-induced dissociation (CID) MS–MS is necessary for partial on-line identification or for dereplication of known constituents.

Flavonoids have been recognised as one of the largest and most widespread classes of plant constituents [6], their dereplication in crude plant extracts is thus crucial for conducting efficient targeted

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isolation procedure [7]. Different papers have dealt with mass fragmentation pathways of flavonoid aglycones under electron impact ionisation [8], and such pathways have also been documented for flavones and flavonols under fast atom bombardment CID-MS–MS [9–11]. Liquid secondary ion tandem mass spectrometry was directly applied on crude extracts for the characterisation of various *O*-diglycosidic flavonoids [12]. All these polyphenols were also successfully analysed in crude plant extracts or in biological fluids by LC–APCI-MS [13–16] and LC–ESI-MS [17]. Furthermore the low-energy CID-MS–MS spectra of various flavonoid aglycones and glycosides in mixtures were described in both LC–ESI-MS–MS [18,19] and LC–APCI-MS–MS [20,21] modes. Recently we have also summarised the possibilities of on-line structure identification of various classes of flavonoid aglycones by LC–APCI-MS–MS and LC–ESI-MS–MS under different low-energy collision regimes [22]. In these different CID-MS–MS studies, it has been demonstrated that fragment ions provide important structural information for flavonoids and can be used to establish the distribution of the substituents between the A- and B-rings.

In crude plant extracts, flavonoids are often present as *O*- or *C*-glycosides. The *O*-glycosides have sugar substituents bonded to a hydroxyl group of the aglycone, whereas the *C*-glycosides have sugar substituents bonded to a carbon of the flavonoid aglycone, generally at position 6-*C* and 8-*C*. A careful study of the fragmentation patterns in CID-MS–MS can also be of a particular value in the determination of the nature and site of attachment of the sugars in *O*- and *C*-glycosides [11,16,23–25].

In complement to LC–MS–MS other hyphenated techniques such as LC–UV-DAD (diode array detection), with post-column addition of UV shift reagents [26], or LC–NMR have proven to be very valuable for the screening of these constituents [27,28].

In this paper, low-energy LC–CID-MS–MS procedures will be studied in more detail for the on-line differentiation of 6-*C* and 8-*C*-glycosidic flavonoid isomers, under typical LC–APCI-MS conditions, on two types of MS instruments: a hybrid quadrupole time-of-flight (Q-TOF) one and an ion-trap (IT) one. These two instruments were chosen in order to obtain a general picture of the differences that may occur between isomers in the MS–MS spectra produced by beam and trap systems. In a beam instrument collisions take place over a very short period of time for each ion as it passes through the neutral gas (argon) and there is a spatial separation of ionisation and collision processes. The precursor ions in a linear beam instrument are accelerated so as to increase the energy transferred at collision. In an ion trap, the ions are constrained within one space for a varying length of time during which, into the same space, some neutral gas is admitted (helium). The ions can be given more energy by accelerating them in their orbits [29,30].

The CID-MS–MS spectra acquired by these different low-energy collision regimes will be compared for the search of diagnostic ions that may be used for the on-line differentiation of *C*-glycoside isomers. The high mass accuracy capabilities of Q-TOF were used for the precise fragment ion assignments in the product ion spectra recorded.

Previous studies on the differentiation of *C*-glycoside isomers have already been reported in positive ion mode by fast atom bombardment (FAB) mass spectrometry in combination with collision-activated dissociation (CAD) and linked scanning at constant ratio of magnetic and electric sector fields (B/E) [24]. Similar studies were also reported on spectra obtained by negative ion FAB-CAD mass analysed ion kinetic energy (MIKE) [31]. As this will be demonstrated in this study, the discriminating ions originating from these high-energy collision processes differ from those obtained at low energy. The results obtained confirm those of our preliminary studies performed on a triple quadrupole instrument with thermospray ionisation (TSP) [23].

We report here on the CID-MS–MS or CID multiple-stage MS (MS^n) spectra recorded in positive and negative ion mode under LC flowing conditions for four widespread *C*-glycosidic flavonoids: vitexin (8-*C*-Glc-apigenin) (**1**), isovitexin (6-*C*-Glc-apigenin) (**2**), orientin (8-*C*-Glc-luteolin) (**3**) and isoorientin (6-*C*-Glc-luteolin) (**4**). A flavonoid containing a *C*-diglycoside, vitexin-2-*O*″-rhamnoside (**5**), has also been studied. The CID fragmentations of different types of precursor ions obtained at

several energies on both IT and Q-TOF instruments are discussed and an evaluation of different LC–MS–MS strategies are given.

2. Experimental

2.1. Solvents and reagents

Acetonitrile (MeCN) was HPLC-grade (Maechler, Basel, Switzerland). Water was prepared by distillation on a Büchi (Flawil, Switzerland) Fontapor 210 distillation instrument. Both solvents were passed through Millipore filters (water: 0.45 μm HA; MeCN: 0.50 μm FH) (Bedford, MA, USA). Acetic acid was purchased from Fluka (Buchs, Switzerland).

2.2. Standards

Pure flavonoid standards (**1** and **5**) were purchased from Roth (Reinach, Switzerland) or were isolated from plants (**2**–**4**) at the Institute of Pharmacognosy and Phytochemistry (Lausanne, Switzerland).

2.3. HPLC analyses

A 10 μl volume of pure flavonoid solutions (1 mg/ml) was directly injected on a C_{18} Guard-PAK column (Waters, Milford, MA, USA) using an isocratic acetonitrile–water (80:20) solvent system containing 0.5% acetic acid at a flow-rate of 0.3 ml/min. Under these conditions the polyphenols eluted in the API source in less than 3 min.

2.4. Ion-trap-MS analyses

IT-MS^n experiments were performed on a LCQ ion-trap mass spectrometer equipped with ESI or APCI interface (Finnigan MAT, San Jose, CA, USA). The HPLC system consisted of an HP-1100 equipped with binary pumps and a photodiode array high-speed spectrophotometric detector (Hewlett-Packard, Palo Alto, CA, USA). The APCI conditions were as follows: vaporiser temperature, 450°C; transfer capillary temperature, 150°C; cone voltage, 0 V and 25 V. Electrospray conditions: spray voltage, 4.5 kV; capillary temperature, 200°C. On both interfaces nitrogen was used as sheath gas.

2.5. Q-TOF-MS analyses

Q-TOF-MS–MS experiments were realised on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK). The APCI conditions were as follows: Corona pin voltage, 5 V; vaporiser temperature, 600°C; nebuliser gas, nitrogen; cone voltage, 26 V; MS scan time 1 s+0.1 s interscan delay; MS–MS scan time 0.5 s+0.1 s interscan delay.

For accurate mass measurements a reference compound, sulfadimethoxine ($[M+H]^+$: m/z 311.0814) (Aldrich, Buchs, Switzerland) was added post-column.

2.6. CID-MS–MS analyses

The MS–MS methods used for IT-MS^n and Q-TOF-MS–MS were both based on scan-dependent type of experiments.

In the IT-MS the most-abundant ion was automatically selected as precursor ion and fragmented up to the MS^4 stage, each successive most-abundant fragment ion being selected again as precursor ion for the next step. The isolation width was set to 2 u. This method was applied in both positive and negative modes at different energy levels (35, 40, 50 and 60%) in separate LC runs. The little amount of helium residing in the trap was used as collision gas. Energy levels on the Finnigan IT-MS are given in % and not in eV since the voltages applied vary according to the m/z value of the precursor ions.

In the Q-TOF-MS–MS scan-dependent MS–MS autoswitch experiments were performed sequentially on the three most abundant ions recorded by LC–APCI-MS. The combination of quadrupole ion selectivity with the full scan sensitivity of the TOF analyser allowed the realisation of each autoswitch MS–MS experiment at five different CID energy levels (20, 25, 30, 35 and 40 eV) during a 3 s period. In this study up to three different co-eluting precursor ions could be selected (out of a maximum of eight co-eluting precursor ions) for further MS–MS experiments. Argon was used as collision gas.

The energy range chosen (CID 35–60% for IT-

MS and CID 20–40 eV for Q-TOF) covered all the settings where CID-MS–MS spectra were in practice recordable on both instruments. At low energy (35% IT, 20 eV Q-TOF) almost no fragmentation was recorded, while at high energy (60% IT, 40 eV Q-TOF) only a general loss in sensitivity without any increase in the number or the abundance of the fragment ions formed was observed.

3. Results and discussion

In order to rapidly evaluate the information generated on-line by LC–Q-TOF-MS–MS or LC–IT-MS^n, the collision-induced dissociation (CID) MS–MS spectra of different *C*-glycosidic flavonoids were recorded. These samples were injected on a C_{18} precolumn and eluted rapidly in the API source of the MS analysers. All the experiments were performed in these flowing conditions in order to mimic the LC elution of these compounds when crude plant extracts are analysed. Both LC–APCI-MS and LC–ESI-MS have been evaluated for the ionisation of flavonoids and protonated and deprotonated molecules were observed in positive and negative ion modes respectively. No significant sodium adduct was produced since the samples were eluted on a C_{18} column prior ionisation.

For the different flavonoids studied, we observed that the MS–MS results obtained with APCI were also applicable to ESI. Indeed in our preliminary study [22] the MS–MS spectra recorded on the $[M+H]^+$ ions of flavonoid aglycones generated either by ESI or APCI were very similar indicating that the energy deposition on the precursor ion by both ionisation methods did not affect significantly the product ion spectra.

APCI was found more convenient for further application to crude plant extract screening since no flow splitting was needed and thus all measurements presented have been performed in this ionisation mode.

Contrary to flavonoid *O*-glycosides, the CID-MS–MS spectra of *C*-glycosides did not generate abundant Y_o^+ aglycone ions but instead characteristic ions of the fragmentation of the *C*-glycoside unit itself [16,32]. Losses of 120 u ($^{0,2}X^+$ $[M+H\text{-}120]^+$) are relatively easy and can be directly observed in the LC–APCI-MS spectra, especially when cone voltage is applied in the APCI source (‘‘source CID on’’ mode). This cleavage is indicative for *C*-glycosides and was also easily observed when ionisation occurs in TSP-MS [23]. A and B ions issued from C-ring cleavage cannot be observed from the first order MS–MS spectra of the $[M+H]^+$ ions.

All these different fragment ions have been studied in more detail in view of their use for the differentiation of 6-*C* and 8-*C* glycoside isomers.

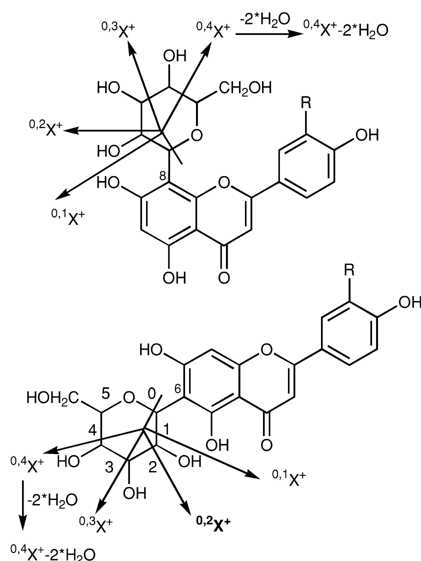
In the discussion of the CID-MS–MS spectra, the fragments recorded have been annotated according to the nomenclature adopted by Mabry and Markham [8], Domon and Costello [32], Claeys and co-workers [10,11] (Fig. 1).

3.1. Choice of the optimal CID collision energy

The choice of the optimum collision energy affects significantly the abundance and also the type of ions generated in the MS–MS spectra. In order to have a rather general view of what is typically occurring in a low-energy collision regime, MS–MS spectra of *C*-glycosidic flavonoids generated at different energies on a hybrid Q-TOF system and on an IT system are compared. The range of CID energies on both instruments were selected between cases where almost no fragmentation of the precursor ions occurs and those where fragments were too weak for valuable interpretation, due to the excess of energy applied. For the flavonoids studied these ranges were between 20 and 40 eV on the Q-TOF system and 35–60% on the IT-MS system. In our previous systematic study on the CID-MS–MS fragmentation of flavonoid aglycones, it has been demonstrated that the MS–MS spectra recorded at a CID energy level of 40% on IT-MS were similar to those obtained with 25 eV on the Q-TOF system [22]. This statement, however, was only valid if no consecutive fragmentation reactions were possible, as in the case of fully hydroxylated flavonoids.

In this study, none of the CID-MS–MS spectra of the protonated flavonoid *C*-glycoside molecules recorded on Q-TOF (CID: 20–40 eV) matched completely those obtained by IT-MS^2 (CID: 35–60%). From a qualitative viewpoint, the characteristic fragment ions of the *C*-glycoside moiety was obtained on both instruments by selecting the $[M+H]^+$ as pre-

C-glycoside unit fragmentation



8-C-glycosidic flavonoids:

R=H: Vitexin (1)
R=OH: Orientin (3)

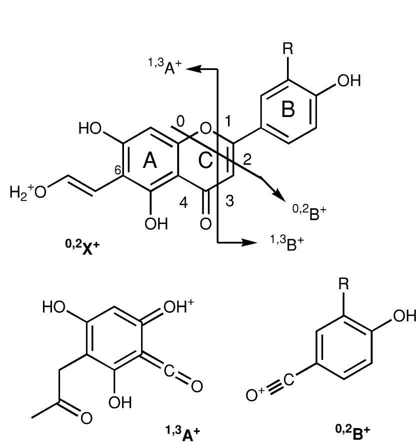
MAIN IONS OBTAINED BY CID-MS-MS OF $[M+H]^+$ OF COMPOUNDS 1-4:

1 + 2 / 3 + 4
433 / 449: $[M+H]^+$
415 / 431: $[M+H-18]^+$: E_1^+
397 / 413: $[M+H-36]^+$: E_2^+
379 / 395: $[M+H-54]^+$: E_3^+
367 / 383: $[M+H-30-36]^+$: $^{2,3}X^+$-2*H_2O
343 / 359: $[M+H-90]^+$: $^{0,3}X^+$
337 / 353: $[M+H-96]^+$: $^{0,4}X^+$-2*H_2O
313 / 329: $[M+H-120]^+$: $^{0,2}X^+$
295 / 311: $[M+H-120-18]^+$: $^{0,2}X^+$-H_2O
283 / 299: $[M+H-150]^+$: $^{0,1}X^+$

6-C-glycosidic flavonoids:

R=H: Isovitexin (2)
R=OH: Isoorientin (4)

Fragmentation of $^{0,2}X^+$ in the case of 6-C isomers*



MAIN IONS OBTAINED BY CID-MS-MS OF $^{0,2}X^+$ FOR COMPOUNDS 2 AND 4:

2 / 4
313 / 329: $^{0,2}X^+$
295 / 311: $^{0,2}X^+$-H_2O
284 / 300: $^{0,2}X^+$-CHO
283 / 299: $^{0,2}X^+$-CH_2O
267 / 283: $^{0,2}X^+$-CH_2O_2
256 / 272: $^{0,2}X^+$-C_2HO_2
255 / 271: $^{0,2}X^+$-2*CHO
195 / 195: $^{1,3}A^+$
177 / 177: $^{1,3}A^+$-H_2O
149 / 149: $^{1,3}A^+$-H_2O-CO
121 / 137: $^{0,2}B^+$

R=H: Isovitexin (2)
R=OH: Isoorientin (4)

* C-ring A fragment ions were not observed in the case of 8-C isomers

Fig. 1. Structures and fragmentation pathways of the C-glycosidic flavonoids studied.

cursor ion: $^{0,4}X^+$-2H_2O $[M+H-96]^+$, $^{0,3}X^+$ [M+H-90]$^+$, $^{0,2}X^+$ $[M+H-120]^+$, $^{0,1}X^+$ $[M+H-150]^+$ [32]. The fragment ion intensities of vitexin (**1**) obtained with all the different collision energies are summarised in Table 1. In the IT-MS^2 spectra, the intensity ratio of the different fragments was not significantly affected if CID was changed from 35 to 60%. In Q-TOF, on the other hand, the intensity of the ions $^{0,2}X^+$ $[M+H-120]^+$ and $^{0,1}X^+$ $[M+H-150]^+$ was strongly dependent on the collision energy applied and these ions were dominating the spectra at high energy levels. As the collision energy seems not to affect the spectral pattern in IT-MS, all the comparison experiments between 6-C and 8-C-glycosidic flavonoids were performed at a CID of 50%, which was found to generate intense fragment ions without

Table 1
CID-MS–MS of *C*-glycosidic flavonoids

Expt.	CID	Prec.	Fragment ions (% RA)[a]										
			$[M+H]^+$	E_1^+	E_2^+	E_3^+	$^{2,3}X^+$-$2H_2O$	$^{0,3}X^+$	$^{0,4}X^+$-$2H_2O$	$^{0,2}X^+$	$^{0,2}X^+$-H_2O	$^{0,1}X^+$	Base peak
(1) Vitexin (M_r 432)													
Q-TOF-MS–MS POS	20 eV	433	433 (100)	415 (95)	397 (70)	379 (20)	367 (40)	343 (10)	337 (20)	313 (60)	295 (5)	283 (20)	433 (100)
	25		433 (15)	415 (40)	397 (55)	379 (20)	367 (30)	343 (20)	337 (20)	313 (100)	295 (10)	283 (55)	313 (100)
	30		–	415 (10)	397 (20)	379 (15)	367 (15)	343 (10)	337 (20)	313 (100)	295 (15)	283 (75)	313 (100)
	35		–	–	397 (5)	379 (5)	367 (5)	343 (3)	337 (15)	313 (75)	295 (20)	283 (100)	283 (100)
	40		–	–	–	–	–	–	337 (15)	313 (70)	295 (35)	283 (100)	283 (100)
$IT-MS^2$ POS	35%	433	–	415 (100)	397 (50)	379 (10)	367 (35)	–	337 (10)	313 (20)	295 (3)	283 (2)	415 (100)
	40		–	415 (100)	397 (60)	379 (20)	367 (50)	343 (2)	337 (15)	313 (25)	295 (3)	283 (5)	415 (100)
	50		–	415 (100)	397 (85)	379 (30)	367 (65)	343 (3)	337 (25)	313 (40)	295 (5)	283 (7)	415 (100)
	60		–	415 (90)	397 (100)	379 (40)	367 (85)	343 (10)	337 (40)	313 (60)	295 (5)	283 (20)	397 (100)
(5) Vitexin 2″-O-rhamnoside (M_r 578)													
$IT-MS^3$ POS (579→433)	50%	433	–	415 (100)	397 (45)	379 (10)	367 (45)	343 (2)	337 (10)	313 (25)	295 (2)	283 (5)	415 (100)
(2) Isovitexin (M_r 432)													
Q-TOF-MS–MS POS	20 eV	433	433 (5)	415 (30)	397 (55)	379 (60)	367 (70)	–	337 (75)	313 (90)	295 (5)	283 (100)	283 (100)
	25		–	–	397 (15)	379 (25)	367 (10)	–	337 (50)	313 (75)	295 (5)	283 (100)	283 (100)
	30		–	–	–	379 (15)	–	–	337 (30)	313 (75)	295 (10)	283 (100)	283 (100)
	35		–	–	–	379 (5)	–	–	337 (10)	313 (60)	295 (15)	283 (100)	283 (100)
	40		–	–	–	–	–	–	–	313 (35)	295 (30)	283 (100)	283 (100)
$IT-MS^2$ POS	50%	433	–	415 (55)	397 (50)	379 (40)	367 (100)	–	337 (80)	313 (50)	–	283 (20)	367 (100)
(3) Orientin (M_r 448)													
$IT-MS^2$ POS	50%	449	–	431 (100)	413 (75)	395 (25)	383 (65)	359 (5)	353 (25)	329 (40)	311 (5)	299 (10)	431 (100)
(4) Isoorientin (M_r 448)													
$IT-MS^2$ POS	50%	449	–	431 (50)	413 (45)	395 (40)	383 (100)	–	353 (80)	329 (40)	–	299 (20)	383 (100)

[a] Fragment nomenclature according to Domon and Costello [32]. RA, relative abundance; POS, positive mode; prec.: precursor ion.

important loss of sensitivity. In Q-TOF, on the other hand, comparisons were still performed at different energy levels.

3.2. Differentiation of 6-C- and 8-C-glycosidic flavonoids in the MS–MS of $[M+H]^+$

For a discrimination between 6-*C* and 8-*C*-glycosidic flavonoids, the low-energy CID spectra of vitexin (**1**) and isovitexin (**2**) were recorded by choosing their protonated molecules as precursor ions on both Q-TOF and IT-MS. A previous paper on FAB high-energy CAD-MS–MS [24] mentioned that these isomers were differentiated based on the characteristic intensity ratio of $E_1^{+\cdot}/E_{1\,iso}^{+}$ (*m/z* 414/416), $^{0,3}X^+$-H_2O/$^{0,3}X^+$ (*m/z* 325/343), $Y^{+\cdot}/Y^+$ (*m/z* 270/271) and $[^{1,5}X_A^+$-$2H]^+/^{1,5}X_A^+$ (*m/z* 179/181). These different couples of ions were not observed in our low-energy CID experiments or were too weak to draw any valuable structural information. Another observation of the FAB-CAD-MS–MS was the preferential losses of water observed for 6-*C* isomers, especially that observed with E_3^+ $[M+H$-$3H_2O]^+$. The more facile loss of an additional molecule of water in the 6-*C* isomers was explained by elimination of water between the 2″-hydroxyl

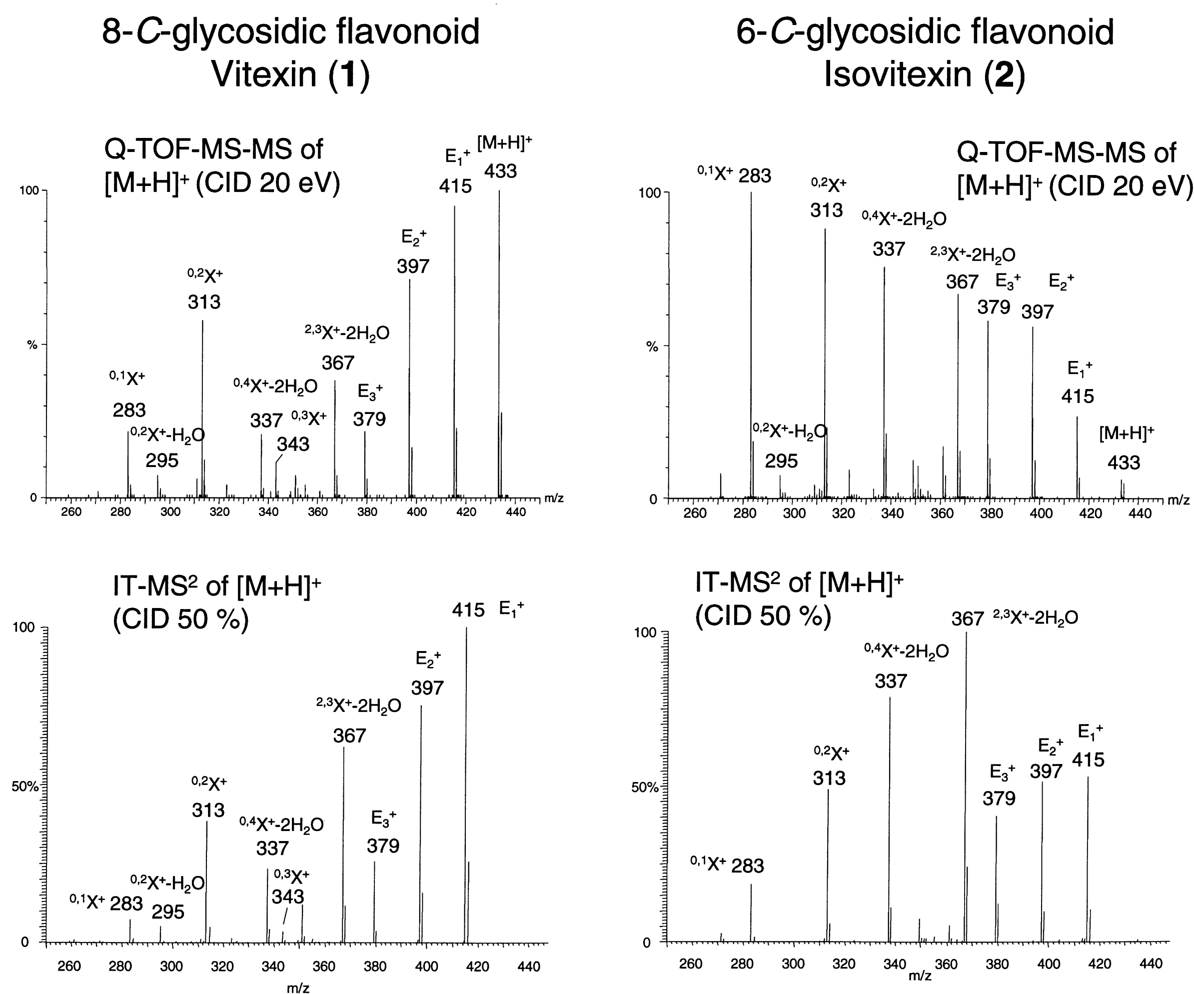


Fig. 2. Q-TOF-MS–MS (CID 20 eV) and IT-MS^2 (CID 50%) spectra of the two *C*-glycoside isomers vitexin (**1**) and isovitexin (**2**), with $[M+H]^+$ as precursor ion (*m/z* 433).

group of the sugar moiety and the 5- or 7-hydroxyl groups of the aglycone part. At low energy, in our experiments, the E_3^+ ion was also found more abundant in the case of 6-*C* than for 8-*C* isomers, but the differences were not striking and the intensity of the E_3^+ ion was also strongly dependent on the different low energies applied in the Q-TOF collision cell (Table 1). At relative low-energy settings (CID: 20 eV, Q-TOF; 35–50%, IT), on the other hand, the E_1^+ ion $[M+H-H_2O]^+$ was the base peak in the spectra of all 8-*C* flavonoid glycosides while its abundance was lower than 50% for the corresponding 6-*C* isomers. In IT the precursor ion of both 6-*C* and 8-*C* isomers was not observed in all spectra recorded (CID 35–50%). In Q-TOF, at a CID energy of 20 eV, the protonated molecule was found much more stable for 8-*C* than for 6-*C* glycosides (Fig. 2).

The fragment abundance profile obtained in the MS–MS spectra differs significantly between 6-*C* and 8-*C* isomers in the given conditions on both mass spectrometers. These spectra could be used for isomer discrimination by comparison with CID-MS–MS databases characteristic of each instrument. A spectral matching of a given flavonoid analysed on both instruments was not possible, as shown in Fig. 2, since important differences in fragment ion abundances were observed between IT and Q-TOF. However, in all MS–MS spectra of protonated molecules, no diagnostic fragment ion, clearly recorded for one of the isomers only, was observed (Fig. 2).

Based on these considerations it was difficult to draw rules for an unambiguous distinction between 6-*C* and 8-*C* isomers in the low-energy CID-MS–MS spectra of their $[M+H]^+$ ions.

3.3. Differentiation of 6-C and 8-C-glycosidic flavonoids in the MS–MS of $[M+H-120]^+$

In order to generate low-energy CID-MS–MS spectra which exhibit more striking differences between 6-*C* and 8-*C* isomers, several precursor ions different from $[M+H]^+$ have been selected, namely: $^{0,4}X^+$-$2H_2O$ $[M+H-96]^+$, $^{0,3}X^+$ $[M+H-90]^+$, $^{0,2}X^+$ $[M+H-120]^+$ and $^{0,1}X^+$ $[M+H-150]^+$. These experiments have demonstrated that in general the MS–MS spectra obtained from these fragment ions were not significantly different between 6-*C* and 8-*C* isomers, except in the case where $^{0,2}X^+$ $[M+H-120]^+$ was used as precursor. This $^{0,2}X^+$ ion is weak when no skimmer voltage is applied in the ion source and becomes the base peak of the spectra when skimmer voltages higher than 25 V are applied. However high skimmer voltages render the observation of the protonated molecule difficult.

In the Q-TOF the fragmentation of the $^{0,2}X^+$ $[M+H-120]^+$ was obtained by selecting directly this ion in the LC–APCI-MS spectra. The abundance of this precursor ion was 100% for all the flavonoid *C*-glycosides studied, when a skimmer voltage of 25 V was applied in the APCI source. The source CID fragmentation provides, in this case, pseudo MS^3 analyses (MS–MS on fragments generated by source CID).

In IT-MS, using the multiple-stage MS–MS possibilities of the trap, the MS–MS spectra of $^{0,2}X^+$ $[M+H-120]^+$ were recorded in the MS^3 mode ($[M+H]^+ \rightarrow {}^{0,2}X^+$ $[M+H-120]^+$) in order to be more specific. No skimmer voltage was applied in this case and MS^1 spectra exhibited almost exclusively protonated molecules. This mode was preferred to the source CID on mode, since it generated protonated molecules in higher abundances and allowed an unambiguous molecular mass assignment in the MS^1 scan.

The IT-MS^3 (433→313) of vitexin (**1**) and isovitexin (**2**) displayed mainly important losses of small molecules which were specific for each isomer. The IT-MS^3 of vitexin (**1**) displayed a single loss at *m/z* 284 (100%) $[^{0,2}X^+$-CHO$]^+$, while isovitexin (**2**) presented three main losses at *m/z* 295 (100%) $[^{0,2}X^+$-$H_2O]^+$, 284 (30%) $[^{0,2}X^+$-CHO$]^+$ and 267 (65%) $[^{0,2}X^+$-H_2O-CO$]^+$ (Table 2) (Fig. 3). These ions were present with all the CID energies tested (35–50%) and the fragmentation pattern was not affected by the change of excitation energy. Besides these ions, only a very weak $^{0,2}B^+$ ion (*m/z* 121) issued from the C-ring cleavage was observed for both isomers. Another weak $^{1,3}A^+$ ion (*m/z* 195) was recorded for the 6-*C* isomers. The other ions of low abundance could not be attributed to any characteristic fragments.

In the Q-TOF the fragmentation of the $^{0,2}X^+$ $[M+H-120]^+$ produced the same main fragments as those reported for the IT-MS^3 experiments. In this case, however, A and B fragment ions were re-

Table 2
CID-MS–MS of the $[M+H-120]^+$ ($^{0,2}X^+$) fragment ion of *C*-glycosidic flavonoids

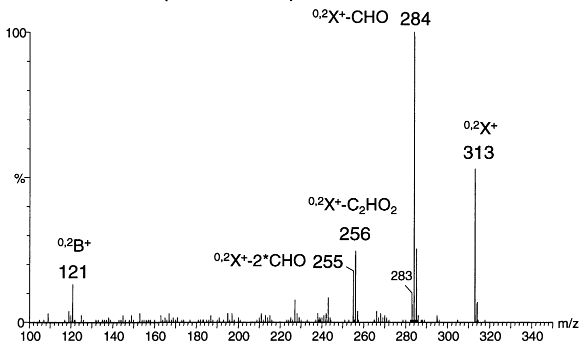
Expt.	CID	Prec.	Fragment ions (% RA)													
			$^{0,2}X^+$	$^{0,2}X^+$-H_2O	$^{0,2}X^+$-CO	$^{0,2}X^+$-CHO	$^{0,2}X^+$-CH_2O	$^{0,2}X^+$-CH_2O_2	$^{0,2}X^+$-C_2HO_2	$^{0,2}X^+$-2*CHO	$^{1,3}A^+$	$^{1,3}A^+$-H_2O	$^{1,3}A^+$-H_2O-CO	$^{0,2}B^+$	$^{1,3}B^+$	Base peak
(1) Vitexin (M_r 432)																
Q-TOF-MS–MS POS (433→313)	20 eV	313	313 (100)	–	–	284 (10)	–	–	–	–	–	–	–	–	–	313 (100)
	25		313 (100)	–	–	284 (40)	–	–	–	–	–	–	–	121 (5)	–	313 (100)
	30		313 (50)	–	–	284 (100)	283 (10)	–	256 (25)	255 (20)	–	–	–	121 (15)	–	284 (100)
	35		313 (15)	295 (2)	–	284 (100)	283 (30)	–	256 (80)	255 (60)	–	–	–	121 (25)	–	284 (100)
	40		313 (2)	–	–	284 (30)	283 (35)	–	256 (55)	255 (100)	–	177 (5)	149 (10)	121 (35)	–	255 (100)
IT-MS^3 POS (433→313)	50%	313	–	–	–	284 (100)	283 (5)	–	–	–	–	–	–	121 (5)	–	284 (100)
(5) Vitexin 2″-O-rhamnoside (M_r 578)																
IT-MS^4 POS (579→433→313)	50%	313	313 (25)	–	–	284 (100)	–	–	–	–	195 (2)	–	–	121 (8)	–	284 (100)
(2) Isovitexin (M_r 432)																
Q-TOF-MS–MS POS (433→313)	20 eV	313	313 (100)	295 (10)	–	–	–	267 (5)	–	–	–	–	–	–	–	313 (100)
	25		313 (100)	295 (30)	–	284 (25)	283 (5)	267 (30)	–	–	195 (2)	177 (5)	149 (5)	121 (25)	–	313 (100)
	30		313 (55)	295 (40)	–	284 (100)	283 (45)	267 (55)	256 (10)	255 (5)	195 (10)	177 (20)	149 (25)	121 (15)	119 (5)	284 (100)
	35		313 (10)	295 (15)	–	284 (65)	283 (100)	267 (30)	256 (15)	255 (25)	195 (5)	177 (25)	149 (55)	121 (30)	119 (10)	283 (100)
	40		–	295 (5)	–	284 (40)	283 (100)	267 (20)	256 (35)	255 (35)	–	177 (15)	149 (45)	121 (40)	119 (10)	283 (100)
IT-MS^3 POS (433→313)	50%	313	–	295 (100)	–	284 (30)	283 (10)	267 (65)	–	–	195 (5)	–	–	121 (5)	–	295 (100)
(3) Orientin (M_r 448)																
Q-TOF-MS–MS POS (449→329)	20 eV	329	329 (100)	–	–	300 (10)	–	–	–	–	–	–	–	–	–	329 (100)
	25		329 (100)	–	–	300 (45)	–	–	–	–	–	–	–	137 (5)	–	329 (100)
	30		329 (75)	–	–	300 (100)	299 (10)	–	272 (30)	271 (20)	–	–	–	137 (25)	–	300 (100)
	35		329 (30)	–	–	300 (100)	299 (40)	–	272 (85)	271 (85)	–	–	–	137 (40)	–	300 (100)
	40		–	–	–	300 (75)	299 (100)	–	272 (75)	271 (100)	–	–	–	137 (100)	–	299 (100)
IT-MS^3 POS (449→329)	50%	329	–	–	–	300 (100)	299 (5)	–	–	271 (10)	–	–	–	137 (5)	–	300 (100)
(4) Isoorientin (M_r 448)																
Q-TOF-MS–MS POS (449→329)	20 eV	329	329 (100)	311 (5)	–	300 (5)	299 (5)	283 (5)	–	–	–	–	149 (2)	–	–	329 (100)
	25		329 (100)	311 (15)	–	300 (30)	299 (10)	283 (25)	–	–	195 (5)	–	149 (5)	137 (5)	–	329 (100)
	30		329 (55)	311 (60)	–	300 (100)	299 (45)	283 (45)	–	271 (15)	–	177 (25)	149 (35)	137 (20)	–	300 (100)
	35		329 (15)	311 (35)	–	300 (100)	299 (50)	283 (30)	272 (35)	271 (25)	–	177 (25)	149 (65)	137 (20)	–	300 (100)
	40		–	–	–	300 (65)	299 (80)	–	272 (30)	271 (50)	–	177 (30)	149 (100)	137 (65)	–	149 (100)
IT-MS^3 POS (449→329)	50%	329	–	311 (100)	–	300 (35)	299 (15)	283 (90)	–	–	195 (10)	–	–	137 (5)	–	311 (100)

POS, positive mode; prec., precursor ion.

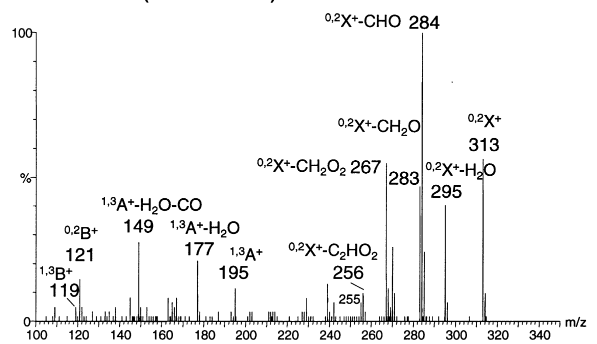
8-*C*-glycosidic flavonoid
Vitexin (**1**)

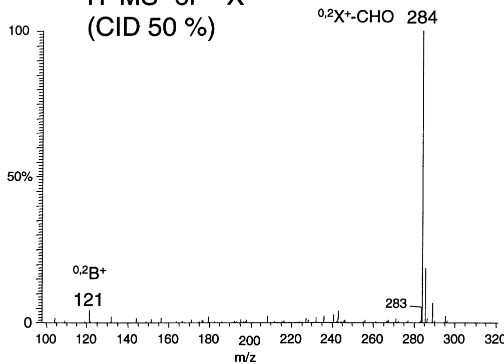
6-*C*-glycosidic flavonoid
Isovitexin (**2**)

Q-TOF-MS-MS of $^{0,2}X^+$ (CID 30 eV)



Q-TOF-MS-MS of $^{0,2}X^+$ (CID 30 eV)





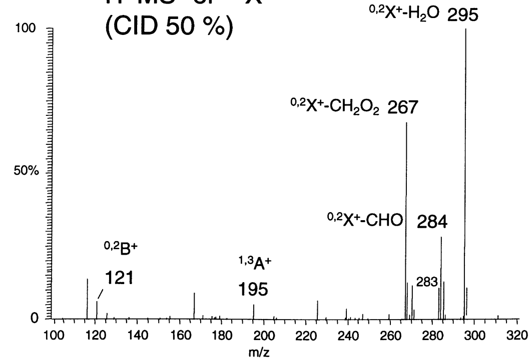


Fig. 3. Q-TOF-MS–MS (30 eV) and IT-MS^3 (CID 50%) (433 →313) spectra of the two *C*-glycoside isomers vitexin (**1**) and isovitexin (**2**), with $^{0,2}X^+$ ($[M+H-120]^+$) as precursor ion (*m/z* 313). For Q-TOF measurements, the precursor ion was generated by source CID (26 V).

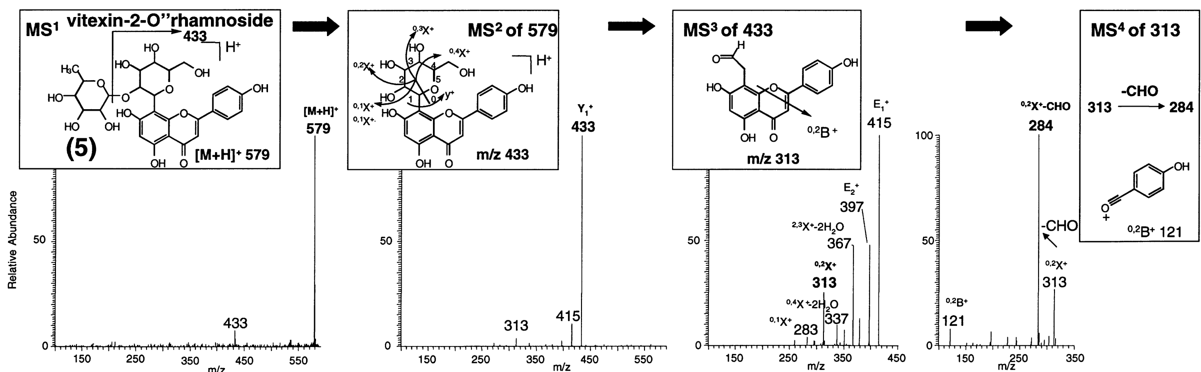


Fig. 4. IT-MS^n experiments (CID 50%) performed on vitexin-2-*O*″-rhamnoside (**5**).

Table 3
CID-MS–MS of *C*-glycosidic flavonoids in negative mode

Expt.	CID	Prec.		Fragment ions (% RA)									
				$[M-H]^-$	E_1^-	E_2^-	E_3^-	$^{0,3}X^-$	$^{0,2}X^-$	$^{0,2}X^-$-H_2O	$^{0,2}X^-$-CO	Other ion	Base peak
(1) Vitexin (M_r 432)													
IT-MS^2 NEG	50%	431	$[M-H]^-$	–	–	–	–	341 (10)	311 (100)	–	–	–	311 (100)
IT-MS^3 NEG (431→311)	50%	311	$^{0,2}X^-$						–	–	283 (100)	–	283 (100)
(2) Isovitexin (M_r 432)													
IT-MS^2 NEG	50%	431	$[M-H]^-$	–	413 (10)	395 (2)	–	341 (60)	311 (100)	–	–	–	311 (100)
IT-MS^3 NEG (431→311)	50%	311	$^{0,2}X^-$						–	293 (2)	283 (100)	–	283 (100)
(3) Orientin (M_r 448)													
IT-MS^2 NEG	50%	447	$[M-H]^-$	–	–	–	393 (2)	357 (65)	327 (100)	–	–	–	327 (100)
IT-MS^3 NEG (447→327)	50%	327	$^{0,2}X^-$						–	–	299 (100)	284 (15)	299 (100)
(4) Isoorientin (M_r 448)													
IT-MS^2 NEG	50%	447	$[M-H]^-$	–	429 (20)	411 (3)	–	357 (100)	327 (60)	–	–	–	357 (100)
IT-MS^3 NEG (447→327)	50%	327	$^{0,2}X^-$						–	309 (3)	299 (100)	284 (20)	299 (100)

NEG, negative mode; prec., precursor ion.

corded. As shown in Fig. 3, a clear difference in the C-ring cleavages between vitexin (**1**) and isovitexin (**2**) was observed. In the case of vitexin (**1**) only a $^{0,2}B^+$ ion was recorded (*m/z* 121) while for isovitexin (**2**) other additional ions $^{1,3}A^+$, $^{1,3}A^+$-H_2O, $^{1,3}A^+$-H_2O-CO at, respectively, *m/z* 195, 177 and 149 were observed (Table 2).

Contrary to IT, changes in CID energy in the Q-TOF (20–40 eV) induce noticeable differences in the abundance of MS–MS fragment ions. At low-energy (20 eV) fragments issued from C-ring cleavage were not distinguishable, while losses of small molecules were already observable. In these spectra the $^{0,2}X^+$ $[M+H\text{-}120]^+$ precursor ion represented still the base peak. By increasing the CID energy (30 eV), the $[^{0,2}X^+-CHO]^+$ became the most abundant fragment ion for both 6-*C* and 8-*C* isomers and the characteristic C-ring fragments of the 6-*C* isomers were clearly observable in the spectra. A further increase in CID energy (35 and 40 eV) still enhanced the abundance of the fragment ions of low mass, but the spectra became more complex and differences between both isomers were less clear. In particular, an ion at *m/z* 283 became more abundant than *m/z* 284 ($[^{0,2}X^+$-$CHO]^+$), probably due to an additional loss of a proton. A setting of 30 eV was finally selected for the best discrimination. Under these conditions the characteristic losses of small molecules and the C-ring cleavage ions were all recorded with a satisfactory abundance (Fig. 3).

In the case of orientin (**3**) and isorientin (**4**) characteristic fragment ions corresponding to those discussed above were also recorded in all the spectra (Table 2).

An IT-MS^n experiment performed on vitexin 2-O''-rhamnoside (**5**) gave similar results as those obtained for vitexin (**1**). In this case, MS^2 performed on the $[M+H]^+$ yielded Y_1^+ at *m/z* 433. The MS^3 spectrum of this latter fragment ion used as precursor (595 →433) was similar to the MS^2 of vitexin (**1**). MS^4 performed on the $^{0,2}X^+$ $[M+H\text{-}146\text{–}120]^+$ gave the same diagnostic ions as those observed for vitexin (**1**) (MS^3) proving that C-glycosylation occurred at position 8 in the case of **5** (Fig. 4).

3.4. Differentiation of 6-C- and 8-C-glycosidic flavonoids in negative CID-MS–MS

For all these *C*-glycosidic flavonoids, negative ion MS–MS CID experiments similar to those performed in the positive mode were realised, but only in the ion-trap system. All the compounds generated intense $[M-H]^-$ ions in the negative LC–APCI-MS spectra. The MS^3 spectra obtained using $^{0,2}X^-$ $[M-H\text{-}120]^-$ as precursor ion presented in this case only

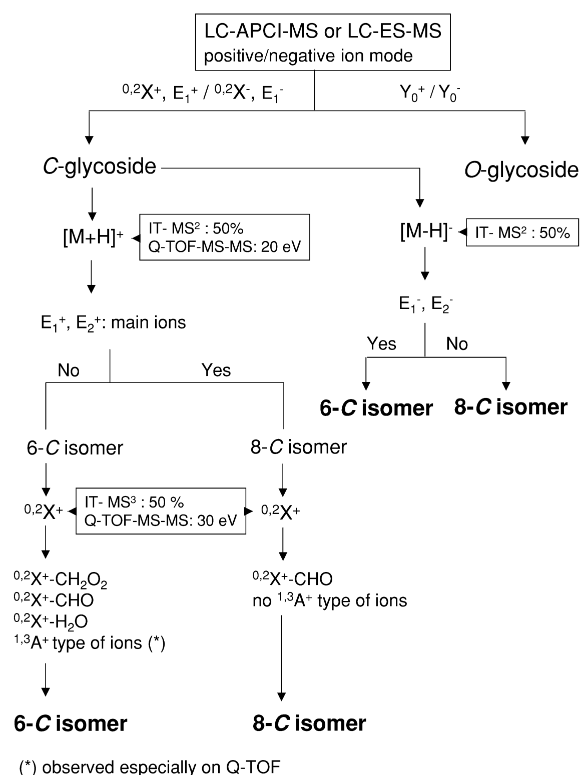


Fig. 5. Guidelines for the identification of isomeric mono-*C*-glycosidic flavonoids based on low-energy CID-MS–MS spectra.

a very stable ion corresponding to the loss of CO for all the isomers. This ion was almost unique in all the MS^3 spectra recorded and did not allow any discrimination between isomers. The MS^2 of $[M-H]^-$ however presented interesting differences. In the spectra of both isovitexin (**2**) and isoorientin (**4**) a clear loss of H_2O was observed (E_1^-), as well as a low abundant E_2^-. These losses were not recorded for the 8-*C* isomers vitexin (**1**) and orientin (**3**). Furthermore, for both 6-*C*-glycosidic flavonoids, the $^{0,3}X^-$ $[M-H\text{-}90]^-$ ions were more abundant than in the spectra of the corresponding 8-*C* isomers. These last results confirmed the observations made previously with negative FAB-CAD-MIKE experiments [31] (Table 3). Differences in ion abundances were also previously described in the negative ion LC–APCI-MS–MS spectra of vitexin and isovitexin obtained with high source and octapole voltage settings on the IT instrument [16]. Low-energy LC–CID-MS–MS performed on a triple quadrupole instrument with ion spray ionisation has been reported for the characterisation of various *C*-glycosidic flavonoids in the crude extract of *Passiflora incarnata* [18]. In this paper however the specific aspect of isomer differentiation has not been investigated.

4. Conclusion

As has been demonstrated, the low-energy LC–CID-MS–MS or LC–MS^n spectra of *C*-glycosidic flavonoids differ from those obtained from high-energy measurements, and the diagnostic ions used for the discrimination between 6-*C* and 8-*C* isomers in the high collision energy mode can not be used on IT or Q-TOF instruments.

The MS–MS spectra of the protonated molecules $[M+H]^+$ yielded different fragmentation profiles but the differences in ion abundance between 6-*C* and 8-*C* isomers do not give enough information for an unambiguous identification since no diagnostic ion only observed in one of the isomer product ion spectra was recorded. In the negative ion mode, a loss of H_2O is specific to 6-*C* glycosides and can be used for discrimination. The most striking differences however appear in the MS–MS spectra recorded by selecting the $^{0,2}X^+$ ($[M+H\text{-}120]^+$) as precursor ion. In this case, the patterns of losses of small molecules are clearly different between isomers. These differences are observed with both IT and Q-TOF instruments. In Q-TOF however, C-ring cleavage fragment ions, difficult to observe in IT, are also specific for the distinction between isomers. An overview of all these observations is given in Fig. 5. These guidelines can be used for the dereplication of such compounds in crude plant extracts.

In the general dereplication process, molecular mass information and informative product ion spectra should be ideally obtained for all constituents in one single LC–MS run. Based on the observations made for *C*-glycosidic flavonoids, one-way to obtain all the desired information would be to use the scan switchable source CID capability of the LC–MS instruments and perform data dependent MS–MS on the $^{0,2}X^+$ ($[M+H\text{-}120]^+$). This approach is mandatory on tandem MS–MS instruments such as Q-TOF and allows the recording of pseudo MS^3 spectra. These analyses can be performed with multiple cone voltage experiments in a single acquisition on fast scanning instruments such as Q-TOF. In IT, the same

strategy can be applied and in this case only scan-dependent MS^2 experiments need to be performed on the $^{0,2}X^+$ ($[M+H-120]^+$ obtained by source CID fragmentation. As discussed the MS^3 of the $^{0,2}X^+$ ($[M+H-120]^+$ obtained by MS^2 of the protonated molecules can also be realised. The specificity of multiple stage experiments may represent an advantage for the analysis of complex mixture containing co-eluting compounds. As shown, MS^n also allows the recording of very clear MS–MS spectra of *C*-diglycosidic flavonoids where $^{0,2}X^+$ can be selected as precursor ion, even at the MS^3 level, for precise discrimination of the *C*-glycosidic isomers.

As the $^{0,2}X^+$ ion abundance may be compound-dependent, it would be an advantage to use an algorithm (precursor ion-120 u) for the automatic selection of $^{0,2}X^+$ in scan-dependent MS–MS measurements. This feature was not available on the instruments used for this study.

The methods presented have been tested in preliminary experiments on various plant extracts either on Q-TOF- or IT-MS. The results were comparable to those described here for pure standards but the selection of the putative precursor ions had to be made manually based on a preliminary LC–MS analysis of the extracts. Further optimisation of the LC–MS–MS automation process is underway, in view of its application in dereplication of flavonoids and related compounds in crude plant extracts. Based on this study instrument-specific CID-MS–MS libraries will be created, together with LC–UV-DAD databases.

Acknowledgements

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