Evaluation of Q-TOF-MS/MS and multiple stage IT-MS^n for the dereplication of flavonoids and related compounds in crude plant extracts

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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 CID MS/MS spectra of ubiquitous flavonoids and related plant constituents have been systematically studied using hybrid quadrupole time of flight (Q-TOF) and ion trap (IT) mass analysers under various CID energy conditions. The results demonstrate that, if for hydroxylated flavonoids the CID MS/MS spectra generated on both instruments are similar, for partially methoxylated derivatives, important differences are observed hampering the creation of MS/MS databases exchangeable between instruments. Generally, fragments issued from C-ring cleavage, corresponding to those classically reported, were recorded but they were more easily observed on a Q-TOF instrument while losses of small molecules were favoured in IT-MS. MS/MS spectra recorded in the positive ion mode were more informative than those obtained from negative ions. For the assignment of flavonoids product ion spectra, on-line accurate mass measurement of all MS/MS fragments was obtained on the Q-TOF, while the multiple stage MS^n capability of the ion trap was used to prove fragmentation pathways.

Introduction

The introduction of LC/MS for 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]. Dereplication refers to a process where known compounds are identified on-line to avoid their re-isolation [4,5].

Electrospray (ES) [6] or Atmospheric Pressure Chemical Ionisation (APCI) [7], are soft ionisation techniques which generate mainly molecular ions for relatively small plant metabolites such as flavonoids. Molecular weight information alone is however not sufficient for the on-line structure determination of natural products and fragment information is necessary for partial on-line identification or for dereplication of known constituents.

In order to generate fragment ions in LC/MS, collision induced dissociation (CID) MS/MS experiments have to be performed. Low energy CID MS/MS spectra can be generated by different means: 1) collisions can be performed in the API source by adjusting ion source lens potentials without parent ion isolation to yield in-source CID spectra; 2) selected precursor ions can be specifically isolated on

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triple-quadrupole systems, on double-focussing magnetic sector instrument, on hybrid quadrupole time of flight instruments or on ion trap analysers to generate CID MS/MS or multiple stage MS^n spectra [8].

In this paper the CID MS/MS and MS^n spectra of various flavonoid aglycones have been evaluated on two types of MS instruments, an hybrid quadrupole time of flight (Q-TOF) and an ion trap (IT). These two instruments were chosen because the CID process in beam and trap systems is not generated in the same way. 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 process. 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 [8,9]. The CID MS/MS spectra acquired by these different low energy collision regimes are compared in this paper in order to evaluate if the same type of structural information is generated.

Flavonoids have been chosen for this study because they have long been recognised as one of the largest and most widespread classes of plant constituents, occurring throughout the higher and lower plants [10]. LC/MS/MS methods for their dereplication in crude plant extracts are thus very important in phytochemical analyses. Different papers have dealt with mass fragmentation pathways of the aglycones under electron ionisation [11]. Recently, fragmentation pathways of flavone and flavonols by fast atom bombardment CID MS/MS have also been documented [12-15]. Fragment ions provide important structural information for flavonoids and are used to establish the distribution of the substituents between the A- and B-rings. A careful study of fragmentation patterns 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 [14].

In complement to LC/MS/MS other hyphenated techniques such as LC/UV-DAD, with post-column addition of UV shift reagents, [16] or LC/NMR have proven to be very valuable for the screening of these constituents [17,18].

Since several papers have already described the fragmentation of flavonoids in static conditions (*i.e.* FAB experiments) we report here on the CID/MS/MS or MS^n spectra recorded in on-line LC conditions for various type of flavonoid aglycones. Both ES and APCI, in positive and negative ion mode, have been used as ionisation methods. An evaluation of the optimum LC/MS/MS strategy is given in view of its application for the routine dereplication of these widespread natural products directly in crude plant extracts. The fragments recorded have been annotated according to the nomenclature adopted by Domon and Costello [19], Claeys and co-workers [13,14].

Experimental

Materials

Pure flavonoids samples (**1-6**, **8**, **10, 14**) were purchased from Roth (Reinach, Switzerland) or were isolated from plants (**7**, **9**, **11-13**) at the Institute of Pharmacognosy and Phytochemistry of Lausanne.

LC conditions

10 µl of the pure flavonoid solutions (1 mg/ml) were directly injected on a C-18 Nova-Pak Guard-Pak pre-column (Waters, Milford, MA, USA) using an isocratic MeCN-H_2O solvent system (80:20) containing 0.5 % acetic acid at a flow rate of 0.3 ml/min. Under these conditions the polyphenols eluted in less than 3 minutes in the API source.

IT-MS conditions

IT-MS^n experiments were performed on a LCQ ion trap mass spectrometer equipped with ES or APCI interfaces (Finnigan MAT, San Jose, CA, USA). The HPLC consisted of an HP-1100 system 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, 450 °C; transfer capillary temperature, 150 °C; cone voltage 25 V. Electrospray conditions: spray voltage, 4.5 kV; capillary temperature, 200 °C. On both interfaces nitrogen was used as sheath gas**.**

Q-TOF-MS conditions

Q-TOF-MS/MS experiments were conducted on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK). The APCI conditions were as follows: Corona pin voltage, 5 V, Vaporiser, 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 ($[M + H]^+$: 311.0814) (Aldrich, Buchs, Switzerland) was added post-column.

CID MS/MS conditions

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

On 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 Da. This method was applied at different energy levels (35, 40, 50 and 60 %) in both positive and negative ion modes in separate LC runs. A complementary experiment with wideband excitation was also performed at an energy level of 60 %. The little amount of helium standing in the trap was used as collision gas. Energy levels on the Finnigan IT-MS are given in % and not in eV

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since the voltages applied vary according to the *m/z* value of the precursor ion. There is no direct correlation available from the manufacturer between this scale of CID energies in % and a scale in eV. In our experience, a CID energy level of 40 % corresponds roughly to 25 eV.

On the Q-TOF-MS/MS scan dependent MS/MS autoswitch experiments were performed sequentially on the three main ions recorded by LC/APCI-MS. The combination of quadrupole ion-selectivity with the full scan sensitivity of the TOF analyser allowed the on-line recording of autoswitch MS/MS experiments at five different CID energies levels (20, 25, 30, 35 and 40 eV) during a three second 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. For accurate mass measurements in MS/MS the $[M + H]^+$ precursor ion or a characteristic fragment ion of the compound of interest was used as lock mass.

The energies range chosen (35-60 %, IT-MS and 20-40 eV, Q-TOF) covered all the range of setting where CID MS/MS was recordable in practice on both instruments. At low energy (35 % IT, 20 eV Q-TOF) practically 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.

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 CID MS/MS spectra of different 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. Polyphenols such as flavone, flavonol, flavanone, flavane, flavanol aglycones have been analysed using both LC/APCI-MS and LC/ES-MS ionisation methods. For most of the experiments however, LC/APCI-MS has been preferred to LC/ES-MS because it was found more robust. Both techniques, however, yielded protonated and deprotonated molecules in positive and negative ion modes respectively for most of the samples studied.

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. The CID energy can be optimised for the enhancement of certain types of ions in a given sample but the goal here was to define conditions that are general for all type of flavonoids and that will yield all the structural information needed in one single LC run for dereplication purposes. In order to have a rather general view of what is typically occurring in a low energy collision regime, MS/MS spectra of flavonoids generated at different energies on a hybrid quadrupole time of flight system (Q-TOF) and on a trap system (IT-MS) are compared. The range of CID energies on both instruments was selected between cases were almost no CID of the precursor occurs and those were fragment ions were too weak for valuable interpretation, due to the excess of energy applied (see experimental).

The discussion about the optimisation of the collision energy is mainly based here on the observation of A and B ions characteristic for the C-ring cleavage of the flavonoids since these are the most interesting fragments for their on-line identification. The other fragments issued from the losses of small molecules (carbon monoxyde, water or ketene) that also occur [13] are not discussed in detail here. A summary of all the A and B fragment ions observed for the flavonoids is presented in figure 1.

Flavones

For this study three flavone aglycones apigenin (**1**), luteolin (**2**) and chrysoeriol (**3**) were selected (Tab. I). For the hydroxylated flavones **1** and **2**, on both instruments, the CID MS/MS spectra of the protonated molecules $[M + H]^+$ generated in the positive ion mode yielded the A and B series of ions characteristic of the C-ring cleavage, namely $^{1,3}A^+$, $^{1,3}B^+$, $^{0,2}B^+$, $^{0,4}B^+$ and $^{0,4}B^+$-H_2O. On the IT-MS, no significant cleavage was observed at 35 % energy and only a weak $^{1,3}A^+$ was observed at *m/z* 153 (4 %). Increase of the CID energy yielded however the complete series of A and B ions for these two flavones with $^{1,3}A^+$ being the most abundant ion of the spectra. At a 50 % CID energy level, the MS/MS spectra recorded on the $[M + H]^+$ ions generated either by ES or APCI ionisation method were very similar indicating that the energy deposition on the precursor ion by both ionisation methods did not affect significantly the product ion spectra.

Q-TOF MS/MS spectra recorded for these two compounds were similar to those obtained on the IT-MS. For example, the spectrum recorded at 40 % CID in IT-MS^2 for luteolin (**2**) matched well that recorded at 25 eV CID on the Q-TOF (Fig. 2).

For chrysoeriol (**3**), an important neutral loss of CH_3· was prevalent in all the spectra at all the different energies tested. In IT-MS^2 no A or B fragment ion could be observed even when high CID energies were applied, the $[M + H - CH_3]^{+\cdot}$ (*m/z* 286) being the only ion observable in the spectra. MS^3 (303->286) performed automatically yielded however the $^{1,3}A^+$ ion at *m/z* 153.

On the Q-TOF analyser however the $^{1,3}A^+$ was observed directly in the CID MS/MS spectra recorded and its abundance was increasing proportionally with the CID energy used. $^{1,3}B^+$ at *m/z* 149 was weak but was clearly recorded at a CID energy of 30 eV. The abundance of this ion declined when higher energies were applied.

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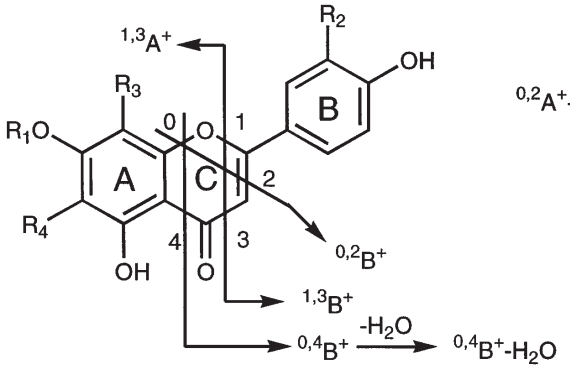
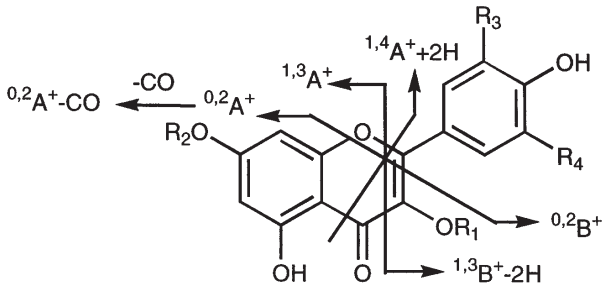
Flavones



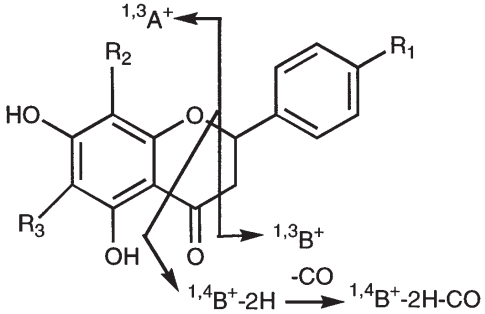
Cpds	R_1	R_2	R_3	R_4
1	H	H	H	H
2	H	OH	H	H
3	H	OCH_3	H	H

Flavonols



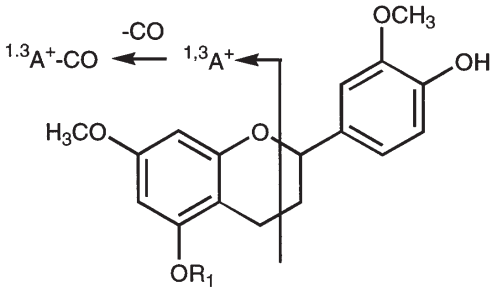
Cpds	R_1	R_2	R_3	R_4
4	H	H	OH	H
5	H	H	H	H
6	H	H	OH	OH
7	CH_3	CH_3	H	H
8	H	H	OCH_3	H
9	CH_3	H	OCH_3	H

Flavanones



Cpds	R_1	R_2	R_3
10	OH	H	H
11	H	CH_3	CH_3

Flavanes



Cpds	R_1
12	H
13	CH_3

Flavanols

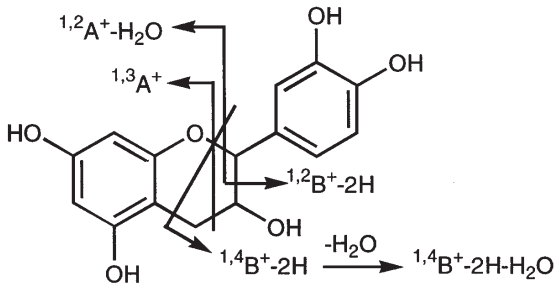


Figure 1. Structures and fragmentation pathways of the flavonoids studied.

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Table I. CID MS/MS of flavones.

Exp.	*CID*[b]	*Prec.*	*Fragment ions (% RA)*					
			$^{1,3}A^+$	*$^{1,3}B^+$*	*$^{0,2}B^+$*	*$^{0,4}B^+$*	*$^{0,4}B^+$-H_2O*	*Main ion*
(1) Apigenin (MW 270)								
QTOF MS-MS POS	25	271	153 (25)	119 (5)	121 (2)	163 (2)	145 (2)	271 (100)
	30		153 (85)	119 (20)	121 (10)	163 (10)	145 (10)	271 (100)
	35		153 (100)	119 (35)	121 (20)	163 (10)	145 (15)	153 (100)
IT POS MS^2	35	271	153 (4)	-	-	-	-	271 (100)
	40		153 (35)	119 (5)	121 (3)	-	145 (5)	271 (100)
	50		153 (100)	119 (15)	121 (10)	163 (5)	145 (15)	153 (100)
	60 w		153 (100)	119 (20)	121 (10)	163 (3)	-	153 (100)
IT NEG MS^{2a}	50	269	151 (20)	117 (10)	-	-	-	225 (100)
(2) Luteolin (MW 286)								
QTOF MS-MS POS	25	287	153 (20)	135 (5)	137 (2)	179 (1)	161 (2)	287 (100)
	30		153 (70)	135 (25)	137 (5)	179 (5)	161 (7)	287 (100)
	35		153 (100)	135 (30)	137 (15)	179 (5)	161 (15)	153 (100)
IT POS MS^2	35	287	153 (3)	-	-	-	-	287 (100)
	40		153 (20)	135 (3)	137 (1)	-	161 (3)	287 (100)
	50		153 (100)	135(20)	137 (5)	179 (10)	161 (25)	153 (100)
	60 w		153 (65)	135 (15)	137 (4)	179 (10)	161 (17)	130 (100)
IT NEG MS^{2a}	50	285	151 (25)	133 (10)	-	-	-	241 (100)
(3) Chrysoeriol (MW 300)								
QTOF MS-MS POS	25	301	153 (1)	149 (1)	-	-	-	286 (100)
	30		153 (3)	149 (1)	-	-	-	286 (100)
	35		153 (10)	-	-	-	-	258 (100)
IT POS MS^2	35	301	-	-	-	-	-	286 (100)
	40		-	-	-	-	-	286 (100)
	50		-	-	-	-	-	286 (100)
	60 w		153 (1)	-	-		-	142 (100)

[a] for reasons of convenience the negative ions have not been mentioned in the headers.
[b] CID energies are indicated in eV (QTOF) or in % (IT); w indicates that wideband excitation was activated.
Prec.: Precursor ion.

Flavonols

The CID MS/MS spectra of the following flavonol aglycones were compared (Tab. II): quercetin (**4**), kaempferol (**5**), myricetin (**6**), 3,7-dimethoxy-kaempferol (**7**) isorhamnetin (**8**), 3-methoxy-isorhamnetin (**9**).

The hydroxylated flavonols (**4-6**) exhibited MS/MS spectra where their main A and B fragments were observable. As in the case of flavones **1** and **2**, $^{1,3}A^+$ and $^{0,2}B^+$ ions were present. Other complementary fragment ions were also recorded $^{1,3}B^+$-2H, $^{1,4}A^+$+2H, $^{0,2}A^+$ and $^{0,2}A^+$-CO (for fragmentation mechanisms see [13]). Unlike for **1** and **2**, A and B fragment ions were already observable at a CID energy of 35 % in IT-MS but they were more clearly distinguished at a CID level of 50 %. The use of higher CID energies resulted only in a loss of the overall sensitivity. As for the hydroxylated flavones, the Q-TOF-MS/MS spectra (CID 25 eV) were rather similar to those obtained by IT-MS^2 (CID 40 %). However, the abundance of the parent ion for all these compounds was radically different: in the Q-TOF-MS/MS spectra (CID 25 eV) the $[M + H]^+$ was the main ion (RA 100 %), while it was not observed anymore in IT-MS^2 (CID 40 %).

As this was already observed for flavones, methoxylated flavonols (**7-9**) behave differently when compared to the hydroxylated derivatives **4-6**. For all these partially methoxylated flavonols the loss of a radical $CH_3^{\cdot}$ was prevalent generating abundant $[M + H - 15]^{+\cdot}$ ions. For isorhamnetin (**8**) the loss of $CH_3^{\cdot}$ at *m/z* 302 represented the main ion of the IT-MS^2 spectra in the complete range of CID energies applied. As shown in figure 3A and 3B, relatively weak A and B fragment ions (< 5 %) were observed. Application

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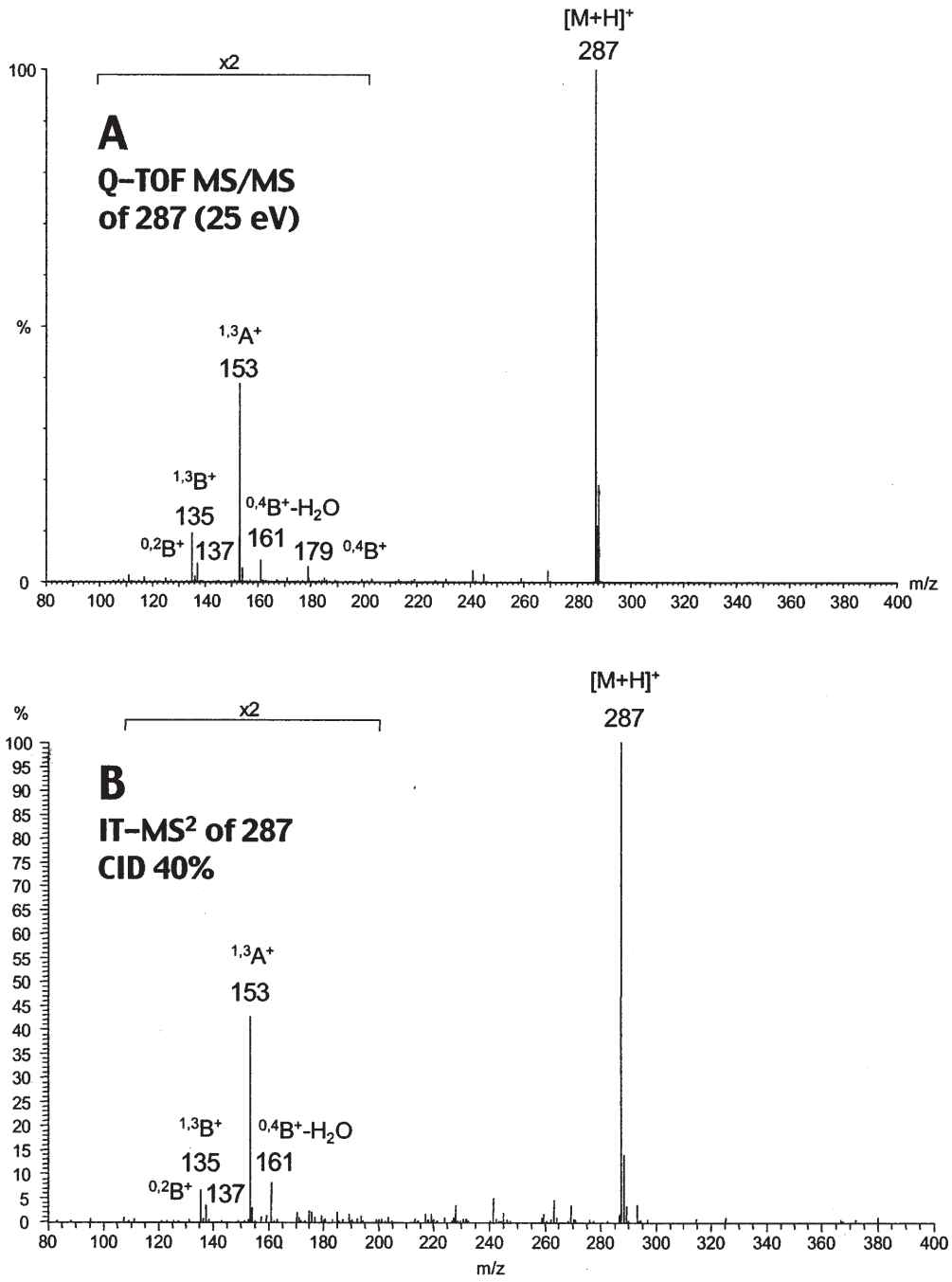

Figure 2. Comparison of A) Q-TOF MS/MS (25 ev) and B) IT-MS² (CID 40 %) spectra of luteolin (2).

of higher CID energies did not increase the abundance of A and B ions but the loss of hydroxyl from *m/z* 302 was favoured. Contrary to IT-MS², on the Q-TOF, increase in CID energy yielded more abundant $^{1,3}A^+$ ions. As shown the MS/MS spectrum of isorhamnetin (**8**) recorded at relatively high CID energy on the Q-TOF (35 eV) (Fig. 3F) differs radically from that obtained in IT-MS (CID 50 %) (Fig. 3B).

These differences of fragmentation pattern can be explained because consecutive reactions are possible in beam instruments while these are not usually observed in trap systems unless important excess of energy is deposited on the precursor ions. For isorhamnetin (**8**), $^{1,3}A^+$ can also be generated from the fragmentation of $[M + H - 15]^{+\cdot}$ as this was proved by IT-MS³ (317->302) (Fig. 3C). In this case, however, information on the B ring was lost since the fragments recorded were issued from a precursor ion without a methoxy group on the B ring. As shown in figure 3F the Q-TOF-MS/MS spectrum obtained for isorhamnetin (**8**) at a high collision energy represent a mixture of ions recorded on both IT-MS² and MS³ spectra.

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Table II. CID MS/MS of flavonols.

Exp.	CID	Prec.	Fragment ions (% RA)						
			$^{1,3}A^+$	$^{1,4}A^+$+2H	$^{0,2}A^+$	$^{0,2}A^+$-CO	$^{1,3}B^+$-2H	$^{0,2}B^+$	Main ion
(4) Quercetin (MW 302)									
QTOF MS-MS POS	20	303	153 (5)	-	-	137 (4)	-	137 (4)	303 (100)
IT POS MS^2	35	303	153 (2)	-	165 (5)	137 (5)	-	137 (5)	257 (15)
	50		153 (3)	127 (5)	165 (20)	137 (10)	149 (5)	137 (10)	257 (100)
	60 w		153 (10)	-	165 (40)	137 (15)	149 (10)	137 (15)	257 (100)
IT NEG MS^2	50	301	151 (60)	-	-	-	-	-	179 (100)
(5) Kaempferol (MW 286)									
QTOF MS-MS POS	25	287	153 (15)	127 (2)	165 (10)	137 (3)	133 (2)	121 (15)	287 (100)
	30		153 (100)	-	165 (45)	137 (20)	133 (10)	121 (35)	153 (100)
	35		153 (100)	-	165 (30)	137 (30)	133 (10)	121 (40)	153 (100)
IT POS MS^2	35	287	153 (5)	-	165 (10)	-	133 (5)	121 (5)	287 (100)
	40		153 (30)	-	165 (45)	137 (3)	133 (13)	121 (20)	241 (100)
	50		153 (45)	-	165 (55)	137 (5)	133 (20)	121 (35)	213 (100)
	60 w		153 (50)	127 (7)	165 (55)	-	133 (25)	121 (50)	258 (100)
IT NEG MS^2	50	285	151 (50)	-	163 (25)	135 (5)	-	-	229 (100)
(6) Myricetin (MW 318)									
QTOF MS-MS POS	20	319	153 (4)	-	165 (4)	-	165 (4)	153 (4)	319 (100)
	25		153 (15)	-	165 (10)	137 (10)	165 (10)	153 (15)	319 (100)
	30		153 (100)	127 (20)	165 (25)	137 (20)	165 (25)	153 (100)	153 (100)
IT POS MS^2	35	319	153 (20)	127 (4)	165 (30)	137 (3)	165 (30)	153 (20)	273 (100)
	40		153 (25)	127 (1)	165 (25)	137 (4)	165 (25)	153 (25)	273 (100)
	50		153 (15)	127 (2)	165 (25)	137 (5)	165 (25)	153 (15)	273 (100)
	60 w		153 (15)	127 (1)	165 (17)	137 (5)	165 (17)	153 (15)	273 (100)
IT NEG MS^2	50	317	151 (35)	-	-	-	-	151 (35)	179 (100)
(7) Kaempferol 3,7-dimethoxy (MW 314)									
QTOF MS-MS POS	25	315	-	-	-	-	121 (2)	-	300 (100)
	30		-	-	-	-	121 (10)	-	300 (100)
	35		-	-	-	-	121 (30)	137 (5)	299 (100)
IT POS MS^2	35	315	-	-	-	-	-	-	300 (100)
	50		-	-	-	-	-	-	300 (100)
	60 w		-	-	-	-	121 (5)	-	272 (100)
IT POS MS^3	50	300	-	-	-	-	121 (5)	137 (5)	272 (100)
IT NEG MS^2	50	313	-	-	-	-	-	-	298 (100)
IT NEG MS^3	50	298	-	-	-	-	-	-	283 (100)
(8) Isorhamnetin (MW 316)									
QTOF MS-MS POS	20	317	153 (2)	-	165 (2)	-	-	151 (1)	317 (100)
	25		153 (15)	-	165 (5)	137 (1)	-	151 (5)	317 (100)
	30		153 (85)	-	165 (10)	137 (5)	-	151 (10)	302 (100)
IT POS MS^2	35	317	153 (2)	-	165 (5)	-	163 (2)	151 (3)	302 (100)
	40		153 (2)	-	165 (5)	-	163 (1)	151 (2)	302 (100)
	50		153 (2)	-	165 (3)	-	-	151 (2)	302 (100)
	60 w		153 (5)	-	165 (4)	137 (2)	163 (2)	151 (3)	285 (100)
IT POS MS^3	50	302	153 (15)	-				-	274 (100)
IT NEG MS^2	50	315	-	-	-	-	-	-	300 (100)
IT NEG MS^3	50	300	151 (70)	-	-	-	-	-	271 (100)
(9) Isorhamnetin 3-methoxy (MW 330)									
QTOF MS-MS POS	25	331	153 (1)	-	-	-	-	151 (1)	316 (100)
	30		153 (4)	-	165 (1)	137 (1)	-	151 (2)	316 (100)
	35		153 (10)	-	165 (2)	137 (2)	-	151 (5)	315 (100)
IT POS MS^2	35	331	-	-	-	-	-	-	316 (100)
	50		-	-	-	-	-	-	316 (100)
	60 w		153 (2)	-	-	-	-	-	301 (100)
IT POS MS^3	50	316	-	-	-	-	-	-	301 (100)
IT NEG MS^2	50	329	-	-	-	-	-	-	314 (100)
IT NEG MS^3	50	314	-	-	-	-	-	-	285 (100)

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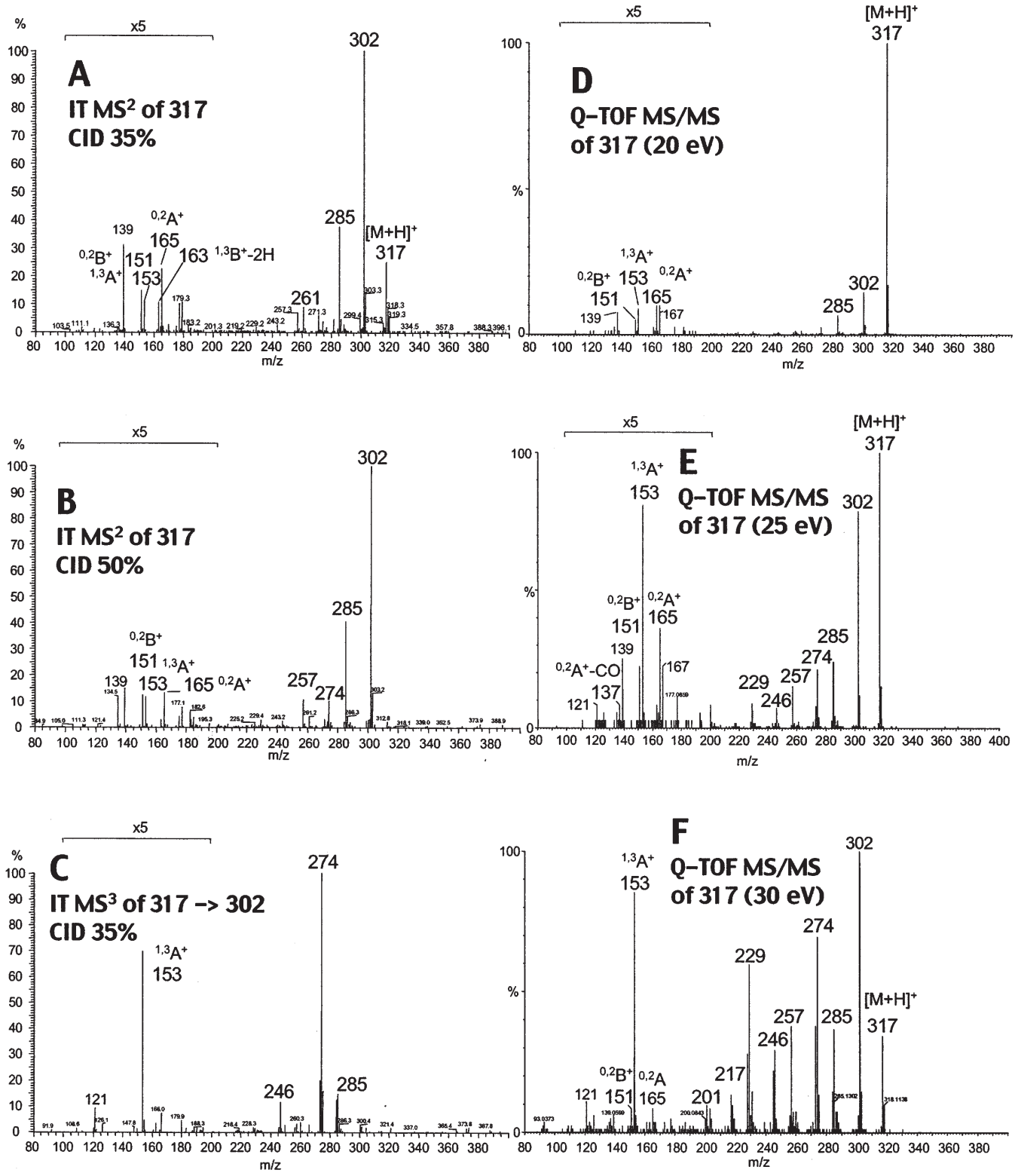

Figure 3. Comparison of different CID MS/MS spectra of isorhamnetin (8). A) IT-MS^2 (CID 35 %), B) IT-MS^2 (CID 50 %), C) IT MS^3 (317 -> 302) (CID 35 %) D) Q-TOF-MS/MS (20 ev) E) Q-TOF-MS/MS (25 ev), F) Q-TOF-MS/MS (30 ev).

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The same type of behaviour was observed for 3-methoxy-isorhamnetin (**9**). In this case, only losses of small molecules were observed in IT-MS^2 in the complete range of CID energies and no $^{1,3}A^+$ and $^{0,2}B^+$ ions could be detected. The C-ring cleavage ions were however recorded on the Q-TOF. In IT-MS^2 the only way to observe a weak $^{1,3}A^+$ ion was to apply wideband excitation with a CID energy level of 60 %. With this MS/MS mode, the ions formed in a window of –20 Da below the precursor ion are excited sequentially and may generate fragments. This feature can be used when important losses of small molecules are observed. For **9** the MS^3 performed on $[M + H – CH_3]^{+\cdot}$ did not yield more information and an abundant ion due to the loss of a second $CH_3^{\cdot}$ was recorded at *m/z* 301.

The same experiments were also performed on a 3,7-dimethoxykaempferol derivative (**7**). In this case also a facile loss of $CH_3^{\cdot}$ was observed on both IT- and Q-TOF-MS. The $^{0,2}B^+$ ion was recorded at *m/z* 121 on Q-TOF but could not be clearly discerned on IT-MS. No ion at *m/z* 167, which could be attributed to $^{1,3}A^+$, was recorded, instead an ion at *m/z* 150 ($C_8H_6O_3$ 150.0349) was present. This ion seems to be specific for this methoxylated flavonol and it was also clearly visible in the MS^3 of the $[M + H – CH_3]^{+\cdot}$.

Flavanones

Two flavanones (**10** and **11**) have been investigated in order to compare their CID MS/MS spectra to those of the flavonoids previously described (Tab. III). In this case, $^{1,3}A^+$ was the most abundant ion of the CID MS/MS spectra on both IT and Q-TOF even when the lowest CID energies were used. Together with $^{1,3}A^+$, corresponding $^{1,4}B^+$-2H and $^{1,4}B^+$-2H-CO were observed at *m/z* 119 (119.0497 C_8H_7O) and *m/z* 147 (147.0446 $C_9H_7O_2$) in the case of naringenin (**10**). The identity of these fragments was precisely assigned by accurate mass measurement on the Q-TOF using the precursor ion as lock mass. According to these results it appears that the cleavage of the C-ring in flavanone is facile and requires only weak CID energies to be observed.

Flavanes

Two different partially methoxylated flavanes (**12** and **13**) were studied (Tab. IV). As for the flavanones, the $^{1,3}A^+$ ion was prevalent in the CID MS/MS spectra recorded on both instruments. This ion was even already clearly observable in the LC/APCI-MS spectra of **12** and **13** but this was not the case for the flavanones **9** and **10**. Beside $^{1,3}A^+$, other ions were observed at *m/z* 121 and *m/z* 125 in the case of **12** for example. On IT-MS, MS^3 (303->153) proved that these two ions resulted from subsequent fragmentation of $^{1,3}A^+$ and not from the C-ring cleavage of **12**. Q-TOF accurate mass measurements allowed the identification of these fragments as $C_7H_9O_2$ (*m/z* 125.0603) and $C_7H_5O_2$ (*m/z* 121.0290). *m/z* 125 was thus assigned to $^{1,3}A^+$-CO and *m/z* 121 was probably issued from a rearrangement of this ion. Similar type of ions were also recorded for **13**. It is also interesting to notice that the two flavanes studied were partially methoxylated and that no loss of $CH_3^{\cdot}$ was observed for these compounds, unlike this was the case for the partially methoxylated flavones and flavonols (**3**, **7**-**9**). These observations indicate that the presence of an α,β unsaturated ketone in the C-ring is necessary for the elimination of CH_3 radicals and confirm also the proposed mechanism of elimination of $CH_3^{\cdot}$ in flavones such as acacetin [13].

Table III. CID MS/MS of flavanones.

Exp.	*CID*	*Prec.*	*Fragment ions (% RA)*				
			$^{1,3}A^+$	*$^{1,3}B^+$*	*$^{1,4}B^+$-2H*	*$^{1,4}B^+$-2H-CO*	*Main ion*
(10) Naringenin (MW 272)							
QTOF MS-MS POS	20	273	153 (100)	-	147 (25)	119 (1)	153 (100)
	25		153 (100)	-	147 (25)	119 (15)	153 (100)
	30		153 (100)	-	147 (15)	119 (10)	153 (100)
IT NEG MS^2	50	271	151 (100)	119 (10)	-	-	151 (100)
(11) Pinocembrin 6,8-dimethyl (MW 284)							
QTOF MS-MS POS	20	285	181 (100)	-	131(50)	-	285 (100)
	25		181 (100)	-	131 (35)	103 (5)	181 (100)
	30		181 (100)	-	131 (30)	103 (10)	181 (100)
IT POS MS^2	35	285	181 (100)	-	131 (60)	103 (10)	181 (100)
	50		181 (100)	-	131 (45)	103 (5)	181 (100)
	60w		181 (100)	-	131 (45)	103 (5)	181 (100)
IT NEG MS^2	50	283	179 (20)	-	-	-	241 (100)

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Table IV. CID MS/MS of flavanes.

Exp.	CID	Prec.	Fragment ions (% RA)		
			$^{1,3}A^+$	$^{1,3}A^+$-CO	Main ion
(12) 4',5-Dihydroxy-3',7-dimethoxyflavan (MW 302)					
QTOF MS-MS POS	25	303	153 (100)	125 (25)	153 (100)
	30		153 (100)	125 (10)	153 (100)
	35		153 (100)	125 (5)	153 (100)
IT POS MS^2	35	303	153 (100)	-	153 (100)
	50		153 (100)	-	153 (100)
	60 w		153 (100)	-	153 (100)
IT POS MS^3	50	153	153 (15)	125 (50)	121 (100)
(13) 4'-hydroxy-3',5,7-trimethoxyflavan (MW 316)					
QTOF MS-MS POS	25	317	167 (100)	139 (10)	167 (100)
	30		167 (100)	139 (25)	167 (100)
	35		167 (100)	139 (45)	167 (100)
IT POS MS^2	35	317	167 (100)	-	167 (100)
	50		167 (100)	139 (1)	167 (100)
	60 w		167 (100)	139 (1)	167 (100)
IT POS MS^3	50	167	-	139 (100)	139 (100)

Flavanols

The CID MS/MS of catechin (**14**) was studied in order to complete this study (Tab. V). In this case, the series of all A and B fragment ions ($^{1,3}A^+$, $^{1,2}B^+$-2H, $^{1,2}A^+$-H_2O and $^{1,4}B^+$-2H) were observed on both instruments. Ions at *m/z* 147 (147.0430 $C_9H_7O_2$) and 119 (119.0495 C_8H_7O) were also clearly discernible in the product ion spectra, they may result from a water loss and a subsequent carbon monoxide loss from the $^{1,4}B^+$-2H ion (*m/z* 165).

Accurate mass measurements on the Q-TOF

As already shown for some fragment ion assignments, an advantage of Q-TOF is that accurate mass measurement can be obtained directly on-line. The use of this feature for mass spectral characterisation of plant metabolites is rather new and its possibilities for on-line accurate MS/MS measurements have not been documented. A first report on the use of LC/ES-TOF-MS has however proved the usefulness of this method for on-line accurate molecular weight determination of cyclodepsipeptides [20].

In order to illustrate the possibilities of accurate MS/MS determination on the Q-TOF by automatic scan dependent autoswitch MS/MS experiments, data obtained for a flavonoid C-glycoside isovitexin (6-*C*-glc-apigenin) are presented in table VI. The molecular formula was measured from the survey scan trace using a reference compound

Table V. CID MS/MS of flavanols.

Exp.	CID	Prec.	Fragment ions (% RA)					
			$^{1,3}A^+$	$^{1,2}A^+$-H_2O	$^{1,2}B^+$-2H	$^{1,4}B^+$-2H	$^{1,4}B^+$-2H-H_2O	Main ion
(14) Catechin (MW 290)								
QTOF MS-MS POS	20	291	139 (100)	151 (3)	123 (45)	165 (20)	147 (30)	139 (100)
	25		139 (100)	151 (3)	123 (50)	165 (5)	147 (35)	139 (100)
	30		139 (100)	151 (2)	123 (70)	165 (5)	147 (40)	139 (100)
IT POS MS^2	35	291	139 (100)	151 (25)	123 (100)	165 (80)	147 (15)	139 (100)
	50		139 (100)	151 (15)	123 (70)	165 (70)	147 (25)	139 (100)
	60 w		139 (100)	151 (20)	123 (70)	165 (70)	147 (20)	139 (100)
IT NEG MS^2	50	289	-	-	-	-	-	245 (100)
IT NEG MS^3	50	245	-	-	121 (5)	-	-	203 (100)

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Table VI. Accurate mass measurements by QTOF MS/MS.

Isovitexin (MW 432)						
M/z measured	*M/z calculated*	*mDa difference*	*ppm difference*	*Molecular formula*	*Experiment*	*Lockmass*
433.1127	433.1135	0.8	1.8	$C_{21}H_{21}O_{10}$	MS	311.0814[a]
313.0716	313.0712	0.4	1.3	$C_{17}H_{13}O_6$	MS	As above
433.1124	433.1135	1.1	2.4	$C_{21}H_{21}O_{10}$	MS/MS of 433	313.0712[b]
283.0605	283.0606	0.1	0.5	$C_{16}H_{11}O_5$	MS/MS of 433	As above
195.0312	195.0293	1.9	9.3	$C_9H_7O_5$	MS/MS of 433	As above
177.0192	177.0188	0.4	2.4	$C_9H_5O_4$	MS/MS of 433	As above
149.0245	149.0238	0.7	4.2	$C_8H_5O_3$	MS/MS of 433	As above
121.0295	121.0289	0.6	4.5	$C_7H_4O_2$	MS/MS of 433	As above

[a]sulfadimethoxine, [b] $^{0,2}X^+$

injected post-column as lock mass. The accurate mass determination could be based only on a very limited number of scans since the TOF was acquiring five MS/MS spectra at five different energies between each survey scan. As shown the molecular formula was assigned with a precision of 1.8 ppm. For accurate mass measurements in the MS/MS mode, the $[M + H - 120]^+$ ion was used for lock mass correction. As shown the molecular formula of the different fragments were assigned with an accuracy of less than 5 ppm with one exception where the 195.0293 fragment ion was of very low abundance.

Conclusion

As shown the CID MS/MS spectra of the flavonoids vary according to the CID energies and the type of instrument used. If, for some compounds at a given energy setting, similar spectra can be generated on both IT and Q-TOF instruments, in other cases important differences are observed, especially when high energy settings are used. In this latter case this will generate consecutive fragmentation in a beam instrument that are not observable in ion trap systems. These differences hamper, at least partially, the use of a common CID MS/MS library for the dereplication of flavonoids with both type of analysers.

Even when a single instrument is considered, the choice of the optimum CID collision energy is difficult to determine since fragmentation is compound dependent. In certain cases, CID energies used are too weak to generate valuable spectra while, in other cases (Q-TOF) too high energies yield consecutive reactions and information on the substitution of A and B fragment ions, issued from C-ring cleavage, may be lost.

As a consequence, it is difficult to define a general strategy which will be optimum for automatic dereplication of all the compounds screened. On the Q-TOF, it is important to run scan dependent MS/MS autoswitch experiments at different energies and it has been observed that all the informative fragments can be recorded in a range of 25-35 eV. In IT-MS the fragments obtained are less dependent on the collision energy used and a CID setting of 40 % represents a good compromise.

As shown, the combined use of IT-MS^n and Q-TOF high resolution MS/MS is very efficient and enables detailed interpretation of the CID MS/MS spectra of the flavonoids recorded on-line. Q-TOF provides accurate molecular formula assignments for all the fragments recorded, while IT-MS^n gives information on the fragmentation pathways. In most cases A and B fragment ions have been recorded providing useful information on the substitution pattern of these compounds.

The CID MS/MS spectra were also systematically recorded in the negative ion mode. The negative IT-MS^n spectra were found generally less informative than positive product ion spectra and were more difficult to interpret. Negative ion CID MS/MS spectra may however constitute an interesting complementary library for confirmation when on-line dereplication based on positive CID MS/MS is ambiguous. For specific results in the negative ion mode see tables I-III and V.

Based on this study instrument-specific CID MS/MS libraries will be created, together with LC/UV-DAD databases, and the proposed LC/MS/MS strategies will be implemented for automatic dereplication procedure of flavonoids and related compounds in crude plant extracts.

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