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Establishing the occurrence of major and minor glucosinolates in Brassicaceae by LC–ESI-hybrid linear ion-trap and Fourier-transform ion cyclotron resonance mass spectrometry

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ABSTRACT

Glucosinolates (GLSs) are sulfur-rich plant secondary metabolites which occur in a variety of cruciferous vegetables and among various classes of them, genus Brassica exhibits a rich family of these phytochemicals at high, medium and low abundances. Liquid chromatography (LC) with electrospray ionization in negative ion mode (ESI-) coupled to a hybrid quadrupole linear ion trap (LTQ) and Fourier transform ion cyclotron resonance mass spectrometer (FTICRMS) was employed for the selective and sensitive determination of intact GLSs in crude sample extracts of broccoli (*Brassica oleracea* L. Var. *italica*), cauliflower (*B. oleracea* L. Var. *Botrytis*) and rocket salad (*Eruca sativa* L.) with a wide range of contents. When LTQ and FTICR mass analyzers are compared, the magnitude of the limit of detection was ca. 5/6-fold lower with the FTICR MS. In addition, the separation and detection by LC–ESI-FTICR MS provides a highly selective assay platform for unambiguous identification of GLSs, which can be extended to lower abundance (minor) GLSs without significant interferences of other compounds in the sample extracts. The analysis of Brassicaceae species emphasized the presence of eight minor GLSs, viz. 1-methylpropyl-GLS, 2-methylpropyl-GLS, 2-methylbutyl-GLS, 3-methylbutyl-GLS, *n*-pentyl-GLS, 3-methylpentyl-GLS, 4-methylpentyl-GLS and *n*-hexyl-GLS. The occurrence of these GLSs belonging to the saturated aliphatic side chain families C_4, C_5 and C_6, presumably formed by chain elongation of leucine, homoleucine and dihomoleucine as primary amino acid precursors, is described. Based on their retention behavior and tandem MS spectra, all these minor compounds occurring in plant extracts of *B. oleracea* L. Var. *italica*, *B. oleracea* L. Var. *Botrytis* and *E. sativa* L. were tentatively identified.

1. Introduction

Plants produce a variety of toxic and repellent secondary metabolites to protect themselves against pathogens (Anantha-krishnan, 2001). Among substances involved in plant defense, glucosinolates (GLSs), abundant in the plants from the Brassicaceae family and in several related plant families, are responsible for diverse physiological effects such as inhibitors of microbial growth, attractants for particular insects, and as deterrents of different herbivores (Halkier, 1999). Intact GLSs are nontoxic, however upon tissue damage (e.g., by cutting or chewing), they come into contact with myrosinases and are hydrolysed into unstable aglycones, which rearrange into a range of bioactive products and sometimes toxic compounds, including isothiocyanates, thiocyanates, nitriles, oxazolidine-2-thiones or epithioalkanes (Agerbirk et al., 2009; Halkier and Gershenzon, 2006; Verkerk and Dekker, 2008). Although certain GLS derivatives have antinutritional properties (Mithen et al., 2000), it is now well established that methionine-derived isothiocyanates can offer substantial protection against cancer (Keum et al., 2004, 2009; Talalay and Fahey, 2001).

The different biological properties of GLSs and their hydrolysis products are the reason why these plant secondary metabolites attract the attention of several investigators (Brown and Morra, 1995; Fenwick et al., 1983; Hanle and Parsle, 1990; Sones et al., 1984; van Poppel et al., 1999). The release of numerous sharp-tasting breakdown products are required to provide protection against many possible herbivores that can afflict a plant, but almost nothing is known about the specificity of major and minor GLSs in plant defense. Because the relative toxicity of different GLS hydrolysis products is dependent both on the target organism and the chemical structure, additional study is necessary to allow accurate identification of GLSs, in order to utilize the potential of these compounds in getting better pest resistance of crop plants (Verkerk

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and Dekker, 2008; Wittstock and Halkier, 2002). To date, more than 200 GLSs structures have been described, although only some of these are commonly found within crop plants. In fact, most plants contain only a limited number of major GLSs (typically six or less) with a few others present in trace amounts (Clarke, 2010; Fahey et al., 2001; Halkier and Gershenzon, 2006; Nour-Eldin and Halkier, 2009).

All GLSs have a common core structure comprising a β-D-thioglucose group linked to a sulfonated aldoxime moiety and a variable side chain derived from amino acids. Generally, they are grouped into aliphatic, aromatic and indole GLSs depending on whether they originate from aliphatic amino acids (methionine, alanine, valine, leucine, isoleucine), aromatic amino acids (tyrosine, phenylalanine) or tryptophan (Clarke, 2010; Fahey et al., 2001; Nour-Eldin and Halkier, 2009). The structural diversity of GLSs is due to chain elongations of protein amino acids before the formation of the glucosinolate core structure and secondary modifications of the GLS side chain (e.g., thiol oxidation, hydroxylation, etc.) and/or the glucose moiety (esterification) (Agerbirk et al., 2001; Agerbirk and Olsen, 2011; Reichelt et al., 2002). As the complexity of the sample increases and the secondary metabolite contents become very low, more sensitive and selective analytical methods are required. Even though mass spectrometry (MS) provides high selectivity between different sample components, the need for a separation step prior to MS detection would aid in reducing matrix interferences and increasing selectivity. High performance-liquid chromatography (HPLC) coupled to MS have been applied for quantification, upon previous identification, of GLSs in Brassicaceae vegetables (Cataldi et al., 2007, 2010; Fabre et al., 2007; Mellon et al., 2002; Mohn et al., 2007; Tolra et al., 2000; Velasco et al., 2011). The structural identity of known and described GLSs is of course only for those which can be isolated and identified by the available methods (Cataldi et al., 2007, 2010; Bialecki et al., 2010; Fabre et al., 2007; Lee et al., 2008; Millán et al., 2009; Mohn et al., 2007; Tolra et al., 2000; Rochfort et al., 2008; Velasco et al., 2011). Although a great deal of GLSs has been reported in recent years, much more awaits discovery before we fully understand how and why plants synthesize these compounds. This may enable us to more fully exploit the potential of these compounds in agriculture and functional foods.

The majority of cultivated plants that contain GLSs belong to the family of Brassicaceae such as Brussels sprouts, cabbage, broccoli and cauliflower, which are major source of these compounds in the human diet. In this work, we present a comprehensive profiling of intact GLSs in some Brassicaceae obtained by using an effective, rapid and selective method based on the electrospray ionization (ESI), linear quadrupole ion trap (LTQ) and Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS). The present method was successfully applied to identify major and minor GLSs, which were found in extracts of broccoli (*Brassica oleracea* L. Var. *italica*), cauliflower (*B. oleracea* L. Var. *Botrytis*) and rocket salad (*E. sativa L.*). This study is a powerful complement to a previously method proposed by our group, based on the fragmentation under collision induced dissociation (CID) of isotopologue peaks, using an optimized reversed-phase liquid chromatography (LC) coupled to electrospray ionization (ESI) and a quadrupole-linear trap (LTQ) mass analyzer (Cataldi et al., 2010).

2. Experimental

2.1. Chemicals

Sinigrin monohydrate from horseradish (99%) was obtained from Sigma–Aldrich (Steinheim, Germany). Progoitrin/epiprogoitrin, glucoraphanin, glucoerucin and glucoiberin were purchased from C2 Bioengineering Aps (Karlslunden, Denmark). Methanol and acetonitrile (ACN), both LC–MS grade, and formic acid (99%) were from Carlo Erba (Milan, Italy). Ultra-pure water was produced using a Milli-Q RG system from Millipore (Bedford, MA, USA). Stock solutions of the analytes were prepared by dissolving them in MeOH/H_2O (70/30, v/v) at a concentration of 1 mg ml^{-1} and stored at −20 °C. Standard solutions for LC–MS analyses were prepared by diluting the stock solution to the desired concentration with MeOH/H_2O (70/30, v/v). Pure nitrogen (99.996%) was delivered to the LC–MS system as sheath gas. The ion-trap pressure was maintained with helium 99.999%, which was used for trapping and for collisional activation of the trapped ions.

2.2. Plant material and sample preparation

Samples of broccoli (*B. oleracea* L. Var. *italica*), cauliflower (*B. oleracea* L. Var. *Botrytis*) and rocket salad (*E. sativa* L.) commercial cultivars were purchased from a local market. The extraction procedure was based on that previously reported by Cataldi et al. (2007, 2010). In brief, 500 mg of ground sample (dry weight) were extracted with 8 ml of 70% (v/v) aqueous methanol solution at 70–80 °C for about 10 min, and sonicated at 80 °C for 10 min. Then, the extract was centrifuged at 5000 rpm (3100*g*) at 4 °C for 10 min and the supernatant was removed using a syringe and filtered through a 0.22 μm nylon filters (Whatman, Maidstone, UK). The extraction procedure was repeated again with 5 ml of solvent; the supernatants were combined and concentrated by evaporation to dryness at 40 °C on a rotary evaporator (Laborota 400-efficient, Heidolph Instruments) and subsequently redissolved in 3 ml of 70% v/v aqueous methanol. When necessary the extracts were diluted and injected into the LC/MS system without further pretreatment.

2.3. ESI-LTQ-FTICR MS instrumentation and LC separation conditions

All experiments were performed using an LC system coupled to a hybrid linear quadrupole ion trap (LTQ) – Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). LC separation was performed at ambient temperature on a Discovery C_{18} column, 250 × 4.6 mm i.d., 5 μm particle size, equipped with a Discovery C_{18} 20 × 4 mm i.d. security guard cartridge (Supelco Inc., Bellefonte, PA, USA). Standard solutions and plant extracts were injected into the column via a 20 μl sample loop. Purified water with the addition of 0.1% formic acid was used as eluent A and acetonitrile as eluent B. The linear gradient profile was programed from 90%:10% (A:B, v/v) linear gradient to 76%:24% (A:B, v/v) in the first 10 min; varied to 40%:60% in the next 2 min and changed to 90%:10% (A:B, v/v) in the next 3 min, and finally the column was reconditioned for 5 min to the initial conditions. Analyses were performed at ambient temperature at a flow rate of 1.0 ml min^{-1}, which was split 4:1 after the analytical column to allow 200 μl min^{-1} to enter the ESI source. Negative ion ESI-MS was chosen for the detection of GLSs. Mass spectrometric conditions were optimized by direct infusion of standard solutions. The instrument was tuned to facilitate the ionization process and to achieve the highest sensitivity. The MS detector was tuned whenever the solvent flow rate conditions were changed, and the electrospray voltage, heated capillary temperature and voltage, tube lens voltage, sheath gas flow rate and auxiliary gas flow rate were optimized until the ion transmission was maximized. The spray voltage was set at −4.60 kV, while the temperature of the ion transfer tube was set at 350 °C and the applied voltage was set at −22 V. The sheath gas (N_2) flow rate used was 80 (arbitrary units) and the auxiliary gas was set to zero (arbitrary units). Full-scan experiments were performed in both the linear trap as well as the ICR cell in the range *m/z* 70–1000. *m/z* Signals were acquired as profile data at a resolution of 100,000

Table 1
Calibration curves of GLSs obtained by LC–ESI-LTQ MS and LC–ESI-FTICR MS in the concentration range 0.01–1.0 mg/L.

GLSs	LC–ESI-LTQ MS			LC–ESI-FTICR MS		
	$Y = a \times C\ (mg/l) + b$	r	LoD (μg/l)[a]	$Y = a \times C\ (mg/l) + b$	r	LoD (μg/l)[a]
Sinigrin	$(3.12 \pm 0.5)10^5 \times C\text{-}(3.0 \pm 1.3)10^3$	0.9997	62	$(9.33 \pm 0.4)10^5 \times C\text{+}(8.3 \pm 1.0)10^3$	0.9999	12
Progoitrin	$(3.86 \pm 0.6)10^5 \times C\text{-}(2.9 \pm 1.7)10^3$	0.9997	65	$(10.1 \pm 0.5)10^5 \times C\text{-}(2.4 \pm 1.2)10^3$	0.9999	11
Glucoraphanin	$(1.64 \pm 0.4)10^5 \times C\text{-}(1.6 \pm 0.92)10^3$	0.9996	72	$(5.42 \pm 0.2)10^5 \times C\text{-}(3.9 \pm 0.61)10^3$	0.9999	12
Glucoerucin	$(3.16 \pm 0.5)10^5 \times C\text{-}(3.9 \pm 1.4)10^3$	0.9995	63	$(10.2 \pm 0.6)10^5 \times C\text{-}(2.2 \pm 1.5)10^3$	0.9999	12
Glucoiberin	$(1.98 \pm 0.4)10^5 \times C\text{-}(3.1 \pm 1.1)10^3$	0.9995	71	$(7.6 \pm 0.3)10^5 \times C\text{-}(8.9 \pm 0.85)10^3$	0.9999	13

[a] LoD was calculated as 3.3 s_a/a, with s_a standard deviation on slope.

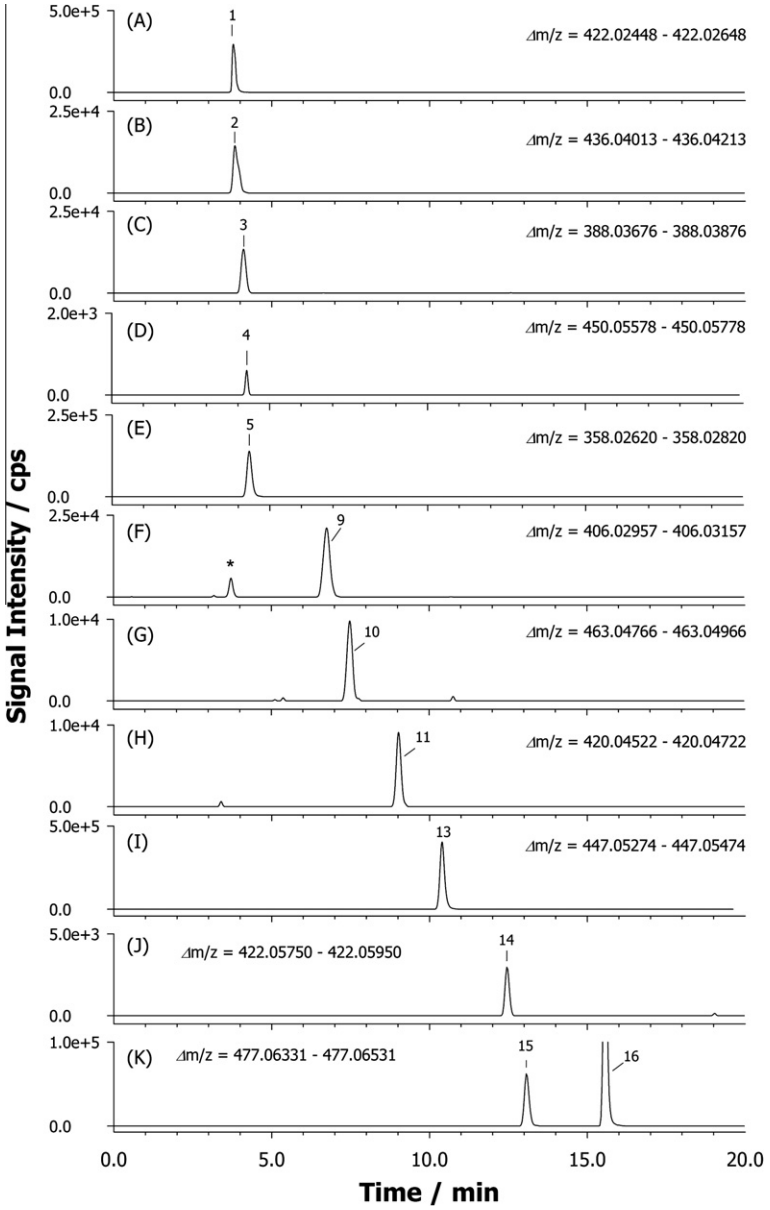

Fig. 1. Extracted ion chromatograms (XICs) by LC/ESI-FTICRMS acquired in negative ion mode of a sample extract of cauliflower (*B. oleracea* L. Var. *Botrytis*) diluted 1:50. The ions monitored are displayed in each trace and correspond to the most abundant deprotonated molecules, $[M-H]^-$, using a restricted window of ±0.0010 m/z unit centered around each selected ion. Data acquisition was made on a LTQ-FT hybrid linear trap/ICR mass spectrometer equipped with a 7-T magnet. Peak numbers correspond to (1) glucoiberin, (2) glucoraphanin, (3) progoitrin/epiprogoitrin, (4) glucoallysin, (5) sinigrin, (9) glucoibervirin, (10) 4-hydroxyglucobrassicin, (11) glucoerucin, (13) glucobrassicin, (14) gluconasturtiin, (15) 4-methoxyglucobrassicin and (16) neoglucobrassicin. Marked peak with an asterisk in plot (F) is due to an interference ion.

(FWHM) at m/z 400. The LTQ and FTICR mass spectrometers were calibrated according to manufacturer's instructions using a solution of sodium dodecyl sulfate (m/z 265) and sodium taurocholate (m/z 514). In addition, the ESI-MS/MS fragmentation performance of different GLSs was investigated. Data acquisition and analyses were accomplished using the Xcalibur software package (version

2.0 SR1 Thermo Electron). Systematic MS/MS experiments were performed by changing the relative collision energy and monitoring the intensities of the fragment ions. All analyses were achieved under automatic gain control (AGC) conditions using helium as the damping as well as the collision gas for MS/MS experiments. The collision energy was varied according to the ion of interest, typically 20–35% (100% corresponding to a 5 V excitation voltage), and was adjusted to get a precursor ion peak of 20% relative intensity or less (when possible).

The simplest method to identify analytes by eXtracted Ion Chromatograms (XICs) in LC–FTMS was used. XIC collects ion intensities falling only within a given mass-to-charge ratio window and appears to be the method of choice for mass analysis leading to very simple chromatograms. The reduction of interferences in the XICs significantly facilitates the identification of potential GLSs, including minor or uncommon ones that otherwise could be missed (Cataldi et al., 2008; Gu et al., 2006; Zhang et al., 2003). Data were collected in full MS scan mode and processed post-acquisition, to reconstruct the elution profile for the ions of interest, with a given *m/z* value and *m/z* tolerance. The chromatographic raw data were imported, elaborated and plotted by SigmaPlot 9.0 (Systat Software, Inc., London, UK).

3. Results and discussion

3.1. Separation and detection of GLSs

With the variety of GLSs in plants which is considerably greater than previously thought, there is a demand to improve GLS identification methods (Clarke, 2010). The major motivations behind the use of high resolution mass spectrometry are to determine as much compounds from the extract samples of interest as possible and to verify the occurrence of major and minor GLSs as well. Evaluating the role of minor GLSs in human health or pest and pathogen resistance will be a formidable undertaking. Recently, our group presented an approach that can be of general applicability for structural elucidation of naturally occurring GLSs in crude plant extracts. Such an approach was based on the selective fragmentation of isotopologues A and A + 2 of the precursor ions in the low resolution linear ion trap (LTQ) MS (Cataldi et al., 2010). The method is very informative, it is suitable for large-scale investigations of plant or vegetable extracts, and because of its ease of implementation in tandem MS experiments, it is also very attractive for detailed structural characterization of GLSs. However, in this work we wish to present a powerful complement to the method previously described, with its main feature of great selectivity and rapid recognition of secondary metabolites in vegetable extracts. Fourier-transform ion cyclotron resonance mass spectrometry (FTICR MS) offers 10–100 times higher mass resolving power than other mass analyzers (Marshall et al., 2002; Schrader and Klein, 2004). For example, only the high resolution and mass accuracy allow to distingue, as deprotonated molecules, glucoiberin (*m/z* 422.02548) and gluconasturtin (*m/z* 422.05850) with the same nominal *m/z*, i.e. 422, especially because they are present in a different concentration level. Glucoiberin is 100 fold more concentrated than gluconasturtin and because their nominal masses would fall within the XIC integration window of ±0.5 units (when LTQ analyser is employed, not shown), only the XIC of glucoiberin could be detected; the same applies to other GLSs with nominal mass at *m/z* 374, 388 and 402 (*vide infra*). The excellent selectivity

Table 2
Glucosinolates occurring in plant extracts of broccoli (*B. oleracea* L. Var. *Italica*), cauliflower (*B. oleracea* L. Var. *Botrytis*) and rocket salad (*E. sativa L.*) identified as $[M-H]^-$ by LC–ESI-FTICR MS.

n[a]	Trivial Name	Synonyms (Other names)	t_R[b]	Molecular Formulae	Monoisotopic calculated value as $[M-H]^-$ (*m/z*)[c]	Mass error (ppm)[d]
1	Glucoiberin	3-Methylsulfinyl propyl-GLS	3.8	$C_{11}H_{21}NO_{10}S_3$	422.02548	+1.2
2	Glucoraphanin	4-methylsulfinylbutyl-GLS	3.9	$C_{12}H_{23}NO_{10}S_3$	436.04113	−0.7
3	Progoitrin/epiprogoitrin	(*R,S*)-2-hydroxy-3-butenyl-GLS	4.0	$C_{11}H_{19}NO_{10}S_2$	388.03776	−1.4
4	Glucoalyssin or glucoallysin	5-Methylsulfinylpentyl-GLS	4.2	$C_{13}H_{25}NO_{10}S_3$	450.05678	−1.9
5	Sinigrin	Prop-2-enyl-GLS	4.3	$C_{10}H_{17}NO_9S_2$	358.02720	−1.4
6	4-(β-D-glucopyranosyl disulfanyl)butyl-GLS	Diglucothiobeinin	4.4	$C_{17}H_{31}NO_{14}S_4$	600.05546	−1.7
7	6-Methylsulfonyl-3-oxo-hexyl-GLS	–	4.5	$C_{14}H_{25}NO_{12}S_3$	494.04662	−0.1
8	Glucosatavin	4-Mercaptobutyl-GLS	6.2	$C_{11}H_{21}NO_9S_3$	406.03057	+1.7
9	Glucoibervirin	3-Methylthiopropyl-GLS	6.8	$C_{11}H_{21}NO_9S_3$	406.03057	+0.4
10	4-Hydroxy-glucobrassicin	4-OH-3-indolylmethyl-GLS	7.4	$C_{16}H_{20}N_2O_{10}S_2$	463.04866	−0.3
11	Glucoerucin	4-Methylthiobutyl-GLS	8.9	$C_{12}H_{23}NO_9S_3$	420.04622	−1.4
12	Dimeric 4-mercaptobutyl-GLS	4-Mercaptobutyl-GLS dimer	10.1	$C_{22}H_{40}N_2O_{18}S_6$	405.02274[e]	+2.0
13	Glucobrassicin	3-Indolylmethyl-GLS	10.5	$C_{16}H_{20}N_2O_9S_2$	447.05374	+0.1
14	Gluconasturtiin	2-Phenylethyl-GLS	12.3	$C_{15}H_{21}NO_9S_2$	422.05850	+0.2
15	4-Methoxy-glucobrassicin	4-Methoxy-3-indolylmethyl-GLS	12.9	$C_{17}H_{22}N_2O_{10}S_2$	477.06431	+0.3
16	Neoglucobrassicin	*N*-Methoxy-3-indolylmethyl-GLS	15.5	$C_{17}H_{22}N_2O_{10}S_2$	477.06431	−0.2
17	1-Methylpropyl-GLS	Glucocochlearin	6.2	$C_{11}H_{21}NO_6S_2$	374.05850	−1.9
18	2-Methylpropyl-GLS	Glucoconringianin	6.4	$C_{11}H_{21}NO_6S_2$	374.05850	−1.8
19	2-Methylbutyl-GLS	Glucojiaputin	9.1	$C_{12}H_{23}NO_9S_2$	388.07415	−0.9
20	3-Methylbutyl-GLS	–	9.6	$C_{12}H_{23}NO_9S_2$	388.07415	−0.1
21	*n*-Pentyl-GLS	Glucokohlrabiin	10.0	$C_{12}H_{23}NO_9S_2$	388.07415	−0.6
22	3-Methylpentyl-GLS	–	13.2	$C_{13}H_{25}NO_9S_2$	402.08980	+0.3
23	4-Methylpentyl-GLS	–	13.7	$C_{13}H_{25}NO_9S_2$	402.08980	−0.2
24	*n*-Hexyl-GLS	Glucoraphasativusain	14.3	$C_{13}H_{25}NO_9S_2$	402.08980	+0.4

[a] *n* is the number used to identify each GLS in the chromatograms of Figs. 1 and 3–5.
[b] Retention time of GLSs eluted under the experimental conditions described in the LC–MS section.
[c] Monoisotopic exact value of deprotonated GLS molecules.
[d] Mass error in part per million (ppm) evaluated as $10^6\times$ (experimental accurate mass – monoisotopic calculated mass)/monoisotopic calculated mass.
[e] *m/z* value of a doubly charged ion.

and mass accuracy of FTICR MS is exploited to assign elemental compositions and thus identify naturally occurring GLSs using an optimized reversed-phase liquid chromatography (LC) coupled to electrospray ionization (ESI) and MS in negative-ion mode.

Using the chromatographic conditions described in Section 2, a comparison was made between the hybrid LTQ and FTICR mass spectrometers. During the evaluation process, mixed samples of an MeOH/H_2O (70/30, v/v) stock solution containing five standard GLSs in the range 0.01–1 mg/l were injected. Five-point calibration curves were plotted as the peak area of analytes versus concentration; all measurements were done in triplicate. Repeatability was assessed by injecting a standard solution (200 μg/l) of GLSs 10 times in the same day. The obtained intraday relative standard deviation (RSD) values were less than 2%, indicating a satisfactory result. The same data evaluated over eight weeks was, in general, better than 12%. In Table 1 are also summarized the limits of detection (LOD) for the investigated GLSs utilizing the same LC–ESI analytical methodology coupled with LTQ or FTICR MS detection. Taking glucoraphanin as an example, an improvement in the LODs up to six-fold was obtained using the ICR detection cell; the estimated value was found to be 72 μg/l and 12 μg/l, using LTQ and FTICR mass analyzers, respectively. As will be demonstrated below, the determined LODs and reproducibility are sufficient to identify GLSs in the examined sample extracts.

3.2. Glucosinolate profiles of Brassicaceae plant extracts

Total ion chromatogram (TIC) provides a good summary of ions being separated/detected, but it works best when the analytes dominate the ion signals in any given retention time window or at least when there is a good signal-to-noise (S/N) ratio and a low-drifting baseline. In the presence of many other ions of similar abundance levels, TIC may become too complex to be informative, thereby making it very difficult to determine the molecular ions of unknown chemical entities. The benefit of using very selective extracted ion chromatograms by FTICR MS, generated with a tight mass-to-charge ratio window of ±0.0010 units around each selected deprotonated molecule (i.e., $[M-H]^-$ ±1.0 mDa), greatly reduce the signal complexity of the total ion current trace (data not shown) allowing us to distinguish all GLSs completely. As illustrated in Fig. 1, 12 chromatographic peaks of GLSs in a sample extract (diluted 1:50, see Section 2) of cauliflower florets (*B. oleracea* L. Var. *Botrytis*) were identified. The hydrophilic character of GLSs does not allow a complete separation of all compounds under reversed-phase LC conditions and those species having a similar side chain (e.g., glucoiberin and glucoraphanin, peaks 1 and 2, respectively) exhibit quite early-eluting peaks. However, the experimental conditions adopted here together with the high selectivity of ICR-MS detection proved to be enough useful to separate a numerous series of GLSs with retention up to 16 min. The major GLSs are: (peak 1) 3-methylsulphinylpropyl-GLS (glucoiberin, *m/z* 422.02600), (peak 5) prop-2-enyl-GLS (sinigrin, *m/z* 358.02670), (peak 13) 3-indolylmethyl-GLS (glucobrassicin, *m/z* 447.05380) and (peak 16) *N*-methoxy-3-indolylmethyl-GLS (neoglucobrassicin *m/z* 477.06421). According to Hodges et al. (2006), glucobrassicin was the major glucosinolate component, followed by glucoiberin, sinigrin, and neoglucobrassicin. Smaller amounts of other GLSs such as (peak 2) 4-methylsulfinylbutyl-GLS (glucoraphanin *m/z* 436.04083), (peak 3) (*R*,*S*)-2-hydroxy-3-butenyl-GLS (progoitrin/epiprogoitrin *m/z* 388.03720), (peak 4) 5-methylsulfinylpentyl-GLS (glucoalyssin *m/z* 450.05593), (peak 9) 3-methylthiopropyl-GLS (glucoibervirin *m/z* 406.03073), (peak 10) 4-hydroxy-3-indolylmethyl-GLS (4-hydroxyglucobrassicin *m/z* 463.04852), (peak 11) 4-methylthiobutyl-GLS, (glucoerucin *m/z* 420.04562), (peak 14) phenylethyl-GLS (gluconasturtiin *m/z* 422.05859) and (peak 15) 4-methoxy-3-indolylmethyl-GLS (4-methoxyglucobrassicin, *m/z* 477.06448) were also recognized in the cauliflower extract. Using less diluted sample extracts, seven new aliphatic GLSs (*vide infra)* were also identified. The identification of all chromatographic peaks was based on accurate mass measurements, comparison with their retention time, and MS/MS fragmentation spectra with those obtained from pure standard solutions when commercially available. Otherwise, the identification based only on accurate mass measurement and interpretation of MS/MS data was performed. In Table 2 are summarized chromatographic and mass spectrometric data of GLSs identified in cauliflower floret extracts. Accurate mass data of GLSs, as deprotonated molecules, with a mass error not greater than 2.0 ppm was found, indicating a very good mass accuracy. Regarding some minor GLSs, tandem MS examination in the LTQ analyzer confirmed their occurrence. As an example, in Fig. 2, are shown the tandem MS spectrum of glucoalyssin at *m/z* 450, collected at the apex of the chromatographic peak (see peak 4 in Fig. 1, plot D), and the MS^3 spectrum of ion at *m/z* 386 selected as precursor ion. As previously reported, $[M-H]^-$ ions of GSLs are decomposed into specific ions when collisionally activated (i.e., collision induced dissociation, CID). The MS/MS spectrum of the deprotonated precursor at *m/z* 450 (Fig. 2, plot A), displays five typical peaks at *m/z* 386, 291, 275, 259, 241 (Cataldi et al., 2010). The most intense one at *m/z* 386 can be rationalized as the neutral loss of methanesulfenic acid (CH_3SOH) from the deprotonated molecule, $[M-H]^-$. Additional insight can be obtained by using the ion trapping and MS^3 capabilities of the LTQ MS analyzer. Such experiments on the precursor ion at *m/z* 386 reveal an interesting product ion spectrum, which exhibits the common fragmentation behavior of GLSs in which the neutral loss of SO_3 (80 Da) plays a role (Fabre et al., 2007; Cataldi et al., 2010; Bialecki et al., 2010). Indeed, the product ions at *m/z* 306, 208, 190 and 144 can be rationalized by loss of neutral molecules as following $306 = [386 - 80]^-$, $208 = [386 - 178]^-$,

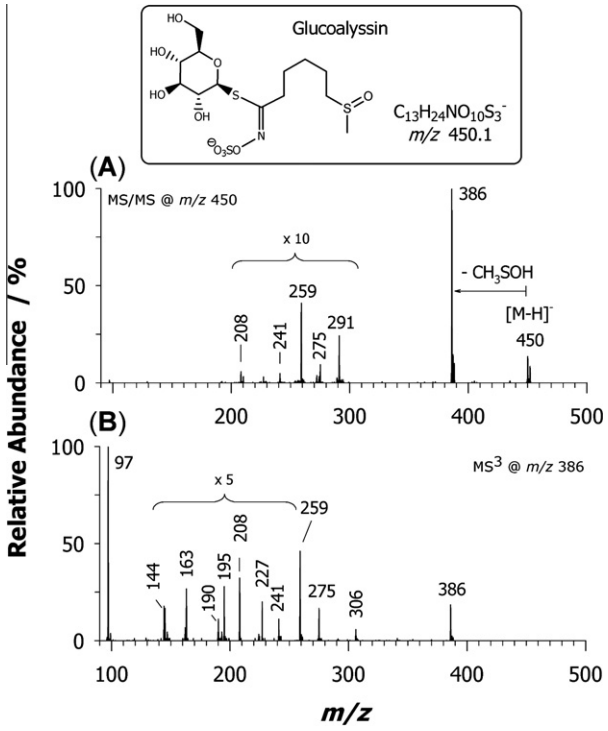

Fig. 2. (A) Product ion spectrum at the apex of the chromatographic peak 4, i.e. glucoallysin, at *m/z* 450 (see Fig. 1). (B) MS^3 spectrum of the precursor ion at *m/z* 386 selected for a further stage of fragmentation. Precursor ions were selected within the linear trap LTQ analyzer and collisionally activated to induce fragmentation. Relative collisional energy in CID equals to 24%. For details, see text.

190 = $[386 - 196]^-$, and 144 = $[386 - 80 - 162]^-$, which are formed upon H-rearrangement from the side-chain R-group by cleavage of the bond on either side of the sulfur with charge retention on the sugar aglycone. The loss of 178, 196 and 162 neutral molecules has been already described in a systematically manner (Cataldi et al., 2010; Fabre et al., 2007), and will not be discussed further. Quantification was not one of our first goals: we wanted to develop a method for the fast and accurate determination of GLSs in *Brassica* genus samples. However, at least three concentration levels may be considered for a 1:50 diluted cauliflower extract: compounds at a relatively high content (1.7–4.8 mg/l), glucoiberin (1), sinigrin (5), glucobrassicin (13), neoglucobrassicin (16); compounds at medium a content (0.1–0.7 mg/l), i.e. glucoraphanin (2), progoitrin/epiprogoitrin (3), glucoibervirin (9), 4-hydroxyglucobrassicin (10), glucoerucin (11) and 4-methoxyglucobrassicin (15) and compounds occurring at a low content (<0.05 mg/l), i.e. gluconasturtiin (14), glucoalyssin (4) and methylpropyl- (17, 18), methylbutyl- (19, 20, 21) and methylpenthyl- (23, 24) GLS isomers (see Fig. 5). The occurrence of these minor GLSs (i.e., from 17 through 23) in our sample extracts will be discussed in more detail below.

An additional example of very selective XICs corresponding to an extract of broccoli (*B. oleracea* L. Var. *italica*) is shown in Fig. 3. The following GLSs were identified: glucoraphanin (2), glucobrassicin (13) and neoglucobrassicin (16) in the concentration range 5.7–24.0 mg/l; glucosatavin (8), glucoallysin (4), gluconasturtiin (14), 4-hydroxyglucobrassicin (10), sinigrin (5), glucoiberin (1) and glucoerucin (11) in the concentration range 0.05–2.0 mg/l; 4-methoxyglucobrassicin (15), and some minor (eight) aliphatic GLSs, detected in less diluted extract samples (i.e., 1:10 or 1:30), having monoisotopic *m/z* values at 374.05850 (17, 18), 388.07415 (19, 20, 21) and 402.08980 (22, 23, 24) at concentration lower than 0.05 mg/l (see Fig. 5, plot B).

In Fig. 4 are shown the XICs of an extract of rocket salad (*E. sativa*). GLSs identified as 4-mercaptybutyl-GLS (8), 4-hydroxyglucobrassicin (10) and dimeric 4-mercaptybutyl-GLS (12) were in the concentration range 1.2–1.7 mg/l; glucoraphanin (2), progoitrin/epiprogoitrin (3), glucoerucin (11), 4-(β-D-glucopyranosyld isulfanyl)butyl-GLS (6), 6-methylsulfonyl-3-oxohexyl-GLS (7) and 4-methoxy-glucobrassicin (15) in a concentration range from 0.13 to 0.9 mg/l; glucoalyssin (4), sinigrin (5) and neoglucobrassicin (16) at concentration lower than 0.05 mg/l. Again, in extracts con-

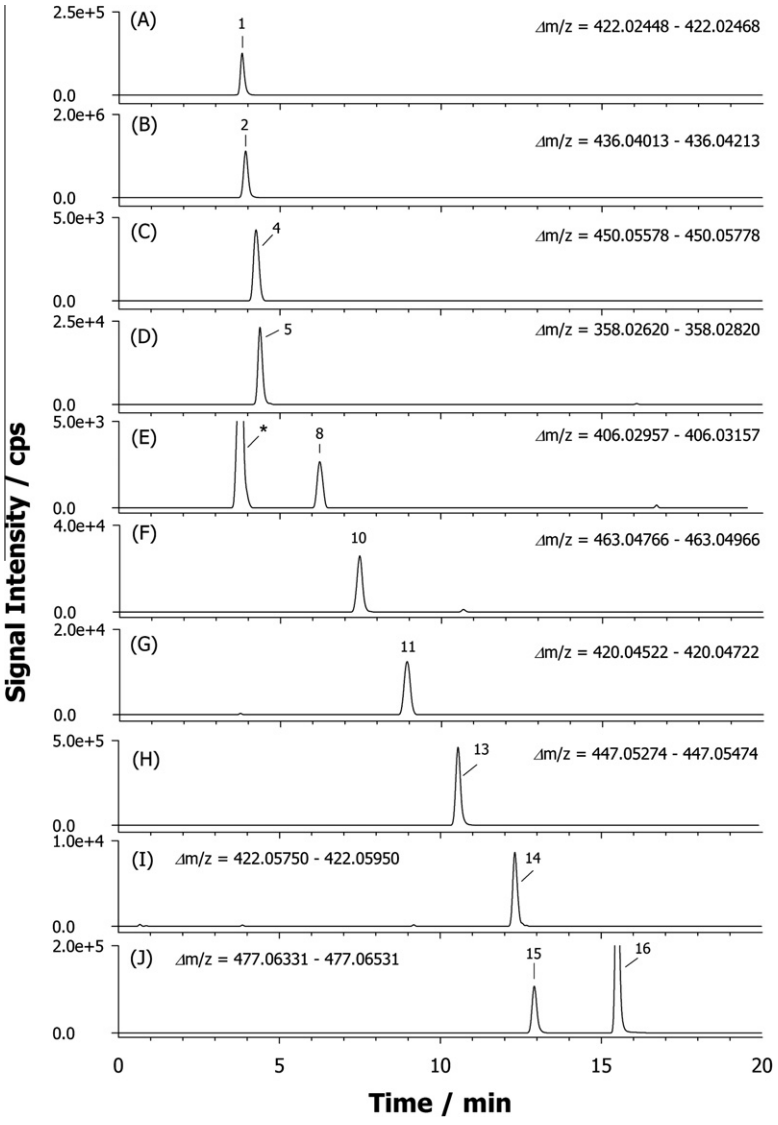

Fig. 3. XICs by LC–ESI-FTICRMS acquired in negative ion mode ($[M-H]^-$) of a an extract of broccoli (*B. oleracea* L. Var. *italic*) diluted 1:50. Peak numbers correspond to (1) glucoiberin, (2) glucoraphanin, (4) glucoalyssin, (5) sinigrin, (8) glucosatavin, (10) 4-hydroxyglucobrassicin, (11) glucoerucin, (13) glucobrassicin, (14) gluconasturtiin, (15) 4-methoxyglucobrassicin and (16) neoglucobrassicin. Peak with asterisk in plot (E) was proved not to be a glucosinolate.

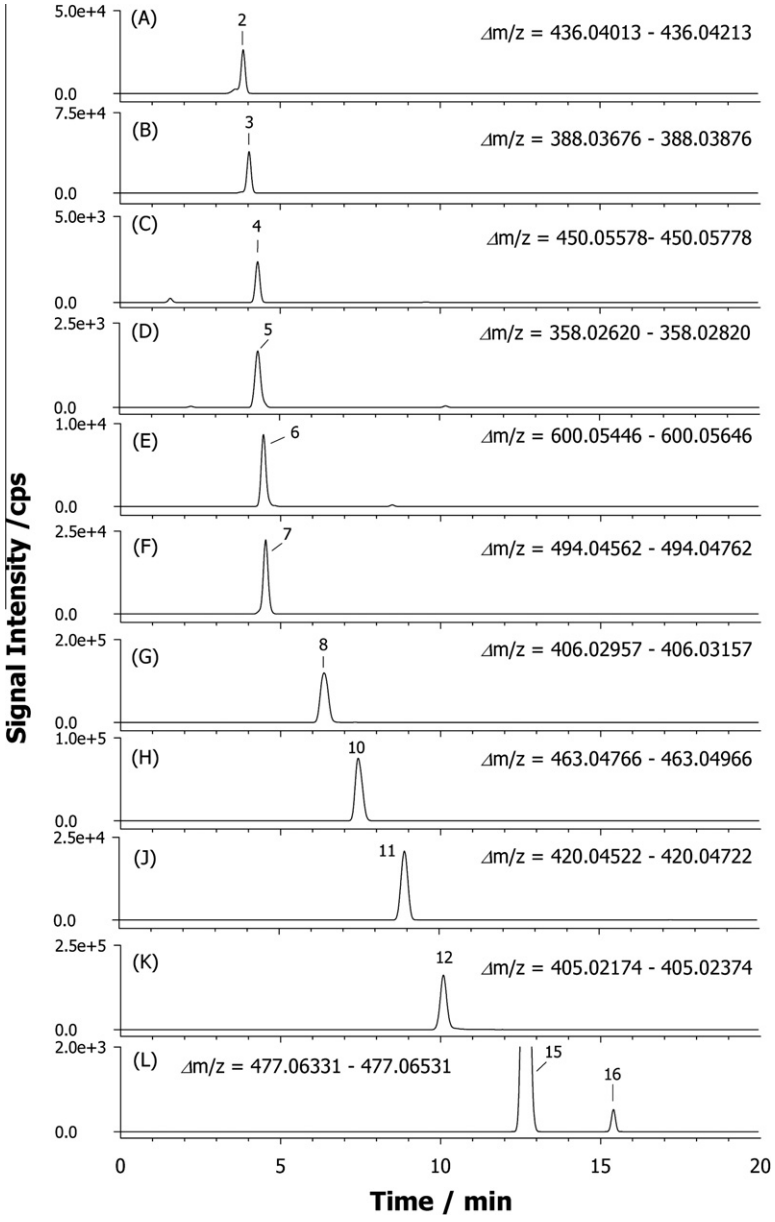

Fig. 4. XICs by LC–ESI-FTICRMS acquired in negative ion mode ($[M-H]^-$) of an extract of rocket salad (*E. sativa* L.) diluted 1:50 except for the XIC trace of sinigrin (plot D) whereby the sample was diluted 1:5. Peak numbers correspond to (2) glucoraphanin, (3) progoitrin/epiprogoitrin, (4) glucoalyssin, (5) sinigrin, (6) diglucothiobeinin, (7) 6-methylsulfonyl-3-oxo-hexyl-GLS, (8) glucosatavin, (10) 4-hydroxyglucobrassicin, (11) glucoerucin, (12) dimeric 4-mercaptobutyl-GLS, (15) 4-methoxyglucobrassicin, and (16) neoglucobrassicin.

centrated or just diluted 1:10, we verified the occurrence of (see below) six minor aliphatic GLSs at *m/z* 374.05779 (17), 374.05783 (18), 388.07390 (21), 402.08993 (22), 402.08971 (23) and 402.08996 (24), which correspond to the same compounds detected in broccoli and cauliflower extracts in concentration lower than 0.05 mg/l. Analysis of this series of compounds at *m/z* 374, 388 and 402, with masses separated by 14 Da, as well as comparison of their chromatographic behavior, provide insights into their relationship as related compounds and common precursor amino acids.

3.3. Occurrence of minor GLSs in Brassicaceae plant extracts

In a previous paper we have reported the occurrence of two minor aliphatic GLSs in rocket salad extracts, which exhibited, as intact deprotonated molecules, a value of 402 *m/z* (Cataldi et al., 2007). Here, the use of more concentrated sample extracts along with a highly selective and sensitive FTICR MS analyser allowed us to identify a greater number of saturated aliphatic GLSs (*vide infra*). Of particular note was the occurrence of low levels of *n*-pentyl and *n*-hexyl GLSs together with their two isomers likely to be methylbutyl and methylpentyl derived from 2-oxo-4-methylhexanoic acid, ('homoleucine') and 2-oxo-4-methylheptanoic acid (dihomoleucine), respectively, and methylpropyl isomers derived from leucine (Mithen et al., 2000). The accepted model for GLS biosynthesis process can be divided into three steps: (i) amino acid chain elongation, in which additional methylene groups are inserted into the side chain, (ii) conversion of the amino acid moiety to the glucosinolate core structure, and (iii) subsequent side chain modifications (Ananthakrishnan, 2001; Halkier, 1999). As reported in Fig. 4, *E. sativa* accumulates principally glucosinolates derived from dihomomethionine, the product of two rounds of chain elongation (Graser et al., 2000). According to the chain elongation of methionine and formation of homomethionine and

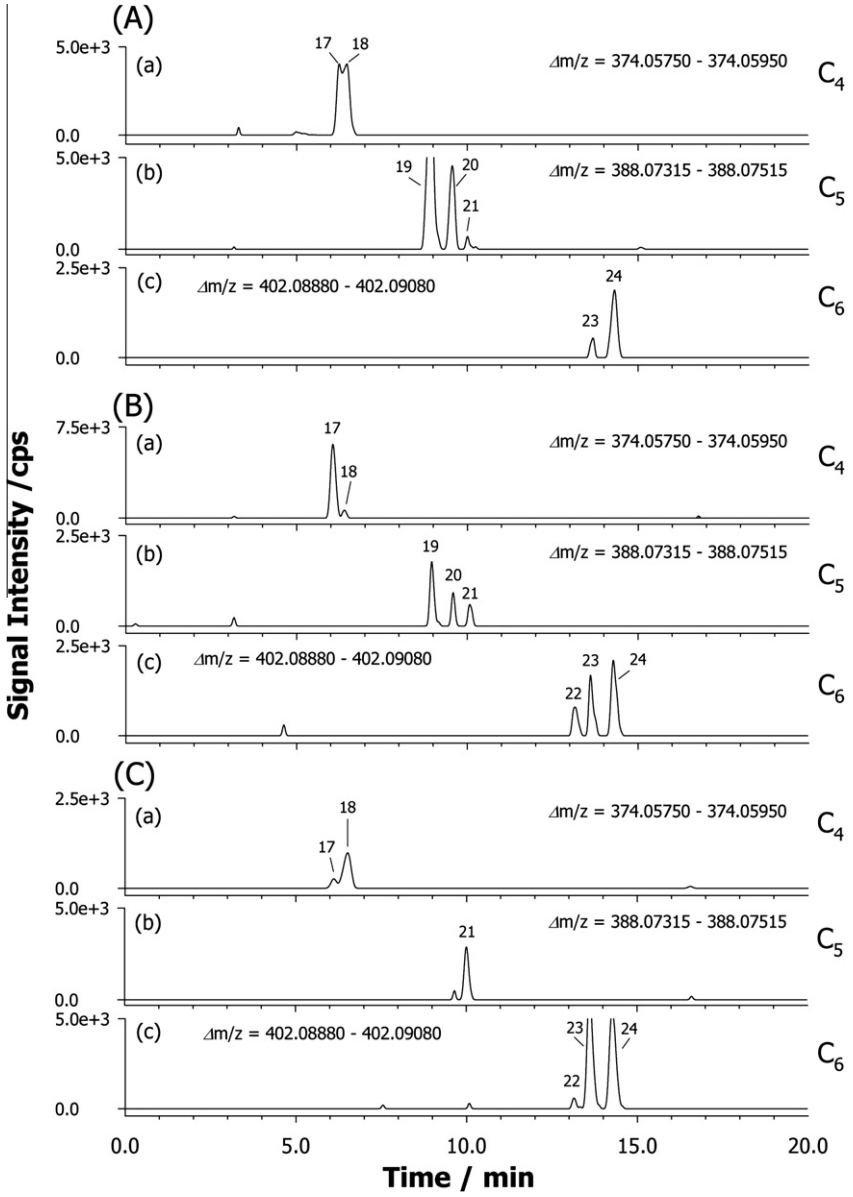

Fig. 5. (A) XICs by LC–ESI-FTICRMS acquired in negative ion mode ($[M-H]^-$) of an extract of cauliflower *B. oleracea* L. Var. *Botrytis*), (B) broccoli (*B. oleracea* L. Var. *italica)* and (C) rocket salad (*E. sativa* L.) diluted 1:10 except for the XIC traces at *m/z* 402.08980 in plot A and at *m/z* 374.05850 in plot C, whereby the sample were not diluted and trace at *m/z* 374.05850 in plot B whereby the sample was diluted 1:30. Peak numbers correspond to (17) 1-methylpropyl-GLS, (18) 2-methylpropyl-GLS, (19) 2-methylbutyl-GLS, (20) 3-methylbutyl-GLS, (21) *n*-pentyl-GLS, (22) 3-methylpentyl-GLS, (23) 4-methylpentyl-GLS and (24) *n*-hexyl-GLS.

dihomomethionine (Gu et al., 2006), we suggest the initial formation of both homo- and dihomoleucine, and leucine as precursors of aliphatic GLSs. Confirmation of glucosinolate structure was on the basis of likely elemental composition estimated from accurate *m/z* values of ions and from the analyses of intact glucosinolates along with the occurrence of diagnostic ions (Cataldi et al., 2010; Mellon et al., 2002).

To systematically demonstrate the ability to detect minor GLSs on all samples examined by LC–ESI-FTICR MS, the XICs results obtained of species at *m/z* 374.05850, 388.07415 and 402.08980 are presented in Fig. 5. These putative aliphatic GLSs, containing side chains of four, five and six carbons, are designed as C_4, C_5 and C_6, respectively. Their corresponding molecular formula as intact molecule (M) should be $C_{11}H_{21}NO_6S_2$, $C_{12}H_{23}NO_9S_2$, $C_{13}H_{25}NO_9S_2$, respectively (see Table 2). While two C_4 GLS isomers were barely separated at retention time of 6.2 and 6.4, peaks 17 and 18, respectively, the three C_5 GLS isomers corresponding to peaks 19, 20 and 21, were tentatively assigned (*vedi infra*), in order of elution, as 2-methylbutyl-GLS, 3-methylbutyl-GLS (i.e., isobutyl-GLS) and *n*-pentyl-GLS, respectively.

Interestingly, there is a second set of peaks, viz. 22, 23 and 24, which should correspond to saturated C_6 isomers, i.e., 3-methylpentyl-GLS, 4-methylpentyl-GLS and *n*-hexyl-GLS, respectively. In the model plant *Arabidopsis thaliana* (Graser et al., 2000) reported the occurrence of 3-methylbutyl-GLS and 4-methylpentyl-GLS as leucine-derived. It is intriguing that the three C_5 side chain GLS isomers occur in both cauliflower and broccoli extracts, while all three C_6 side chain GLS isomers occur in both broccoli and rocked salad extracts. Mass spectrometry approaches cannot accurately distinguish structural-isomer C_4, C_5 and C_6 aliphatic GLSs. Sufficient concentrations of GLSs required for NMR analyses were not available from extractions of vegetable material. Their retention has been suggested on the basis of the fact that branched-chain compounds are usually eluted more rapidly than their correspond-

ing linear isomers. In the proposed reverse-phase LC procedure, the 2/3-methyl- isomers eluted first, then the 3/4-methyl- branched isomers, and finally the linear ones. The separation of C_5 and C_6 GLS isomers is satisfying with chromatographic resolution ($R > 1.5$).

We wish to mention that other compounds possess the same nominal mass at *m/z* 402.1 (exact monoisotopic calculated value *m/z* 402.05341) corresponding to a chemical formula of $C_{12}H_{21}NO_{10}S_2$; these putative GLSs are recently reported by Clarke (2010) in the most comprehensive list of structures, with more than 400 different GLSs. Unfortunately, among saturated C_4, C_5 and C_6 isomers similar tandem MS spectra were observed, making assignment of isomer compounds difficult. Typical MS/MS spectra of aliphatic C_4, C_5 and C_6 isomers are illustrated in plots A, B and C of Fig. 6, respectively. Whereas the C_4 isomer was collected from *B. oleracea* L. Var. *Botrytis* (see peak 17), the C_5 isomer was from *B. oleracea* L. Var. *italica* (peak 20) and the C_6 isomer was taken from a sample extract of *E. sativa* (peak 22). As can be seen, in the MS/MS spectrum at *m/z* 388 (plot B), typical peaks at *m/z* 275, 259, 227, 195 and 97 are observed (Cataldi et al., 2010). Only peak at *m/z* 210 is due to the loss of a common neutral loss (178 Da) and is related to the aliphatic side chain containing a saturated C_5 chain. The same rationale applies to the MS/MS spectra at *m/z* 374 and 402 (plots A and C), which exhibits the common fragmentation behavior of GLSs including the neutral loss of SO_3. The neutral loss of 196 and 178 give rise to peaks at *m/z* 178/206 and 196/224, C_4 and C_6 GLSs, respectively. We wish to mention that the absence of aliphatic C_7 side chain GLSs (i.e., *m/z* 416.10545) was also ascertained.

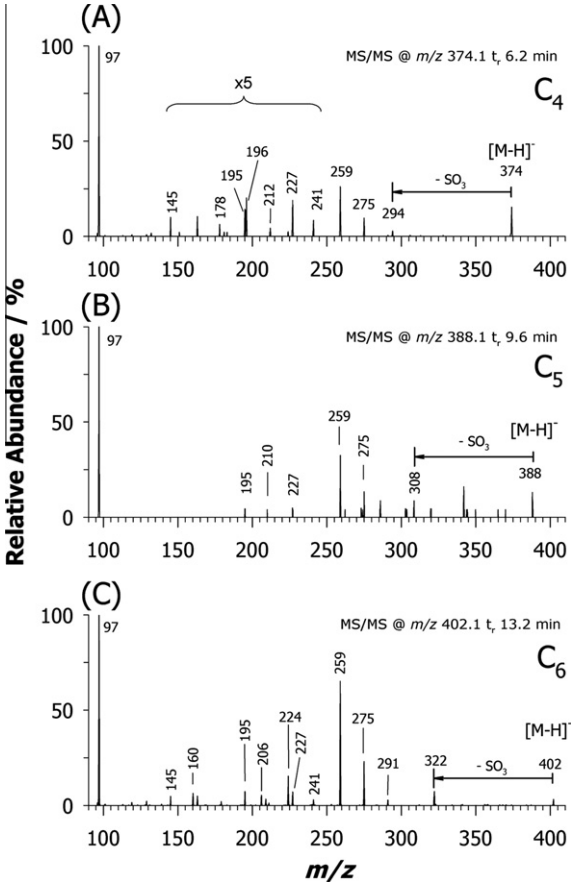

Fig. 6. Product ion spectra in the LTQ MS analyser of peaks at *m/z* 374 (A), (see peak 17 of Fig. 5A, plot a), at *m/z* 388 (B), (peak 20 of Fig. 5B, plot b) and at *m/z* 402 (C), (peak 22 of Fig. 5B, plot c). Each CID tandem MS spectrum is representative of GSLs possessing saturated C_4, C_5 and C_6 side chains. A relative collisional energy in CID equal to 25% was applied.

4. Conclusions

Using a very selective method based on LC–ESI-LTQ and Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS), we have demonstrated that it is possible to analyze major and minor GLSs in crude sample extracts of broccoli (*B. oleracea* L. Var. *italic)*, cauliflower (*B. oleracea* L. Var. *Botrytis*) and rocket salad (*E. sativa* L.) with high sensitivity and very good mass accuracy. We identified some minor GLSs that have been previously suggested in the literature, but not yet reported from Brassicaceae species, which are probably derived from leucine as amino acid precursor, upon side chain elongation.

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