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Investigation of Glucosinolate Profile and Qualitative Aspects in Sprouts and Roots of Horseradish (*Armoracia rusticana*) Using LC-ESI–Hybrid Linear Ion Trap with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry and Infrared Multiphoton Dissociation

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ABSTRACT: Within the family of Brassicaceae, an important source of glucosinolates (GLSs) is represented by horseradish (*Armoracia rusticana* P. Gaertner, B. Meyer & Scherbius), cultivated for its roots, which are grated fresh or processed into a sauce and used as a condiment. The characteristic pungent flavor of the root depends on the abundance of the bioactive GLS molecules. In crude plant extracts (sprouts and roots) of an accession of horseradish largely diffused in the Basilicata region (southern Italy), which develops many sprouts and produces white, fiery, and sharp-flavored marketable roots, we characterized the GLS profile by LC-ESI-LTQ-FTICR-MS and IRMPD. In sprouts and roots we identified 16 and 11 GLSs, respectively. We confirmed the presence of sinigrin, 4-hydroxyglucobrassicin, glucobrassicin, gluconasturtin, and 4-methoxyglucobrassicin and identified glucoiberin, gluconapin, glucocochlearin, glucoconringianin, glucosativin, glucoibarin, 5-hydroxyglucobrassicin, glucocapparilinearisin or glucobrassicanapin, glucotropaeolin, and glucoarabishirsutain, not previously characterized in horseradish. Of particular note was the presence of the putative 2-methylsulfonyl-oxo-ethyl-GLS.

KEYWORDS: *Armoracia rusticana, glucosinolates, morpho-agronomic traits, LC-FTICR MS, IRMPD*

INTRODUCTION

Glucosinolates (GLSs) are secondary metabolites mainly found in plants belonging to the order of Capparales, with particular reference to several species of the Brassicaceae family.[1]

The renowned healthy potential of these species has been largely attributed to the content of GLSs, and is a consequence of their enzymatic degradation due to myrosinase release of bioactive molecules containing nitrogen and sulfur, named isothiocyanates (ITCs).[2] The literature largely reports the antiseptic properties of ITCs in relation to their important role in plant resistance versus fungi and bacteria[3,4] and nematodes and other pathogens.[5,6] Currently, the scientific interest in GLSs is also related to their possible protective properties against cancer in humans.[7,8] These wide biological properties of GLSs and their hydrolysis products stimulate the attention of several investigators on these secondary metabolites.

Within the family of Brassicaceae, a range of GLS sources is represented by the horseradish (*Armoracia rusticana* P. Gaertner, B. Meyer & Scherbius),[9] a popular spice[10] cultivated for over 2000 years for its pungent fleshy root, which is grated and used primarily as a dish condiment.[11] Some German studies have investigated the effect of horseradish on nonspecific urinary tract infections and the antibacterial action of its essential oils.[9] Compounds found in horseradish, as in many others species of Brassicaceae, have antibacterial, antifungal, and insecticidal activities.[12] The characteristic pungent flavor of the root depends on the abundance of GLSs and ITCs, to which are attributed to the above-mentioned health properties; its richness in glucosinolates makes it a possible cancer-preventive component in the diet.[13]

The root system consists of a long, white, cylindrical or tapering main rhizome that can be grown to about 60 cm in sandy soils. Several thin lateral roots also develop around the main rhizome and near the collar of the crown. Undisturbed root systems can reach a depth of 3–4 m with a lateral spread of about 1 m.[9] Horseradish roots exhibit distinct polarity with a proximal end, or point of attachment, to the main root that sprouts, producing new vegetation, and a distal end. The leaves grow on the rhizome crown from various shoots.[14] Usually, horseradish is harvested in late October–November once the foliage has been killed by frost, continuing through the winter and early spring months.[9,14]

The main areas of commercial cultivation are Central Europe, North America (California, Illinois, and Wisconsin), Russia, Caucasus, Asia, and some regions 1000 m above sea level within tropical countries. In Italy this plant grows particularly in the northern areas, in the southern Lazio region, and in the Basilicata region.[15] Some authors[15,16] have described the local uses of horseradish in the Basilicata region either as a condiment to

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prepare traditional dishes or for medical purposes as a remedy for rheumatism, headaches, sinuses, coughs, and bronchitis.

The approved modern therapeutic applications for horseradish are based on its long history of use in well-established systems of traditional medicine, pharmacological studies in animals, and its well-documented chemical composition.[9] According to above reports, the commercial value of the horseradish root is a function of morphological characteristics (i.e., diameter, length, dry weight) and flavor, associated with the presence of GLSs. Despite these properties, very little is known about the agronomic performance and composition of GLSs in horseradish, and few studies are available on the GLS profile in plants of *A. rusticana*. Some authors reported the presence of GLSs such as sinigrin, gluconasturtiin, glucobrassicin, neoglucobrassicin, progoitrin, gluconapin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin in tissues (roots and leaves) of horseradish plants grown both in vitro and in open fields and analyzed as desulfoglucosinolates by using liquid chromatography methods.[10,17] Although the glucosinolate composition of horseradish roots and black mustard seeds has been described,[18] the presence of new or putative GLSs remains to be characterized. The range of GLSs identified to date in *Brassica* species has been recently reviewed by Clarke.[1] The structural identity of GLSs is known and described only for those which can be isolated and identified by the traditional methods. The complexity of GLS composition in the plant sample increases and the concentration of each secondary metabolite becomes very low; hence, sensitive and selective analytical methods are required for their identification.

Cataldi et al.[19] developed a new LC-MS method well suited for the rapid and efficient analysis of intact and, thus, negatively charged GLSs, exemplarily for the determination of the major and minor GLS profile in crude extracts of *E. sativa*. Because the structural interpretation of the GLSs is still a major bottleneck in glucosinolate identification, Cataldi et al.[20] and Lelario et al.[21] developed new improved methods that illustrate the capability of mass spectrometry for the characterization of representative extracts of different plants belonging to the Brassicaceae family.

The purposes of this study were (i) to record the main morphological and yield traits of an accession of horseradish largely diffused and consumed in the Basilicata region of southern Italy and (ii) to identify intact GLSs in crude plant extracts of different portions (sprouts and roots) by using reversed-phase liquid chromatography coupled with electrospray ionization and a hybrid quadrupole linear ion trap and Fourier transform ion cyclotron resonance mass spectrometry (LC-ESI-LTQ-FTICR). To provide structural information of GLSs, precursor ion isolation within the linear ion trapping cell and subsequent fragmentation were induced by infrared multiphoton dissociation (IRMPD).

MATERIALS AND METHODS

Plant Material and Extract Preparation. The experiments was carried out in 2010−2011 growth year on plants of an accession of *A. rusticana* from a horseradish field established at the Institute of Plant Genetics, National Research Council, Thematic Centre for the Preservation of Mediterranean Biodiversity, located in Policoro (MT) (40° 17′ 30″ N, 16° 65′ 16″ E), where numerous accessions previously collected in the internal areas of the Basilicata region[14] were vegetatively multiplied starting in the spring of 2009.

The accession chosen for our study consisted of root cuttings, transplanted in April 2010 under the soil in a single row (130 cm between rows and 70 cm in the row). Irrigation, plant protection, and weed control were carried out according to local practices.

In September 2010, morphological traits (plant height, length and width of leaf and petiole) were measured on four plants at the end of the vegetative growth period.

In February 2011, four plants were harvested and morphological and agronomic traits were recorded (number of sprouts, root number, length and diameter, total marketable roots, and dry matter). Then, plant samples were separated into sprouts and roots, cleaned with distilled water, and dried with paper towels, and then quickly frozen at −80 °C to inactivate myrosinase.[19,20] Before analysis, sprouts were lyophilized[22,23] and ground to a fine powder using a laboratory mill, whereas roots were quickly frozen in liquid nitrogen to allow crushing and then lyophilized and ground into fine powder.

GLSs were extracted following the procedure described by Cataldi et al.[20,21] Briefly, dried plant powder (500 mg) was weighed and blended with 8 mL of a 70% v/v aqueous methanol solution at 70−80 °C, stirred for about 10 min by vortex, 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 with a syringe and filtered through a 0.22 μm nylon filter (Whatman, Maidstone, UK). The extraction procedure was repeated again with 5 mL of solvent and the supernatant was added to the first extract. The joined extract was concentrated by evaporation to dryness at 40 °C on a rotavapory (Laborota 400-efficient, Heidolph Instruments) and finally redissolved in 1.5 mL of 70% v/v aqueous methanol. The extracts, when necessary, were diluted and afterward injected in the LC-MS system without further treatment.

Chemicals. Sinigrin monohydrate from horseradish (99%) was obtained from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile (ACN), both of LC-MS grade, were obtained from Carlo Erba (Milan, Italy). Ultrapure water was produced using a Milli-Q RG system from Millipore (Bedford, MA, USA).

Instrumental and Separation Conditions. Analyses of GLSs were performed using a Surveyor LC system coupled to a Finnigan LTQ FT, a 7 T hybrid linear ion trap FTICR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a 20 W continuous CO_2 laser (Synrad, Mukilteo, WA, USA; 10.6 μm).

Instrumental control, data acquisition, and data processing were performed using the Xcalibur software package (version 2.0 SR1 Thermo Electron). The FTICR mass spectrometer was operated in negative ion mode. The LC system was simultaneously connected to the mass spectrometer and the UV-DAD detector by a laboratory-made splitter with a split ratio of 1:3. Analyses were carried out at ambient temperature on a Discovery C18 column, 250 × 4.6 mm i.d., 5 μm particle size (pore size = 180 Å), equipped with a Discovery C18 20 × 4 mm i.d. security guard cartridge (all from Supelco Inc., Bellefonte, PA, USA). Standard solutions and plant extracts were injected into the column via a 20 μL sample loop. The gradient elution of mobile phase was prepared by mixing 0.1% HCOOH water solution (solvent A) and ACN (solvent B). The gradient program was 90% A−10% B, linear gradient to 76% A−24% B in the first 10 min; changed to 40% A−60% B in the next 2 min; changed to 90% A−10% B in the next 3 min; final reconditioning of the column for 5 min to the initial conditions. The cycle time was 20 min, and the flow rate was 1.0 mL min^{-1}. The negative ion mode for ESI-MS and tandem MS analyses was selected, working under the following experimental conditions optimized by direct infusion of standard solutions into the ionization source: ESI needle voltage, −4.60 kV; capillary voltage, −22 V; temperature of the heated capillary, 350 °C; sheath gas (N_2) flow rate, 80 (arbitrary units). Full-scan experiments were performed in both the linear trap as well as the ICR cell in the range of m/z 50−1000. Mass-to-charge ratio signals (m/z) were acquired as profile data at a resolution of 100,000 (FWHM) at m/z 400. The automatic gain control (AGC) ion population target in full-scan MS was 5,000,000 for FTICR MS. The maximum ion injection time was 200 ms for FTICR. IRMPD with different pulses in terms of duration, that is, from 100 to 220 ms, at a laser power of 100 arbitrary units was applied.

The LTQ and FTICR mass spectrometers were calibrated in negative ion mode according to the manufacturer's instructions using a solution of sodium dodecyl sulfate (m/z 265) and sodium taurocholate (m/z 514). The ESI-FTICR mass spectra obtained were used to characterize the GLS ionization behavior. In addition, the ESI-IRMPD MS/MS fragmentation

performance of different GLSs was investigated. Data were collected in full MS scan mode and processed postacquisition, to reconstruct the elution profile for the ions of interest, with a given m/z value and ±0.001 Da of tolerance.

The chromatographic raw data were imported, elaborated, and plotted by SigmaPlot 9.0 (Systat Software, Inc., London, UK).

The identification of GLSs was obtained by studying the common fragments of glucosinolates, according to the method of Cataldi et al.[19,20] The facile deprotonation of these secondary metabolites obviously renders them amenable to negative ion mass spectrometry. All GLSs exhibit $[M - H]^-$ as the predominant ion, which corresponds to the deprotonation of the sulfate group. Moreover, dissociation of $[M - H]^-$ yields abundant product ions and side chain structurally informative fragments, which would be of great value for a correct assignment of known and unknown GLSs.

Among all types of GLSs found in sprouts and roots of horseradish, only one compound was identified with an authentic standard, sinigrin, which is commercially available. The identification of other GLSs was based only on interpretation of MS/MS data and measured accurate masses observed under LC/ESI-FTICR MS, according to the method of Cataldi et al.[19] For each glucosinolate found in different portions of horseradish, we determined the retention time (t_R), monoisotopic accurate value as $[M - H]^-$ ion (m/z), and tandem MS spectra of the $[M - H]^-$ ion (m/z).

RESULTS AND DISCUSSION

Horseradish is a semicultivated species largely diffused in theBasilicata region, where there is a strongly rooted culinary tradition related to the use of this plant, especially during the carnival and Easter period.

In the internal areas of such regions, horseradish can be found in home gardens and also in open fields, principally recognized as a semicultivated species, and can be produced in an annual or perennial production system; it is frequently planted in the early spring, and the roots are harvested beginning in late winter and usually continuing to early spring.

Morphological and Yield Traits of Selected Horseradish Ecotype. Thirteen morphological and agronomic traits of the horseradish are shown in Table 1: the first five traits describe the morphological characteristics of the plant during the vegetative stage, whereas the others are related to the agronomic characteristics of the roots and sprouts at the harvest.

Table 1. Morphological and Yield Traits of Horseradish (Mean Value ± SE (n = 4))

trait and units	
plant height (cm)	95 ± 0.3
leaf length (cm)	75 ± 0.3
leaf width (cm)	19 ± 0.3
petiole length (cm)	22 ± 0.4
petiole width (cm)	0.7 ± 0.3
sprouts on the crown (no.)	11 ± 1.0
marketable roots per plant (no.)	2 ± 0.3
primary root length (cm)	23 ± 0.7
primary root diameter at top (cm)	6 ± 1.0
primary root diameter at base (cm)	2 ± 0.6
dry matter root (%)	37 ± 0.1
total fresh weight of roots per plant (g)	1056 ± 6.7
marketable fresh weight of roots per plant (g)	733 ± 1.3

Plants showed a vigorous *habitus* and reached an average height of 95 cm, with well-developed leaves having a long petiole of 22 cm, on average, and two primary roots, with a length, diameter at the top, and diameter at the base of 23, 6, and 2 cm, respectively, and a great number of sprouts on the crown. The total fresh weight of roots, on average, was 1056 g per plant, 70% of which was the weight of marketable fresh root and 37% the root dry matter. Literature information on root yield in the open field is very scarce, and results are often not in agreement with each other.[9]

During the vegetative stage, the accession selected for this experiment showed a vigorous growth with long and thick leaves, healthy looking with a bright green color. It was early growing and resistant to major disease such as *Verticillium* spp. and *Fusarium* spp., which can cause the complex of internal discoloration of horseradish roots, reducing its commercial value.[15]

The accession developed many sprouts and produced white, fiery, and sharp-flavored marketable roots. Such characteristics make this accession suitable for the agricultural and food commercial sector.

Identification of Glucosinolates' Profile in Sprouts and Roots of Horseradish. As all Brassicaceae, horseradish produces various sulfur-containing glucosides, that is, glucosinolates.[10] In fact, glucosinolates occur in numerous vegetables and species such as cabbage, broccoli, cauliflower, cress, mustard, and horseradish. Intact glucosinolates and their breakdown products possess chemo-ecological functions and serve both as defense mechanisms against herbivores and pathogens and as attractant to specialized toxin-tolerant insects. Moreover, interest in the role of dietary glucosinolates has been stimulated by the observation of cancer chemopreventive properties of certain breakdown products.[24]

Fourier transform ion cyclotron resonance (FTICR) mass spectrometry is one of the techniques that can provide high mass accuracy and high mass resolution.[24] Accurate molecular weight, elemental composition, and structural information can be achieved from FTICR. Infrared multiphoton dissociation (IRMPD) is a dissociation technique often used with FTICR for MS/MS analysis. No collision gas is required for IRMPD; instead, the 20 W CO_2 laser is used to irradiate the ions to form fragments. The fragments may continue to acquire some energy from the infrared laser pulse and further fragment to ions of lower masses.

Previous studies showed that GLSs form characteristic fragments during MS^2 analysis by ion trap and FTICR-IRMPD mass spectrometers.[19,20,26]

Very little is known about the GLS composition of horseradish.[17] Our results confirmed the presence of major GLSs found in a few previous papers.[10,17,18] In addition, we have found different compounds that have shown m/z values, mass spectra, and retention times similar to those reported by several authors for other GLSs in different species of Brassicaceae such as rocket salad[19,20] and other edible *Brassica* species.[1,21]

The selective extracted ion chromatograms (XICs) of GLSs identified in sprouts and roots obtained by FTICR MS are shown in retention time order, respectively, in Figures 1 and 2.

In Table 2 are shown the GSLs found in these samples indicated by peak number used to identify each GLS in the XICs of Figures 1 and 2, common name, side chain name (R), retention time (t_R), molecular formula, monoisotopic exact values as $[M - H]^-$ ion (m/z), and mass error (ppm) obtained by using LC-ESI-FTICR MS, whereas in Table 3 are shown the main IRMPD MS/MS product ions (accurate m/z) and mass error (ppm) of each identified GSL.

As shown in Figure 1, in sprouts have been identified 16 main peaks corresponding to different GLSs with various retention times ranging from 3.8 to 16.7 min and monoisotopic nominal values ranging from m/z 358 to 478. In particular, we have recognized glucoiberin (peak 1), sinigrin (peak 2), gluconapin

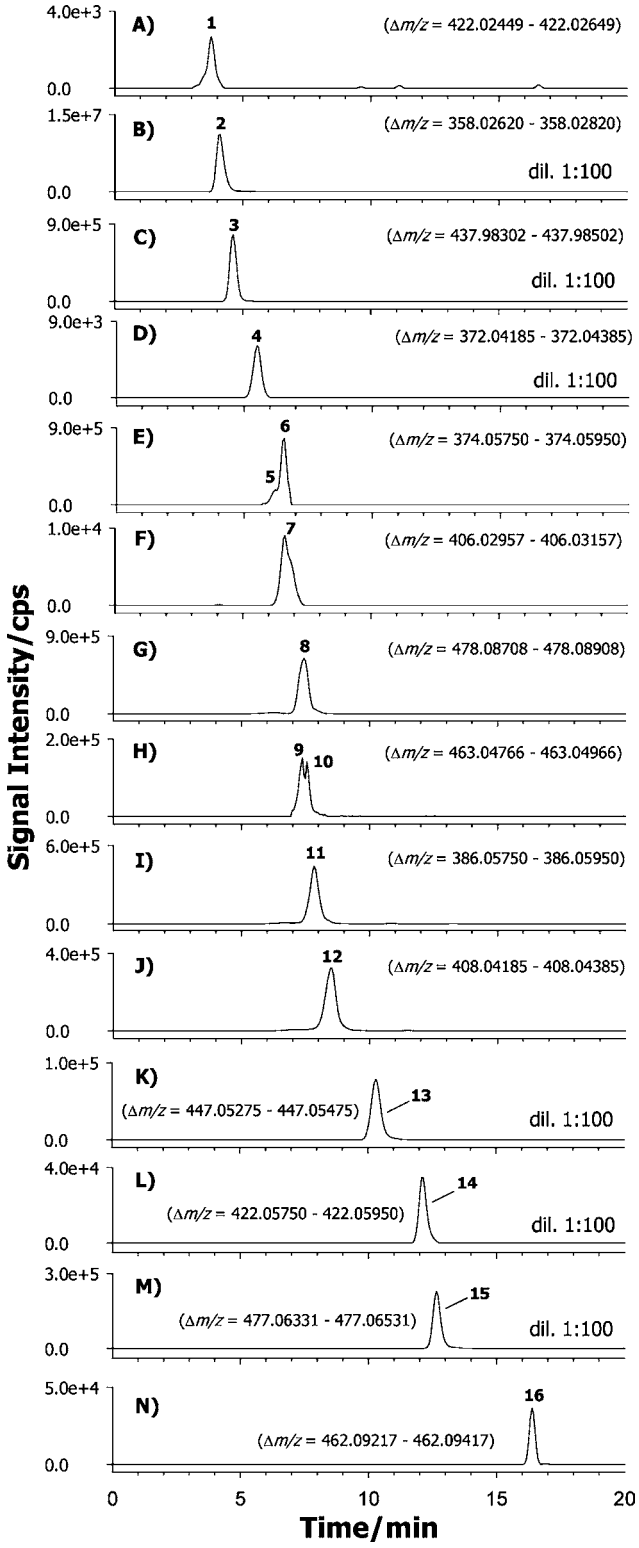

Figure 1. XICs by LC/ESI-FTICRMS acquired in negative ion mode of sprout crude extract of *A. rusticana*. Ions monitored are displayed in each trace (A–N) and correspond to the most abundant deprotonated molecules, $[M - H]^-$, using a restricted window of m/z ±0.0010 unit centered around each selected ion. Peaks: (1) glucoiberin; (2) sinigrin; (3) 2-methylsulfonyl-oxo-ethyl-GLS; (4) gluconapin; (5) glucocochlearin; (6) glucoconringianin; (7) glucosativin; (8) glucoibarin; (9) 4-hydroxyglucobrassicin; (10) 5-hydroxyglucobrassicin; (11) glucocapparilinearisin or glucobrassicanapin; (12) glucotropaeolin; (13) glucobrassicin; (14) gluconasturtiin; (15) 4-methoxyglucobrassicin; (16) glucoarabishirsutain. Numbers in parentheses indicate the range of monoisotopic value as [M − H]− ion (m/z).

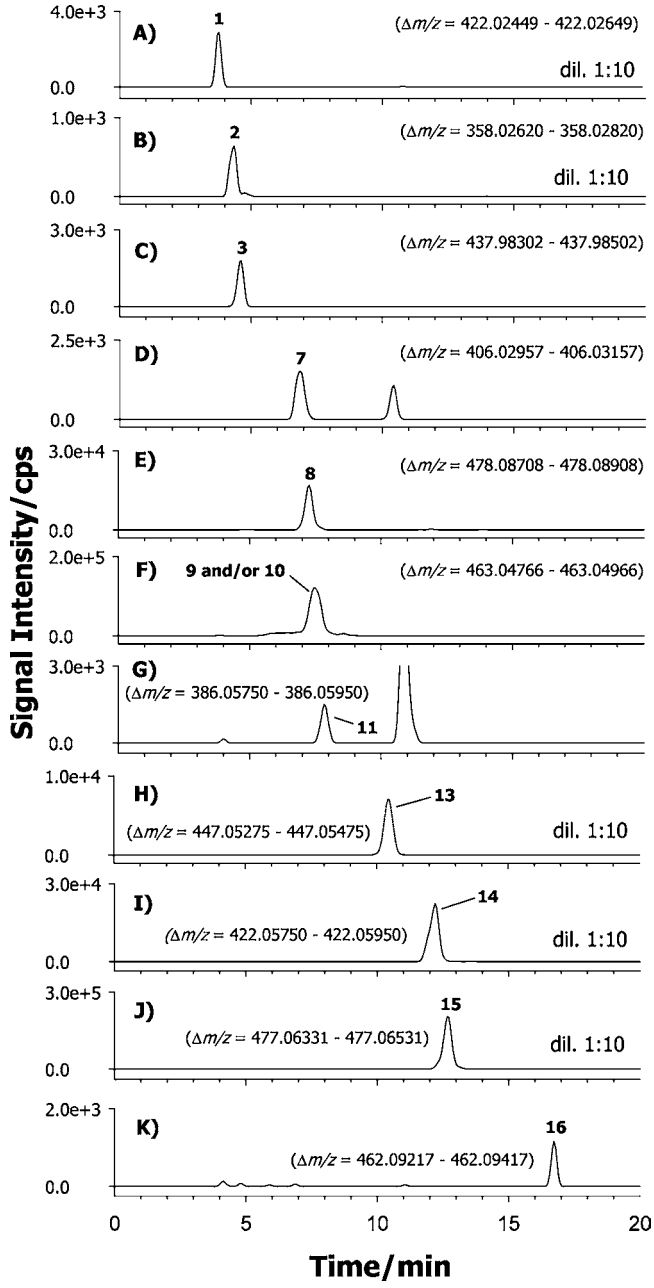

Figure 2. XICs by LC/ESI-FTICRMS acquired in negative ion mode of root crude extract of *A. rusticana*. Ions monitored are displayed in each trace (A–N) and correspond to the most abundant deprotonated molecules, $[M - H]$, using a restricted window of m/z ±0.0010 unit centered around each selected ion. Peaks: (1) glucoiberin; (2) sinigrin; (3) 2-methylsulfonyl-oxo-ethyl-GLS; (7) glucosativin; (8) glucoibarin; (9) 4-hydroxyglucobrassicin and/or (10) 5-hydroxyglucobrassicin; (11) glucocapparilinearisin or glucobrassicanapin; (13) glucobrassicin; (14) gluconasturtiin; (15) 4-methoxyglucobrassicin; (16) glucoarabishirsutain. Numbers in parentheses indicate the range of monoisotopic value as $[M - H]^-$ ion (m/z).

(peak 4), glucocochlearin (peak 5), glucoconringianin (peak 6), glucosativin (peak 7), glucoibarin (peak 8), 4-hydroxyglucobrassicin (peak 9), 5-hydroxyglucobrassicin (peak 10), glucocapparilinearisin or glucobrassicanapin (peak 11), glucotropaeolin (peak 12), glucobrassicin (peak 13), gluconasturtiin (peak 14), and 4-methoxyglucobrassicin (peak 15), which are the typical glucosinolates reported in the literature. According to Clarke,[1] it is difficult to give a name to some GLSs, because the

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Table 2. Glucosinolates Identified in Different Portions of Horseradish Plants by LC-ESI-FTICR MS[a]

peak	GLS common name	R side chain	t_R, min	molecular formula	monoisotopic calcd value as $[M-H]^-$ ion, m/z	mass error, ppm[b]
1	glucoiberin	3-methylsulfinyl propyl	4.3	$C_{11}H_{21}NO_{10}S_3$	422.02549	−1.30
2	sinigrin	2-propenyl	4.4	$C_{10}H_{17}NO_9S_2$	358.02720	−0.59
3	2-methylsulfonyl-oxo-ethyl-GLS	2-methylsulfonyl-oxo-ethyl	4.6	$C_{10}H_{17}NO_{12}S_3$	437.98402	−1.21
4	gluconapin	3-butenyl	5.5	$C_{11}H_{19}NO_9S_2$	372.04285	−1.30
5	glucocochlearin	1-methylpropyl	6.2	$C_{11}H_{21}NO_9S_2$	374.05850	1.00
6	glucoconringianin	2-methylpropyl	6.4	$C_{11}H_{21}NO_9S_2$	374.05850	−0.70
7	glucosativin	4-mercaptylbutyl	6.5	$C_{11}H_{21}NO_9S_3$	406.03057	1.35
8	glucoibarin	(*R*)-7-(methylsulfinyl) heptyl	7.3	$C_{15}H_{29}NO_{10}S_3$	478.08808	−0.10
9	4-hydroxyglucobrassicin	4-hydroxy-3-indolyl methyl	7.4	$C_{16}H_{20}N_2O_{10}S_2$	463.04866	−1.50
10	5-hydroxyglucobrassicin	5-hydroxy-3-indolyl methyl	7.5	$C_{16}H_{20}N_2O_{10}S_2$	463.04866	−1.70
11	glucocapparilinearisin or glucobrassicanapin	3-methyl-3-butenyl or pentenyl	7.8	$C_{12}H_{21}NO_9S_2$	386.05850	−0.20
12	glucotropaeolin	benzyl	8.5	$C_{14}H_{19}NO_9S_2$	408.04285	−1.10
13	glucobrassicin	3-indolylmethyl	10.3	$C_{16}H_{20}N_2O_9S_2$	447.05375	−1.50
14	gluconasturtiin	2-phenylethyl	12.1	$C_{15}H_{21}NO_9S_2$	422.05850	−1.20
15	4-methoxyglucobrassicin	4-methoxy-3-indolylmethyl	12.7	$C_{17}H_{22}N_2O_{10}S_2$	477.06431	−1.20
16	glucoarabishirsutain	7-(methylthio)heptyl	16.7	$C_{15}H_{29}NO_9S_3$	462.09317	−1.20

[a]Peak number, common name, side chain name (R), retention time (t_R), molecular formula, monoisotopic value as $[M-H]^-$ ion (m/z), mass error (ppm). [b]Mass error in parts per million, ppm = 10^6 × (accurate mass − exact mass)/exact mass.

Table 3. IRMPD FTICR MS Product Ions Obtained from the Deprotonated Ions of the Glucosinolates Identified in Different Portions of Horseradish Plants[a]

peak	compound	precursor ion	main IRMPD MS/MS product ions, accurate m/z; and mass error (ppm)[b]
1	glucoiberin	422	358.02771 (1.42); 259.01318 (0.98); 241.00256 (0.83); 96.96014 (0.39)
2	sinigrin	358	274.99030 (0.79); 259.01327 (1.33); 241.00223 (−0.54); 195.03329 (0.11); 179.99740 (1.02); 161.98670 (0.25); 116.01759 (0.28); 96.96015 (0.49); 74.99106 (0.68)
3	2-methylsulfonyl-oxo-ethyl-GLS	438	358.02764 (1.23); 259.01324 (1.21); 241.00312 (1.08); 195.97420 (−0.87); 179.99746 (1.35); 96.96015 (0.49)
4	gluconapin	372	292.08652 (1.68); 274.99039 (1.12); 259.01324 (1.21); 241.00269 (1.37); 194.01308 (1.10); 138.97079 (0.88); 130.03331 (0.77); 96.96016 (0.59); 74.99104 (0.42)
5	glucocochlearin	374	294.10189 (0.71); 274.99009 (0.03); 259.01321 (1.09); 241.00232 (−0.17); 96.96001(−0.95); 74.99099 (−0.25)
6	glucoconringianin	374	294.10197 (0.99); 274.99015 (0.25); 259.01337 (1.71); 241.00225 (−0.46); 96.96011 (0.08); 74.99104 (0.42)
7	glucosativin	406	259.01312 (0.75); 241.00272 (1.49); 96.96013 (0.28); 74.99103 (0.28)
8	glucoibarin	478	414.09042 (1.52); 358.02756 (1.60); 274.99033 (0.90); 259.01306 (0.52); 241.00200 (−1.49); 96.96012 (0.18); 74.99107 (0.82)
9	4-hydroxyglucobrassicin	463	285.01823 (−1.58); 267.00805 (−0.26); 259.01304 (0.44); 96.96004 (−0.64)
10	5-hydroxyglucobrassicin	463	285.01853 (0.53); 267.00825 (0.49); 259.01314 (0.83); 96.96014 (0.39)
11	glucocapparilinearisin or glucobrassicanapin	386	224.00614 (−0.36); 274.99033 (0.90); 259.01318 (0.98); 208.02867 (−1.87); 195.03342 (0.78); 144.04910 (1.67); 96.96014 (0.39); 74.99104 (0.42)
12	glucotropaeolin	408	274.99027 (0.98); 259.01306 (0.52); 230.01062 (0.65); 96.96016 (0.59); 74.99110 (1.22)
13	glucobrassicin	447	274.99035 (0.97); 269.02430 (2.00); 259.01349 (1.83); 241.00280 (1.83); 205.04450 (1.90); 138.97079 (0.86); 96.96017 (0.70); 74.99104 (0.42)
14	gluconasturtiin	422	342.10164 (0.12); 274.99031 (0.83); 259.01316 (0.90); 244.02839 (−0.49); 241.00356 (0.83); 195.03394 (−1.18); 180.04942 (0.89); 96.96017 (0.70); 74.99104 (0.42)
15	4-methoxyglucobrassicin	477	274.99030 (0.79); 259.01316 (0.90); 195.03342 (0.78); 96.96015 (0.49); 74.99106 (0.68)
16	glucoarabishirsutain	462	138.97089 (1.58); 96.96010 (−0.03); 74.99105 (0.55)

[a]Peak number, compound, isolated precursor ion, IRMPD FTICR MS product ions (accurate m/z), and mass error (ppm). [b]Mass error in parts per million (ppm) evaluated as 10^6 × (experimental accurate mass − monoisotopic calculated mass)/monoisotopic calculated mass.

LC-MS cannot discriminate between the numerous GLS isomers. Among these GLSs, the species at m/z 374.05850 (exact value), peaks 5 and 6, not already reported in horseradish, can correspond to glucocochlearin and glucoconringianin, two GLS isomers with respective retention times of 6.2 and 6.4 min, already reported by Lelario et al.[21] as found in other Brassicaceae plants. Similarly, two isomers barely separated at retention times of 7.4 and 7.5 min, peaks 9 and 10, were assigned, respectively, in order of elution to 4-hydroxyglucobrassicin and 5-hydroxyglucobrassicin.[19] By using accurate masses, the common names of the other GLSs, peaks 3 and 16, have been also tentatively assigned to 2-methylsulfonyl-oxo-ethyl-GLS and glucoarabishirsutain, respectively. Of particular note was the occurrence of putative 2-methylsulfonyl-oxo-ethyl-GLS at m/z 437.98402 (peak 3), with the molecular formula $C_{10}H_{17}NO_{12}S_3$, as reported in Table 2.

The XIC signals of sinigrin (peak 2), 2-methylsulfonyl-oxo-ethyl-GLS (peak 3), gluconapin (peak 4), glucobrassicin (peak 13), gluconasturtiin (peak 14), and 4-methoxyglucobrassicin (peak 15) were acquired using a sample extract diluted 1:100 with the mobile phase.

Interestingly, only 6 of the 16 GLSs found in sprouts (sinigrin, gluconasturtin glucobrassicin, gluconapin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin) have been already reported by other authors in horseradish.

In roots, as reported in Figure 2, we have identified 11 GLSs: glucoiberin (peak 1); sinigrin (peak 2); 2-methylsulfonyl-oxo-ethyl-GLS (peak 3); glucosativin (peak 7); glucoibarin (peak 8); 4-hydroxyglucobrassicin (peak 9) and/or 5-hydroxyglucobrassicin (peak 10); glucocapparilinearisin or glucobrassicanapin (peak 11); glucobrassicin (peak 13); gluconasturtiin (peak 14); 4-methoxyglucobrassicin (peak 15); and glucoarabishirsutain (peak 16). Others GLSs shown in Table 2 were present in trace amounts.

The XIC signals of glucoiberin (peak 1), sinigrin (peak 2), glucobrassicin (peak 13), gluconasturtiin (peak 14), and 4-methoxyglucobrassicin (peak 15) were acquired using a sample extract diluted 1:10 with the mobile phase.

The GLSs tentatively assigned by using only accurate masses and retention time values have been confirmed by fragmentation studies using IRMPD as a dissociation technique.

Accurate mass data of main IRMPD MS product ions (Table 3) were obtained as deprotonated molecules, with a mass error not greater than 1.9 ppm, indicating a very good mass accuracy. Such a fragmentation was found to be very useful in terms of structural identification of all precursor ions $[M - H]^-$, including GLSs reported here for the first time; in fact, these product ions constitute the basis for identification of GLSs by matching their m/z values with those reported for known GLSs. For MS/MS experiments, the molecular ions were isolated in the linear ion trap, accelerated toward the ICR cell, and dissociated. Some similar fragmentation patterns of GLSs were observed, indicating the common structures among these compounds. The typical fragments of GLS ions with exact m/z 96.96010, 195.03327, 259.01292, 274.99008, and 241.00236, which correspond to the fragment ions from the glycone side chain, namely, HSO_4^-, $C_6H_{11}O_5S^-$, $C_6H_{11}O_9S^-$, $C_6H_{11}O_8S_2^-$, $C_6H_9O_8S^-$, respectively, were found in spectra examined as they share a common structure. Other characteristic fragments including $[M - 196 - H]^-$, $[M - 178 - H]^-$, $[M - 162 - H]^-$, $[M - 242 - H]^-$, and $[M - 80 - H]^-$ were very informative for correct molecular identification of GLSs.

However, there were unique fragmentations reflecting the structural characteristics that can be used to identify these compounds.

In Figures 3 and 4 are reported the IRMPD MS spectra of all main glucosinolate ions reported in Figures 1 and 2. Each GLS showed well-defined characteristic fragments.

Particularly, in Figure 3 are reported GLSs with retention times ranging from 3.8 to 7.3. In Figure 3A is shown the IRMPD-FTICR mass spectrum of glucoiberin, precursor ion isolated at m/z 422 (exact m/z 422.02594), with production of four well-defined characteristic fragments having mass errors not greater than 1.42 ppm. The predominant species in the spectrum is the $[HSO_4]^-$ ion at m/z 96.96014, being a good clue that the compound belongs to the GLS family. The presence of an ion at m/z 358.02771 confirmed the nature of the R group of this compound. In Figure 3B is shown the precursor ion of sinigrin at m/z 358 (exact m/z 358.02720) and well-defined product ions at m/z 274.99030, 259.01327, 241.00223, 195.03329, 179.99740, 161.98670, 116.01759, 96.96015, and 74.99106 with mass errors not greater than 1.33 ppm. In Figure 3C are shown the characteristic fragments of 2-methylsulfonyl-oxo-ethyl-GLS at m/z 438 ion (exact m/z 437.98402), the product ions of which are formed by loss of neutral molecules; each well-defined fragment gives a good indication about the occurrence of this putative GLS, not already reported. In Figure 3D is displayed the precursor ion peak of gluconapin at m/z 372 (exact m/z 372.04285), and its product ion spectrum fragmented with a mass error not greater than 1.68 ppm. Figure 3E reports the precursor ion of glucoconringianin at m/z 374 (exact m/z 374.05850) and its well-defined product ions with a mass error not greater than 1.09 ppm. The IRMPD spectrum of glucocochlearin, an isomer of glucoconringianin, was very similar to this spectrum (data not shown). Typical fragment ions are obtained by precursor ion at m/z 406 (exact m/z 406.03057) as shown in Figure 3F. The predominant species in the spectrum, the $[HSO_4]^-$ ion at m/z 96.96013, and the presence of ions at m/z 259.01312, 241.00272, and 74.99103 indicate that it is a glucosinolate, and we tentatively assigned the name as glucosativin, on the basis of retention time. At the end, in Figure 3G are indicated the precursor ion at m/z 478 (exact 478.08808) and common fragments with a mass error not greater than 1.60 ppm. In addition, the presence of ion fragments at m/z 414.09042 and 358.02756 corresponding to $[M - CH_4OS - H]^-$ and $[M - C_5H_{12}OS - H]^-$, respectively, confirmed the R group nature.

In Figure 4 are reported IRMPD MS spectra of GLSs with retention times ranging from 7.5 to 16.7.

Figure 4A shows the fragmentation of 5-hydroxyglucobrassicin as precursor ion with characteristic fragments, namely, HSO_4^- at m/z 96.96014, $C_6H_{11}O_9S^-$ at m/z 259.01314, $[M - 178 - H]^-$ at 285.01853, and $[M - 196 - H]^-$ at m/z 267.00825, with mass errors not greater than 0.83 ppm. 4-Hydroxyglucobrassicin showed similar fragmentation (data not reported). The IRMPD MS spectrum (Figure 4B) of the precursor ion at m/z 386 (exact m/z 386.05850) is rich in well-defined characteristic ions, for example, $[M - 242 - H]^-$ at m/z 144.04910 and $[M - 162 - H]^-$ at m/z 224.00614, with mass errors not greater than 0.98 ppm. Figure 4C shows the IRMPD MS spectrum of glucotropaeolin, precursor ion at m/z 408 (exact m/z 408.04285), and its correspondent characteristic ions. Figure 4D illustrates the high-resolution IRMPD mass spectrum of the deprotonated glucobrassicin at m/z 447 with characteristic common fragments. Of particular importance was the presence of a fragment ion at m/z 138.97079 corresponding to the neutral loss of $C_4H_8O_4$[20] from typical fragment $[C_6H_{11}O_9S]^-$ at m/z 259.01340. Many typical fragment ions are obtained by IRMPD fragmentation of gluconasturtiin, precursor ion at m/z ion 422 (exact m/z 422.05850), as shown in Figure 4E. Figure 4F reports the precursor ion at 477 (exact m/z 477.06431) and its well-defined product ions with a mass error not greater than 0.90 ppm. Finally, Figure 4G shows the precursor ion at m/z 462 (exact m/z 462.09317) and its corresponding characteristic ions; in particular, the predominant species in the spectrum are the $[HSO_4]^-$ ion at m/z 96.96010 and fragment ions at m/z 74.99105 and 138.97089; we tentatively assigned the name as glucoarabishirsutain, but further investigations are necessary.

All data give a complex profile of naturally occurring glucosinolates in the roots and sprouts of horseradish by using the LC-ESI-FTICR MS based on electrospray ionization (ESI) and IRMPD fragmentation. Results obtained can contribute to a better understanding of glucosinolates profile in the *A. rusticana* plant.

Among GLSs, the signals of sinigrin and 4-methoxyglucobrassicin were more intense in sprout and root extracts, respectively. By comparing the GLS signal intensities of these two portions of horseradish, sprouts were found to be richer in

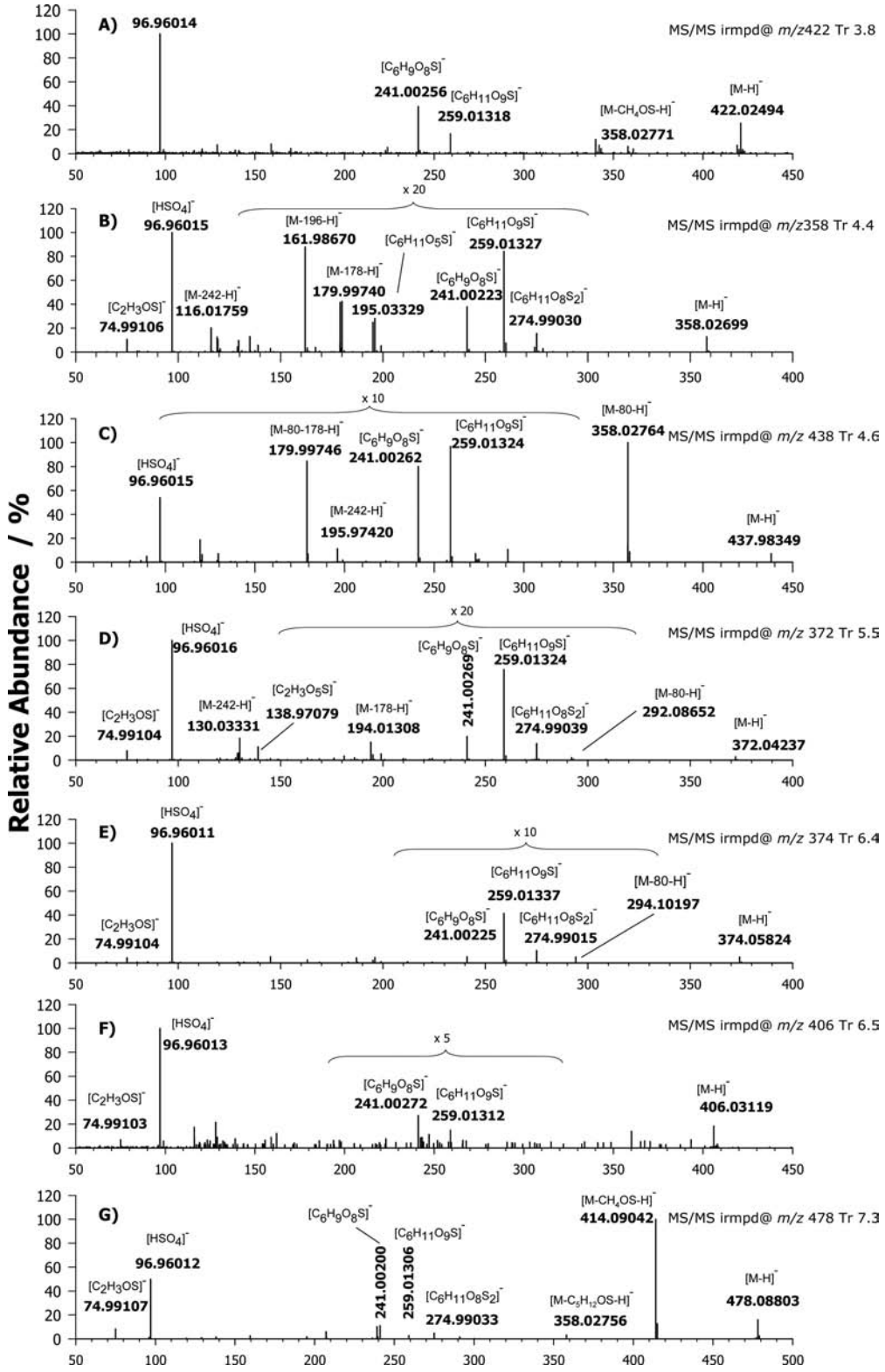

Figure 3. High-resolution IRMPD mass spectra of the deprotonated GLSs identified in *A. rusticana*: glucoiberin at *m*/*z* 422; sinigrin at *m*/*z* 358; 2-methylsulfonyl-oxo-ethyl-GLS at *m*/*z* 438; gluconapin at *m*/*z* 372; glucoconringianin at *m*/*z* 374; glucosativin at *m*/*z* 406; glucoibarin at *m*/*z* 478.

GLSs with respect to roots. These results are in partial agreement with Redovnikovic et al.,[10] who reported that sinigrin, being >80% of total GLSs, was quantitatively dominant in plantlets.

Such a difference of sinigrin signal intensity between sprouts and roots is probably due to a defense role against pathogens, which feed on green tender tissues. In any case, Blazevic and Mastelic[27] reported that GLSs can be found in the roots, seeds, leaves, and stems of the plant and that the youngest tissues contain the highest amounts. Moreover, GLSs have several functions in the plant: plant defense against fungal diseases and pest infestation, sulfur and nitrogen metabolism, and growth regulation. In addition, according to Bellostas et al.,[28] after ripe

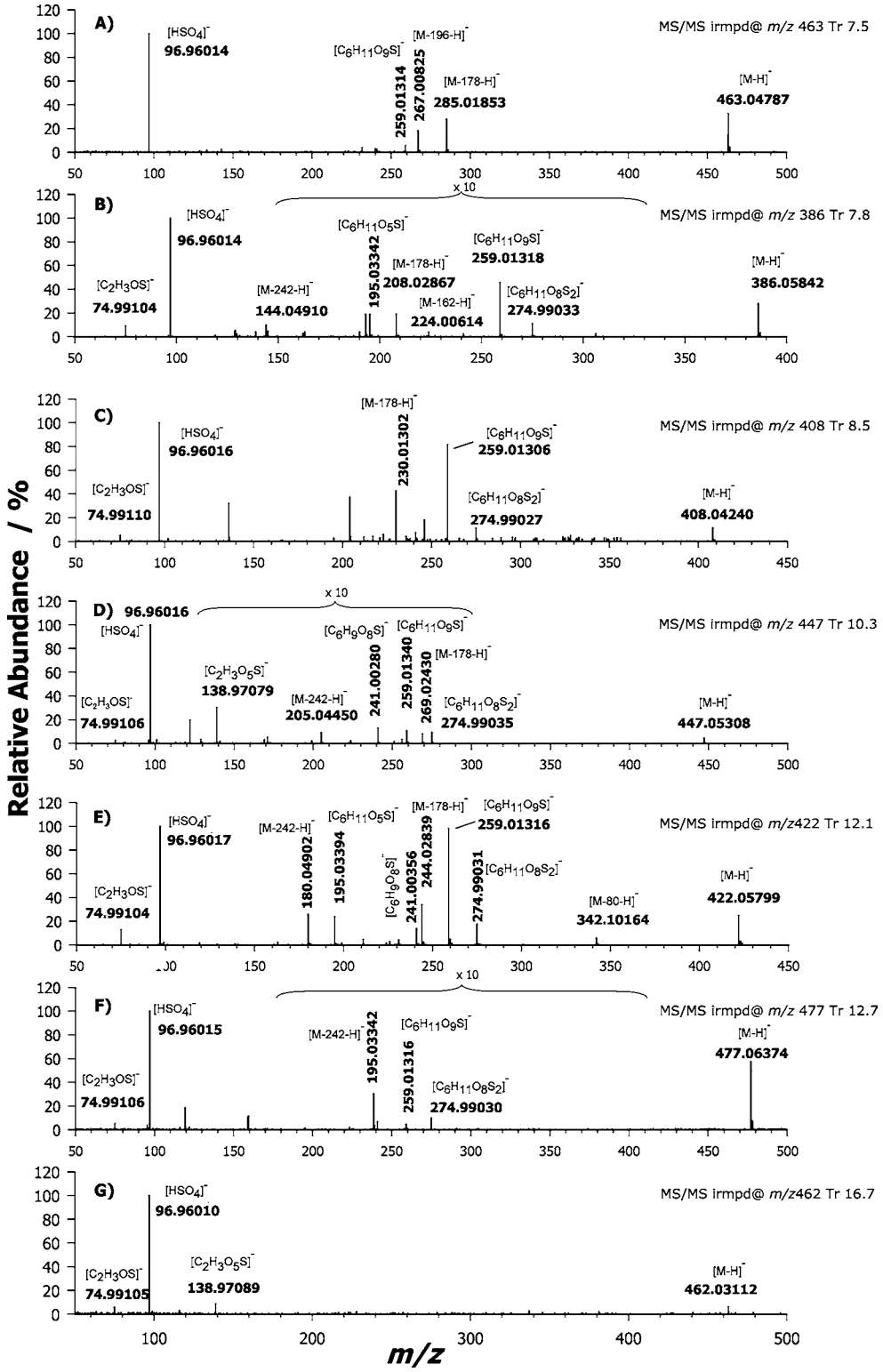

Figure 4. High-resolution IRMPD mass spectra of the deprotonated GLSs identified in *A. rusticana*: 5-hydroxyglucobrassicin at *m*/*z* 463; glucocapparilinearisin or glucobrassicanapin at *m*/*z* 386; glucotropaeolin at *m*/*z* 408; glucobrassicin at *m*/*z* 447; gluconasturtiin at *m*/*z* 422; 4-methoxyglucobrassicin at *m*/*z* 477; glucoarabishirsutain at *m*/*z* 462.

seeds, sprouts of some Brassicaceae plants contain the highest concentration of these compounds and are therefore a good source of GLSs for chemoprotection. The concentration of potentially health-beneficial GLSs for a given species has been found to be greater in sprouts than in fully grown plants. However, studies of the GLSs' content of the growing sprouts are scarce, and much of the literature still focuses on either fully grown plants of *Brassica oleracea* crops or seeds.[28]

Several studies have examined changes in total and individual glucosinolates as a function of tissue age.[29] Cleemput and Becker[30] suggested that vegetative parts mainly provide precursors and that the final step for GLS synthesis occurs in

the seed, perhaps as a consequence of the specialized physiology or defense need of this organ.[25] Among GLSs, independent of organs, Magrath et al.[31] reported that mainly genetic factors determine the sinigrin content, whereas environmental factors have a significant influence on glucobrassicin and neoglucobrassicin contents.

Our results highlight the complex profile of the GLSs in both portions, roots and sprouts, of the plant of horseradish, and the identification of some GLSs not previously characterized yet. Further studies are required to quantify the content of these molecules that are responsible for the typical flavor, which is one of the main characteristics for making a high-quality product together a high marketable root yield, as requested for the agro-industrial and pharmaceutical sectors.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Clarke, B. Glucosinolates, structure and analysis food. *Anal. Methods* **2010**, *2*, 310−325.

(2) Fahey, J. W.; Zalcmann, A. T.; Talalay, P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* **2001**, *56*, 5−51.

(3) Hashem, F. A.; Saleh, M. M. Antimicrobial components of some cruciferae plants (*Diplotaxis harra* Forsk. and *Erucaria microcarpa* Boiss.). *Phytother. Res.* **1999**, *13*, 329−332.

(4) Beevi, S. S.; Mangamoori, L. N.; Dhand, V.; Ramakrishna, D. S. Isothiocyanate profile and selective antibacterial activity of root, stem and leaf extracts derived from *Raphanus sativus* L. *Foodborne Pathogens Dis.* **2009**, *6* (1), 129−136.

(5) Lazzeri, L.; Tacconi, R.; Palmieri, S. In vitro activity of some glucosinolates and their reaction products toward a population of the nematode *Heterodera schachtii*. *J. Agric. Food Chem.* **1993**, *41*, 825−829.

(6) Mari, M.; Bertolini, P.; Pratella, G. C. Non-conventional methods for the control of post-harvest pear diseases. *J. Appl. Microbiol.* **2003**, *94*, 761−766.

(7) Shapiro, T. A.; Fahey, J. W.; Wade, K. L.; Stephenson, K. K.; Talalay, P. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. *Cancer Epidemiol., Biomarkers Prevention* **2001**, *10*, 501−508.

(8) Traka, M.; Mithen, R. Glucosinolates, isothiocyanates and human health. *Phytochem Rev.* **2009**, *8*, 269−282.

(9) Shehata, A.; Mulwa, R. M. S.; Babadoost, M.; Uchanski, M.; Norton, M. A.; Skirvin, R.; Walters, S. A. Horseradish: botany, horticulture, breeding. In *Horticultural Reviews, 35*; Janick, J., Ed.; Wiley: Hoboken, NJ, USA, 2009; Vol. *4*, pp 221−261.

(10) Redovniković, R. I.; Peharec, P.; Marijana, K.; Karmela, D.; Brkić, K.; Vorkapić-Furač, J. Glucosinolate profiles, myrosinase and peroxidase activity in horseradish (*Armoracia lapathifolia* Gilib.) plantlets, tumour and teratoma tissues. *Food Technol. Biotechnol.* **2008**, *46* (3), 317−321.

(11) Sampliner, D.; Miller, A. Ethnobotany of horseradish (*Armoracia rusticana*, Brassicaceae) and its wild relatives (*Armoracia* spp.): reproductive biology and local uses in their native ranges. *Econ. Bot.* **2009**, *63* (3), 303−313.

(12) Tedeschi, P.; Leis, M.; Pezzi, M.; Civolani, S.; Maietti, A.; Brandolini, V. Insecticidal activity and fungitoxicity of plant extracts and components of horseradish (*Armoracia rusticana*) and garlic (*Allium sativum*). *J. Environ. Sci. Health, Part B* **2011**, *46* (6), 486−490.

(13) Wedelsbäck, B. K.; Olsson, K. M. Introduction and use of horseradish (*Armoracia rusticana*) as food and medicine from antiquity to the present: emphasis on the Nordic countries. *J. Herbs, Spices Med. Plants* **2011**, *17* (3), 197−213.

(14) Walters, A. S.; Wahle, E. A. Horseradish production in Illinois. *HortTecnology* **2010**, *20* (2), 267−276.

(15) Sarli, G.; De Lisi, A.; Agneta, R.; Grieco, S.; Ierardi, G.; Montemurro, F.; Negro, D.; Montesano, V. Collecting horseradish (*Armoracia rusticana*, Brassicaceae): local uses and morphological characterization in Basilicata (southern Italy). *Genet. Resour. Crop Evol.* **2011**, DOI: 10.1007/s10722-011-9730-5.

(16) Pieroni, A.; Quave, C. L. Traditional pharmacopoeias and medicines among Albanians and Italians in southern Italy: a comparison. *J. Ethnopharmacol.* **2005**, *101*, 258−270.

(17) Li, X.; Kushad, M. M. Correlation of glucosinolates content to myrosinase activity in horseradish (*Armoracia rusticana*). *J. Agric. Food Chem.* **2004**, *52*, 6950−6955.

(18) Rosa, E. A. S.; Heaney, R. K.; Fenwick, G. R.; Portas, C. A. M. Glucosinolates in crop plants. In *Horticultural Reviews, 19*; Janick, J., Ed.; Wiley: Hoboken, NJ, USA, 1997; Vol. *3*, pp 99−115.

(19) Cataldi, T. R. I.; Rubino, A.; Lelario, F.; Bufo, S. A. Naturally occurring glucosinolates in plant extracts of rocket salad (*Eruca sativa* L.) identified by liquid chromatography coupled with negative ion electrospray ionization and quadrupole ion-trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21* (14), 2374−2388.

(20) Cataldi, T. R. I.; Lelario, F.; Orlando, D.; Bufo, S. A. Collision-induced dissociation of the A + 2 isotope ion facilitates glucosinolates structure elucidation by electrospray ionization-tandem mass spectrometry with a linear quadrupole ion trap. *Anal. Chem.* **2010**, *82* (13), 5686−5696.

(21) Lelario, F.; Bianco, G.; Bufo, S. A.; Cataldi, T. R. 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. *Phytochemistry* **2012**, *73* (1), 74−83.

(22) Mithen, R. F.; Dekker, M.; Verkerk, R.; Rabot, S.; Johnson, I. T. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. *J. Sci. Food Agric.* **2000**, *80*, 967−984.

(23) Moreno, D. A.; Carvajal, M.; López-Berenguer, C.; García-Viguera, C. Chemical and biological characterisation of nutraceutical compounds of broccoli. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1508−1522.

(24) Mohna, T.; Cutting, B.; Ernst, B.; Hamburger, M. Extraction and analysis of intact glucosinolates—a validated pressurized liquid extraction/liquid chromatography−mass spectrometry protocol for *Isatis tinctoria*, and qualitative analysis of other cruciferous plants. *J. Chromatogr., A* **2007**, *1166*, 142−151.

(25) Li, W.; Hendrickson, C. L.; Emmett, M. R.; Marshall, A. G. Identification of Intact proteins in mixtures by alternated capillary liquid chromatography electrospray ionization and LC ESI infrared multi-photon dissociation Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **1999**, *71*, 4397.

(26) Bianco, G.; Lelario, F.; Battista, F. G.; Bufo, S. A.; Cataldi, T. R. I. Identification of glucosinolates in capers by LC-ESI using a hybrid linear ion trap with Fourier-transform ion cyclotron resonance mass spectrometry (LC-ESI-LTQ-FTICR-MS) and infrared multiphoton dissociation. *J. Mass Spectrom.* **2012**, DOI: 10.1002/jms.2996.

(27) Blazevic, I.; Mastelic, J. Glucosinolate degradation products and other bound and free volatiles in the leaves and roots of radish (*Raphanus sativus* L.). *Food Chem.* **2009**, *113*, 96−102.

(28) Bellostas, N.; Kachlicki, P.; Sørensen, J. C.; Sørensen, H. Glucosinolate profiling of seeds and sprouts of *B. oleracea* varieties used for food. *Sci. Hortic.* **2007**, *114*, 234−242.

(29) Brown, P. D.; Tokuhisa, J. G.; Reichelt, M.; Gershenzon, J. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry* **2003**, *62*, 471−481.

(30) Cleemput, S.; Becker, H. C. Genetic variation in leaf and stem glucosinolates in resynthesized lines of winter rapeseed (*Brassica napus* L.). *Genet. Resour. Crop Evol.* **2011**, DOI: 10.1007/s10722-011-9701-x.

(31) Magrath, R.; Herron, C.; Giamoustaris, A.; Mithen, R. The inheritance of aliphatic glucosinolates in *Brassica napus*. *Plant Breed.* **1993**, *111*, 55−72.