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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

Filomena Lelario [a], Giuliana Bianco [b], Sabino A. Bufo [a], Tommaso R.I. Cataldi [c,*]

[a] Department of Agriculture, Forestry and Environment, University of Basilicata, Potenza, Italy
[b] Department of Chemistry, University of Basilicata, Potenza, Italy
[c] Department of Chemistry, University Aldo Moro of Bari, Bari, Italy

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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

* Corresponding author. Address: Università degli Studi di Bari "Aldo Moro", Dipartimento di Chimica, Campus Universitario, Via E. Orabona, 4, 70126 Bari, Italy. Tel./fax: +39 805442015.
E-mail address: tommaso.cataldi@chimica.uniba.it (T.R.I. Cataldi).

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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