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Identification and quantification of glucosinolates in different tissues of *Raphanus raphanistrum* by liquid chromatography tandem-mass spectrometry

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ABSTRACT

This study aims to identify and quantify the glucosinolates from different parts of wild radish *R. raphanistrum* (leaves, flowers, fruits, roots) using liquid chromatography-tandem mass spectrometry. Glucoraphenin is the predominant compound, accounting for about 87% (w/w) of total glucosinolate content, followed by glucobrassicin, glucoraphasatin and glucoraphanin (153 mg 100 g^{-1}, 149 mg 100 g^{-1} and 141 mg 100 g^{-1} FW, respectively) in fruits; followed by glucoraphasatin (3 mg 100 g^{-1} FW) in flowers and by glucobrassicin, 4-hydroxyglucobrassicin and glucoraphasatin (145 mg 100 g^{-1}, 27 mg 100 g^{-1} and 24 mg 100 g^{-1} FW, respectively) in leaves. In roots the major glucosinolate is glucoraphasatin (56 mg 100 g^{-1} FW) followed by the glucoraphenin and methoxyglucobrassicin (16 mg 100 g^{-1} and 7 mg 100 g^{-1} FW, respectively). Principal component analysis allowed the discrimination of fruit samples from other parts of the plant for the majority of glucosinolates and the fruits are highlighted as sources of glucosinolates. The results are interesting given that wild radish is one of the most important weeds of crops in the Mediterranean region and is popular for home vegetable production and for its employment in human nutrition both as a food as well as for medicinal purposes.

1. Introduction

Raphanus genus, belonging to the family of Brassicaceae, consists of two species: *Raphanus sativus* L. (edible radish) and *Raphanus raphanistrum*, which includes three subspecies: *R. r. raphanistrum*, *R. r. landra* and *R. r. rostratus*. Wild radish (*Raphanus raphanistrum* L.) is an annual broadleaf plant widely distributed in the southeast of United States, (Schroeder, 1989) in south of Australia, southern Europe and across the Mediterranean region. *R. raphanistrum* is also an edible plant commonly consumed in the Mediterranean region in countries such as Italy (Conti et al., 2005; Scott et al., 2002). All subspecies of *R. raphanistrum* are found in the Mediterranean area. The plant and its subspecies are known in human nutrition both as a food as well as for medicinal purposes. In fact, the young leaves and the aerial parts, slightly spicy, are consumed fresh or cooked as a salad, with olive oil and lemon juice like spinach, or boiled, like those of cultivated radish (*Raphanus sativus*), broccoli (*Brassica oleracea*) or edible brassicaceae, to prepare side dishes or in vegetable and legume soups (Hedge, 1965). In some areas, the roots are consumed grated or boiled or fried like radish. Moreover, *R. raphanistrum* is a component of a typical Sardininan dish, namely "Ramolaccio", a soup composed of 18 wild herbs of Barbagia. (Atzei, 2003). Moreover the aerial parts of the plant are used in traditional medicine for their anti-rheumatic and hypoglicemic activity and for the treatment of various ailments such as gastrointestinal diseases (Conforti et al., 2008). Anti-inflammatory and antioxidant activity of hydro-alcoholic extracts of *R. raphanistrum* has also been reported (Conforti et al., 2011; El and Karakaya, 2004; Küçükboyaci et al., 2012; The Local Food-Nutraceuticals Consortium, 2005). In addition, wild radish showed biofumigation potential when aqueous extracts and soil-incorporated air-dried biomass were tested in controlled enviroments (Bones and Rossiter, 1996; Norsworthy, 2003; Rosa et al., 1996).

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Previous studies on the chemical composition of radish have reported the presence of numerous beneficial compounds (polyphenols, anthocyanins) and, in particular, the majority of the studies have been conducted on a class of phytochemicals, characteristic of cruciferous vegetables, known as glucosinolates (GLs) (Hanlon and Barnes, 2011).

Glucosinolates are thioglucoside compounds, that contain a sulfated aldoxime moiety and a variable side chain derived from amino acids, more than 200 different side-groups have been identified (Agerbirk and Olsen, 2012; Deng et al., 2015; Franco et al., 2016). Although glucosinolates themselves possess limited biological activity, their enzymatic degradation by myrosinase leads to the formation of a number of biologically active compounds including ionic thiocyanate, isothiocyanates, nitriles, oxazolidinethione, epithionitriles and organic thiocyanates. Glucosinolates hydrolysis products are thought to be responsible for the characteristic pungent smell and flavour of this class of vegetables (Bennett et al., 2002; D'Antuono et al., 2009; Deng et al., 2015). In particular, it has been demonstrated that isothiocyanates can induce phase 2 detoxification enzymes and inhibit phase 1 activation enzymes, and this activity can be a key element in reducing risk of cancer (Kusznierewicz et al., 2013). It has been also demonstrated that all GL derivatives are not equal in their biological potential. Consequently, the identification, but also the quantitative determination, of individual glucosinolates in plant and plant tissues is extremely important (Kusznierewicz et al., 2013; Maldini et al., 2014; Rosa et al., 1996). Several HPLC–MS based methods have recently been used for the analysis of intact glucosinolates (Bennett et al., 2004; Maldini et al., 2012; Maldini et al., 2014). These methods can be used for both qualitative and quantitative analysis, especially for the characterization of GLs in less explored species, where the presence of unknown structures may require more sophisticated identification techniques (Gratacos-Cubarsi et al., 2010; Maldini et al., 2012; Maldini et al., 2014; Mellon et al., 2002; Tian et al., 2005).

The aim of this work was to identify and quantify the occurrence of glucosinolates in the different parts of *R. raphanistrum* i.e. in the leaves, flowers, fruits and roots. To the best of our knowledge, there are no studies in literature that report analysis, either qualitative and quantitative, on "glucosinoloma" of the radish *R. raphanistrum* grown in La Maddalena (Sardinia, Italy). In this paper, glucosinolates were analysed and determined in different samples with the use of ultra-high performance liquid chromatography-triple quadrupole/linear ion trap tandem mass spectrometry (UHPLC-QTRAP/MS/MS). Furthermore, the quantitative results were analysed by principal component analysis (PCA) to compare extracts from different tissues and to identify the variables responsible for the differences and the similarities among samples. The reason for analysing this specie is that it could be a good source of glucosinolates, since wild radish is one of the most important weeds of crops in the Mediterranean region and is popular for home vegetable production and for its employment in human nutrition both as a food as well as for medicinal uses.

2. Material and methods

2.1. Materials

LC–MS grade acetonitrile, methanol and formic acid, were obtained from Sigma-Aldrich Chemical Company (St Louis, MO). HPLC grade water (18 mΩ) was produced using a Millipore Milli-Q (Bedford, MA, USA) purification system. Glucosinolate standards (glucobrassicin, glucoraphanin, glucoiberin, gluctropaeolin, gluconapin, progoitrin, glucoerucin and glucoraphenin potassium salts) were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Standard purity was more than 80%.

2.2. Plant material and extraction

Three samples (biological triplicates) of mature wild *Raphanus raphanistrum* were collected in July 2014 in Spargiotto island, La Maddalena archipelago, Sardegna region (Italy), and authenticated by Doctor M. Chessa. Voucher specimens were dried and deposited at the Erbarium Sassa of Sassari University (n° 60). The plants were gently harvested, without cutting, with whole roots and transported in cold water from the field to the laboratory. Plants were separated into leaves, flowers, fruits and roots, giving 12 biological samples, and immediately frozen in liquid nitrogen and stored at −80 °C to avoid hydrolysis of the GLs. Samples were stored for no more than one week before sample preparation. On the day of extraction, each sample (12) was rapidly ground to a fine powder in a Waring blender cooled with liquid nitrogen and, approximately, 1 g was weighed into several 50 mL tubes and promptly extracted at 70 °C for 30 min under vortex mixing, using 25 mL methanol: water (70:30 v/v); sample to solvent ratio (1:25 w/v). The samples were centrifuged at 4000 rpm for 30 min (4 °C) and the supernatants were collected. After collection the solvent was entirely removed using a rotary evaporator under vacuum at 40 °C. The samples were reconstituted using ultrapure water and filtered through 0.20 μm syringe PVDF filters (Whatman International Ltd., UK). The measurements were done in triplicate on 3 different samples, each of them obtained by combining material coming from at least 3 different plants.

2.3. ESI–MS and ESI–MS/MS analyses

MS analysis was performed using an ABSciex (Foster City, CA, USA) API4000 Q-Trap spectrometer operating in the negative ion mode (ion spray voltage at −4500 V). The experimental conditions were optimised by infusing a standard solution of Glucoraphanin (1 $\mu g\,mL^{-1}$ in methanol:water 50:50 (v/v)) into the source (flow rate: 10 $\mu L\,min^{-1}$). The declustering potential (DP) was set at −70 eV, the entrance potential (EP) was set at −12.2 eV, the collision energy (CE) was set at −28.5 eV and the collision cell exit potential (CXP) was set at −21.8 eV.

2.4. HPLC–ESI–MS and HPLC–ESI–MS/MS analyses

Qualitative UHPLC-ESI–MS/MS analysis was performed using an UHPLC system interfaced to an ABSciex (Foster City, CA, USA) API4000 Q-Trap instrument in ion trap mode. Liquid chromatography was performed with a Flexar UHPLC AS system (Perkin-Elmer, USA) consisting of degasser, Flexar FX-10 pump, autosampler and PE 200 column oven. Five microliters of each sample were injected into a XSelect CSH C18 column (Waters, Milford, MA) (100 × 2.1 mm i.d., 2.5 μm d) (kept at 47 °C) and eluted at 300 $\mu L\,min^{-1}$ with mobile phase A (H_2O containing 0.1% formic acid) and mobile phase B (acetonitrile containing 0.1% formic acid) according to the following gradient: 0–8 min, from 0% to 6% B; 8–25 min, linear gradient 6–10% B; 25–35 min, linear gradient 10–40%. IDA (Information Dependent Acquisition) was used to perform the qualitative analysis. The IDA method included: IDA criteria (specifying the charge state and the mass range), enhanced MS scan, enhanced resolution, enhanced product ion scan or MS/MS scan. The source temperature was held at 450 °C, and MS parameters were those optimised for the ESI–MS and ESI–MS/MS analyses. MS data were acquired using Analyst software (1.6.2 version), and extracted ion fragmentograms (XIC) were analysed in order to identify glucosinolates from their deprotonated molecular ions and retention time.

Quantitative on-line HPLC-ESI–MS/MS analyses were performed in Multiple Reaction Monitoring (MRM) mode (using the mass spectrometer working as a triple quadrupole analyser).