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Analytical Methods

Optimized extraction, separation and quantification of twelve intact glucosinolates in broccoli leaves

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ABSTRACT

A new method has been developed and validated to determine twelve intact glucosinolates (glucoiberin, GIB; glucoraphanin, GRA; glucoerucin GER; gluconapin, GNA; glucotropaeolin, GTL; glucobrassicin, GBC; gluconasturtiin, GST; glucoalyssin, ALY; 4-hydroxyglucobrassicin, 4-OH; 4-metoxyglucobrassicin, 4ME; neoglucobrassicin, NEO; sinigrin, SIN) in broccoli leaves using liquid chromatography (LC) coupled to diode array (DAD) and electrospray ionization mass spectrometry (ESI-MS) detection. An extraction procedure has also been proposed and optimized by means of statistical analysis (the Box–Behnken design and analysis of variance); this is based on the deactivation of myrosinase using a microwave and heated water. Low limits of detection and quantification were obtained, ranging from 10 to 72 μg/g with DAD and 0.01 to 0.23 μg/g with ESI-MS, and the resulting recovery values ranged from 87% to 106% in all cases. Finally, glucosinolates were analyzed in broccoli leaf samples from six different cultivars (*Ramoso calabrese Parthenon, Marathon, Nubia, Naxos* and *Viola*).

1. Introduction

Numerous epidemiological studies indicate that Brassica vegetables in general, and broccoli (*Brassica oleracea* L. var. *italica*) in particular, protect humans against cancer, since they are rich sources of glucosinolates as well as of other phytochemicals (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Glucosinolates are β-thioglucoside *N*-hydroxysulfates, with a side chain (R) derived from amino acids and a sulfur-linked β-D-glucopyranose moiety (see Fig. 1 and Table 1), and some of these compounds appear to be protective against chemical carcinogens (Tolrà, Alonso, Poschenrieder, Barceló, & Barceló, 2000). Moreover, certain metabolic breakdown products of glucosinolates, particularly isothiocyanates and nitriles, have been shown to both modify xenobiotic metabolizing enzymes that protect DNA from damage and induce cell cycle arrest and apoptosis (Lund, 2003). A large body of research into anticarcinogens has focused on a single bioactive component within broccoli, namely, sulforaphane, which is formed by hydrolysis of a glucosinolate (glucoraphanin, GRA). For these reasons, it can be concluded that the chemoprotective effect of broccoli is related with glucosinolate content. It is, therefore, necessary to determine the vegetable's glucosinolate content and profile, as each of the glucosinolates from broccoli generates a specific breakdown product, which possesses different biological properties.

Intensive broccoli cultivars are associated with the production of considerable waste products, mainly leaves that are discarded, even though they may have a similar composition to the edible parts of the plant. These by-products could complement animal feed or be used as a source of nutraceuticals, which would reduce environmental impact and, at the same time, increase economic value. In the scant published scientific literature dealing with the nutritional content of broccoli leaves, as compared with the parts normally consumed, studies have been made of amino acids (Arnáiz et al., 2012), fatty acids (Manzano et al., 2011), vitamins, phenolic compounds, minerals (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010) and glucosinolates (Branca, Li, Goyal, & Quiros, 2002; Domínguez-Perles et al., 2010; Hennig, Verkerk, Bonnema, & Dekker, 2012; López-Berenguer, Martínez-Ballesta, García-Viguera, & Carvajal, 2008; Sasaki, Neyazaki, Shindo, Ogawa, & Momose, 2012).

Glucosinolates are usually determined according to the presence (intact or non-intact desulfo-derivatives) of a sulfate group. Desulfo-glucosinolates have been analyzed previously (Branca et al., 2002; Hennig et al., 2012) following extraction with heated methanol and a water mixture (70:30, v/v, Hennig et al., 2012) or with methanol (Branca et al., 2002). Extracts were subsequently absorbed on a solid support and subjected to enzymatic desulfation. The extraction methods employed with intact glucosinolates mainly involved the use of heated mixtures of methanol and water (70:30, v/v) for 30 min (Domínguez-Perles et al., 2010; López-Berenguer et al., 2008) or 0.1% formic acid in water and methanol (20:80 v/v), subsequent to autoclaving with boiling water, for

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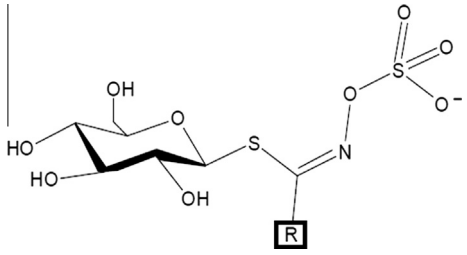

Fig. 1. General chemical structure of glucosinolates.

10 min (Sasaki et al., 2012). All these treatments include heating, which is necessary to deactivate the myrosinase enzyme and obtain a better extraction of intact glucosinolates. In addition, intact glucosinolates have been extracted from edible parts (sprouts, florets, heads, stems and roots) using heated water (Jones, Frisina, Winkler, Imsic, & Tomkins, 2010), methanol (Song & Thornalley, 2007) or water and methanol mixtures (Domínguez-Perles, Moreno, Carvajal, & García-Viguera, 2011; Fernández-León et al., 2013; Lelario, Bianco, Bufo, & Cataldi, 2012; López-Berenguer et al., 2008; Pérez-Balibrea, Moreno, & García-Viguera, 2011a; Tian, Rosselot, & Schwartz, 2005; Wang et al., 2011).

Determining intact glucosinolates has been achieved by means of liquid chromatography (LC) in reversed phase mode (C_{18} columns) with ultraviolet or diode array (LC-DAD) (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Jones et al., 2010; López-Berenguer et al., 2008; Pérez-Balibrea, Moreno, & García-Viguera, 2011b; Pérez-Balibrea et al., 2011a; Sasaki et al., 2012; Wang et al., 2011) and electrospray ionization-mass spectroscopy (LC–ESI-MS) (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Domínguez-Perles, Martínez-Ballesta, Riquelme, Carvajal, & García-Viguera, 2011; Domínguez-Perles, Moreno, Carvajal, & García-Viguera, 2011; Domínguez-Perles, Moreno & García-Viguera, 2012; Fernández-León, Fernández-León, Lozano, Ayuso, & González-Gómez, 2012; Fernández-León, Fernández-León, Lozano, Ayuso, & González-Gómez, 2013; Fernández-León et al., 2013; Lelario, Bianco, Bufo, & Cataldi, 2012; López-Berenguer et al., 2008; Pérez-Balibrea, Moreno, & García-Viguera, 2010; Pérez-Balibrea et al., 2011a; Pérez-Balibrea et al., 2011b; Song & Thornalley, 2007; Tian et al., 2005; Wang et al., 2011).

Our aim, therefore, was to develop a new, robust LC-DAD–ESI-MS method, which was sensitive and as rapid as possible, to separate and determine twelve intact glucosinolates from broccoli leaves; to our knowledge this is the largest number of intact glucosinolates studied in broccoli leaves to date. Moreover, we wish to propose and optimize, by means of a statistical analysis (Box–Behnken design and analysis of variance), a specific, efficient, rapid, cheap and environmentally-friendly extraction procedure based on the deactivation of myrosinase by heating. For these reasons, two different extraction procedures involving the use of microwave (MW) or a conventional oven were tested in order to select the best choice for extracting intact glucosinolates from broccoli leaves. Moreover, this is the first study where an extraction procedure for intact glucosinolates has been developed and optimized in broccoli leaves. In addition, the use of an MW and statistical analysis to optimize the extraction procedure of intact glucosinolates in this matrix has not been previously described. Accordingly, the proposed method was validated and applied in an analysis of glucosinolates in broccoli leaf samples from six cultivars (*Ramoso calabrese, Parthenon, Marathon, Nubia, Naxos* and *Viola*) to determine differences in glucosinolate content and to corroborate the argument that broccoli leaves could be a potential source of glucosinolates.

2. Materials and methods

2.1. Reagents and materials

Glucoiberin (GIB), glucoraphanin (GRA), glucoerucin (GER), gluconapin (GNA), glucotropaeolin (GTL), glucobrassicin (GBC), gluconasturtiin (GST) and sinigrin (SIN), which was used as the external standard (ES), were purchased from Phytoplan Diehm & und Neuberger GmbH (Heidelberg, Germany). SIN was used as an external standard to quantify the glucosinolates from broccoli where individual standards were not available (glucoalyssin, ALY; 4-hydroxyglucobrassicin, 4-OH; neoglucobrassicin, NEO; 4-metoxyglucobrassicin, 4ME), as has normally been the case (Domínguez-Perles, Moreno, Carvajal, & García-Viguera, 2011; Pérez-Balibrea et al., 2011a). Rapeseed certified ERM® BC367 reference material, from Sigma Aldrich Chemie Gbmh (Steinheim, Germany), composed of GNA, GBN, ALY, 4-OH, NEO, GST, GBC, SIN and another four glucosinolates (napoleiferin, brassicanapin, progoitrin and epiprogroitrin), largely absent in broccoli, was used to optimize separation in preliminary tests. This certified material was also employed to identify and confirm the presence of ALY, 4-OH, and NEO due to the absence of individual standards for these glucosinolates. LC grade methanol and acetonitrile were both supplied by Lab-Scan Ltd. (Dublin, Ireland), whilst formic acid was purchased from Sigma Aldrich Chemie Gbmh. Syringe filters (17 mm, Nylon 0.45 μm) were purchased from Nalgene (Rochester, NY, USA), quantitative filter paper (Albet 140) was supplied by Albet-Hahnemuehle S.L. (Barcelona, Spain), and ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA,USA). An Eppendorf Centrifuge 5810R (Eppendorf-Netheler–Hinz GmbH, Hamburg, Germany), a vacuum filter system (Supelco, St. Louis, MO, USA), and an R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), were used for all the extractions. A drying oven from Selecta (Barcelona, Spain) was also employed, as well as a Vortex mechanical mixer from Heidolph (Schwabach, Germany). A microwave model MG-3924W from LG electronics (Madrid, Spain) was used for sample treatment. The samples were ground with a Moulinette chopper device from Moulinex (Paris, France).

2.2. Standard solutions

Standard stock solutions were prepared by dissolving approximately 3 mg of each glucosinolate in 10 mL of ultrapure water to a final concentration of approximately 300 mg/L. These solutions were further diluted with ultrapure water to prepare the intermediate and working solutions. Broccoli leaf samples (50 mg) were spiked with different amounts of the available glucosinolate standards for calibration at different concentrations (between LOQ and 400 μg/g (ESI-MS) or 4000 μg/g (DAD)). These matrix-matched standards were extracted by means of the same procedure as described for samples (Section 2.4). Each quality control (QC) sample was prepared with 50 mg of broccoli leaf samples spiked after sample treatment, with the same amounts of glucosinolates as those used in the calibration studies. The concentrations of different QC samples were as follows: low QC level – 0.1 μg/g for GNA, GTL, GER and GST or 0.3 μg/g for GIB, SIN, GRA and GBC (ESI-MS) and 80 μg/g (DAD); medium QC level – 20 μg/g (ESI-MS) and 500 μg/g (DAD); and high QC level – 400 μg/g (ESI-MS) and 4000 μg/g (DAD). Certified rapeseed material was also spiked and extracted using the same approach. All standard solutions were stored in glass containers and kept in the dark at +4 °C, where they were determined to be stable for more than two weeks (data not shown).

2.3. Plant material

Fresh broccoli leaf samples (*Ramoso calabrese*) were harvested after six months' growth from seed. They were dried at 60 °C in