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Determination of sinigrin, sinalbin, allyl- and benzyl isothiocyanates by RP-HPLC in mustard powder extracts

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ABSTRACT

A reversed phase-high performance liquid chromatography method was developed to quantify sinigrin, sinalbin, allyl isothiocyanate and benzyl isothiocyanate present in aqueous and freeze-dried yellow and Oriental (brown) mustard extract samples using two pre-treatment methods (autoclaving, boiling) to prevent degradation by myrosinase. The lowest detection limits for sinigrin and sinalbin were 0.05 mg/L and for allyl- and benzyl isothiocyanate were 2 mg/L. The methods developed make it possible to quantify both the glucosinolates (sinigrin, and sinalbin) and their hydrolysis products (allyl- and benzyl isothiocyanate) with the same mobile phase, and only require adjustment of the wavelength and a change in the ratio of the high performance liquid chromatography mobile phase solvents (tetrabutylammonium hydrogen sulphate and acetonitrile). The use of a single method yielded accurate and rapid results for the four compounds (sinigrin, sinalbin, allyl- and benzyl isothiocyanate). Autoclaving of both yellow and brown mustard powder before glucosinolate extraction did not consistently improve the amount of sinalbin and sinigrin recovered over boiling treatments because the thermal stability of myrosinase proved problematic in glucosinolate recovery. Nonetheless, the highest extract yields found were 4.06 g/100 g for sinigrin and 2.57 g/100 g for sinalbin, respectively, which represented over 94 g/100 g extract yield of sinigrin from the Oriental mustard powder.

1. Introduction

Chronic diseases such as breast (Ambrosone et al., 2004), colon (Hara et al., 2003), liver and gastrointestinal (Uhl et al., 2004) cancers can be lowered through consumption of food items or ingredients containing glucosinolates from the *Brassicaceae* family (Fahey, Zalcmann, & Talalay, 2001; Rosa, Heaney, Fenwick, & Portas, 1997; Sasaki & Takahashi, 2002; Verhoeven, Goldbohm, Van Poppel, Verhagen, & Van den Brandt, 1996; Wattenberg, 1992; Zhang & Talalay, 1994). Glucosinolates are known to work as anti-cancer (glucoraphanin) or sometimes antinutritional (goitrin) agents, and include an array of naturally occurring bioactive compounds generated by their hydrolysis (Wallsgrove, Doughty, & Bennett, 1999). The latter include glucose, sulphate, isothiocyanates, thiocyanates, and nitriles. Isothiocyanates, the major glucosinolate breakdown by-product play roles in protecting plants from diseases caused by bacterial or fungal infection and insects (Haramoto & Gallandt, 2004). Allyl isothiocyanate (AIT), as a sinigrin (SNG) hydrolysis by-product (Gil & MacLeod, 1980) is used to control plant fungal diseases (Mojtahedi, Santo, Wilson, & Hang, 1993). The effect of the glucosinolates and their metabolites on human health has been studied (Manson et al., 1997), and their antimicrobial and bioactive properties have been established against spoilage and pathogenic microorganisms contaminating food products (Luciano & Holley, 2009). As members of the *Brassicaceae* family, mustard species contain glucosinolates which can be converted by endogenous myrosinase in plant tissue to yield isothiocyanates which give mustard its flavour. The type(s) of glucosinolates present are known to be cultivar dependent (Kushad et al., 1999; Tsao, Yu, Potter, & Chiba, 2002). For example: SNG is found at higher concentrations in *Brassica juncea* var. *rugosa* and *Brassica oleracea* (Carlson, Daxenbichler, VanEtten, Kwolek, & William, 1987); glucoraphanin is the major glucosinolate present in broccoli (Kushad et al., 1999), and sinalbin (SNB) is the major glucosinolate found in yellow mustard (*Sinapis alba*). Benzyl isothiocyanate (BIT) was identified as an autolytic hydrolysis product in *S. alba* macerate (pH 7–8, 40 °C, 1 h), by Cole (1976). BIT is potentially anti-tumorigenic (lung and oesophagus in humans) and works as an antimetastatic agent (Lai et al., 2010).

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There is value in using AIT and BIT in food preservation (Kamii & Isshiki, 2009). Allyl isothiocyanate, allyl cyanide (Pechácêk, Velíšek, & Davídek, 2000) and BIT can be produced from mustard glucosinolate hydrolysis and can be converted by metabolic action into benzyl amine (Tang, Bhothipaksa, & Frank, 1972) or allyl amine (Combourieu, Elfoul, Delort, & Rabot, 2001). The direct and indirect determination of glucosinolates or their metabolites (AIT, BIT, SNG, and SNB) by HPLC is more convenient than by GC which requires trimethyl derivatization of the glucosinolates (Cai et al., 2004; Kiddle et al., 2001; Song, Morrison, Botting, & Thornalley, 2005). However, separation of the glucosinolate from isothiocyanate peaks in the same chromatogram by HPLC is difficult. Therefore, the aims of this study were: 1. to determine the concentrations of SNG, SNB, AIT and BIT in extracts of mustard powder (brown/oriental or yellow) subjected to autoclaving/boiling or boiling treatments to inactivate endogenous myrosinase; and 2. to develop a simplified method using reversed phase-HPLC to sequentially determine the glucosinolate breakdown products during the same run.

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN) of HPLC grade, benzyl isothiocyanate and sinigrin (allyl glucosinolate) were from Sigma–Aldrich (St. Louis, MO, USA). Allyl isothiocyanate was purchased from Acros-Organics (Morris Plains, NJ, USA). Sinalbin (p-hydroxybenzyl glucosinolate.H_2O,K^+) was purchased from C-2 Bioengineering (Copenhagen, Denmark) and tetrabutyl ammonium hydrogen sulphate (TBA) was from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Mustard flour and ground mustard seeds

Two pre-treatments (boiling and autoclaving) were tested by RP-HPLC to extract the SNG and SNB from six mustard samples: Two yellow mustard powder samples coded 615 and 106 consisting of cold or deodorized (myrosinase-inactivated) and hot or spicy (with active myrosinase) types were provided by G.S. Dunn Ltd. (Hamilton, ON, Canada). One sample each of yellow mustard seed, oriental mustard seed powder and whole ground Oriental (hot) brown mustard were provided by Agriculture and Agri-Food Canada (AAFC, Guelph, ON, Canada). The sixth sample was hot Oriental brown mustard powder which was provided by Sakai Oriental Spice (Calgary, AB, Canada). The seed samples were freshly ground before use in a coffee bean grinder (Black and Decker, Smart Grind, Towson, Maryland, USA) in 100 g lots to 74 μm particle size (by graduated sieve measurement).

2.2.1. Autoclaving

A 2 cm thick layer of each mustard powder sample for autoclave treatment was placed in an aluminium foil tray (30 cm × 80 cm). Mustard types treated were: hot yellow mustard seeds from G.S. Dunn; hot brown mustard powder from Sakai Spice, and freshly ground mustard seeds from AAFC. These were placed in an autoclave (SV-120, Amsco, Greenbush, NY, USA) for 15 min at 115 °C in the liquid mode (no drying step).

2.2.2. Boiling and extraction

SNG or SNB were extracted from mustard flour using the method of Prestera et al. (1996) with modification. A 20 g sample of either non-autoclaved (hot or spicy mustard) or autoclaved (deodorized or cold mustard) were added to 200 ml boiling distilled water (100 ± 1 °C) and the mixture (99°C ± 1 °C) was stirred for 10 min at 350 rpm to inactivate the enzyme myrosinase present in the non-heat-treated mustard samples and extract the glucosinolates. The mixtures were cooled at room temperature from 100 °C to 70 °C in 10 ± 2 min and let stand for 4 h at 70 ± 4 °C. They were then centrifuged at 17000 × *g* for 15 min at 4 °C and filtered through Whatman no. 4 paper into 50 mL screw-capped tubes. The mixtures were boiled again, but for 30 min (sample temperature 96 ± 1 °C) to clarify the extracts, and centrifuged for 15 min (17000× *g*, 4 °C). The supernatants were identified as concentrated aqueous extracts.

2.2.3. Evaporation and freeze drying

The entire amount of supernatant generated from a 20 g sample was filtered through Whatman no. 4 paper, concentrated to 10 mL using a rotary evaporator (IKA, RV10 digital, Santa Clara, CA, USA) and freeze-driedfor 48 h in a Genesis 25-XL freeze drier (Genesis Engineering and Technical Service Co., Valley Cottage, NY, USA).The freeze-dried samples and 2 mL of the concentrated extracts were placed separately in screw-capped 25 ml bottles and stored at 4 °C until HPLC analysis (about 7 days).

2.2.4. Analysis by reversed phase-liquid chromatography (RP-HPLC)

Freeze-dried and concentrated mustard extracts were analysed separately and evaluated by HPLC for SNG, SNB, AIT, and BIT levels using an external standard. Samples of 10 μl were injected and passed through an analytical Gemini-NX C18 reversed phase column (150 × 4.6 mm, 5 μ) protected by a security guard Gemini C18, 4 × 3.0 mm column (Phenomenex, Torrance, CA, USA). The HPLC system (Waters Corporation, Milford, MA, USA) consisted of a Waters model 486 detector (wavelength set at 227 nm), Waters 600E system controller and Waters LC-module 1 Millennium software (version 32) was used to process the data. Before use solvents (ACN and TBA) were filtered through a 0.45 μm Millipore nylon filter (Fisher Scientific, Nepean, ON, Canada),and degassed for 5 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, USA), and degassing was continued during the run using the built-in helium (He) degassing unit of the HPLC.

2.2.5. Sinigrin, sinalbin, allyl isothiocyanate and benzyl isothiocyanate analyses

Both SNG and SNB were analysed after the C18 column was equilibrated for 3 h with a mobile phase which consisted of 80 mL (0.02 mol/L) TBA and 20 mL ACN with detection at 227 nm. The AIT and BIT were separated with the same buffer but at a ratio of ACN to TBA of 60:40 and with detection at 244 nm. The flow rate was set at 1.0 ml/min and the equilibrations used were 50 min before analysis of samples and 20 min after changing the solvent ratio and the wavelength. Standard curves were constructed using pure SNG, SNB, AIT, and BIT. The detection limits for SNG, SNB, AIT, and BIT as well as the correlation coefficients were determined for the two tested glucosinolates and their metabolites.

2.2.6. Sinigrin, sinalbin, allyl and benzyl isothiocyanate calibration curves

The retention times on the selected column were used to characterize the peaks of the pure standards. The amount of glucosinolates and their metabolites in samples were calculated from the curves of corresponding SNG, SNB, AIT, and BIT standards injected at different concentrations and after peak areas were integrated. The calibration curves for SNG, SNB, AIT, and BIT were prepared in the range of 25–570, 25–680, 9.55–100, and 11.02–200 mg/L, respectively. The linearity of the curves and the respective correlation coefficients were computed from the peak area at each reference standard concentration, and the concentrations of the injected reference standards were calculated as mg/L. The value shown for each standard is based on the mean of 3 measurements of freeze-dried aqueous extracts