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Analytical Methods

Chromatographic fingerprints and quantitative analysis of isoflavones in Tofu-type soybeans

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ABSTRACT

Tofu-type soybeans can differ from conventional soy varieties in seed size, seed composition, flavour and nutrition. Using HPLC coupled with ESI-MS and PDA detection, a total of 19 isoflavones were detected and identified from Tofu-type soybean seeds, more than previously reported forms from many other soybeans. An HPLC/UV fingerprint study was performed for qualitative evaluation, which enabled the isoflavone profile of Tofu-type soybeans to be characterised and differentiated from other vegetative soybeans by similarity comparison. To meet the frequent quantitative application for isoflavone contents, a simple, precise and reliable method using HCl hydrolysis during sample extraction and LC/UV for the detection was developed and validated to quantitate total isoflavones in soybeans, and then applied to determine the total isoflavone contents of different Tofu-type soy varieties grown in different field locations over two growing seasons. A range of total isoflavone contents for Tofu-type soybeans was established to confirm the use of this analytical approach for quality control applications.

1. Introduction

Soybeans and soy-based food products long considered as a nutritional component of Asian diets, are now gaining acceptance for their potential health-promoting properties. Soybeans are rich in proteins, minerals, vitamins and omega-3 fatty acids, which are each associated with the prevention of heart diseases and other chronic diseases (Kwon, Daily, Kim, & Park, 2010; Nair, Leitch, Falconer, & Garg, 1997). Isoflavones from soybeans contribute to the properties that may lower risk of cardiovascular diseases (Anderson, Johnstone, & Cook-Newell, 1995; Arjmandi et al., 1996), provide neuronal protection (Ma et al., 2010; Sarkaki, Badavi, Aligholi, & Moghaddam, 2010), and potentially prevent hormone-dependent breast and prostate cancers by acting as phytoestrogens, which may perform a broader spectrum of action with lower associated side effects (Cos et al., 2003; Denis, Morton, & Griffiths, 1999; Lee, Gourley, & Duffy, 1991; Wang & Murphy, 1994). These attributes make the compounds attractive as potential candidates for complementary and alternative therapy.

The major isoflavones in soybeans include daidzein, glycitein, genistein, their glycosides, glycoside malonates and glycoside acetates, in which the predominant isoflavone forms in soybeans and non-fermented soy products are glycoside malonates, 6″-*O*-malonylgenistin and 6″-*O*-malonyldaidzin (Kudou et al., 1991). Several research groups have reported 14–16 isoflavones as detected from different types of soy seeds by LC/MS (Fang, Yu, & Badger, 2004; Gu & Gu, 2001; Wu, Wang, Sciarappa, & Simon, 2004). Interestingly, Bowey et al. reported that soy aglycones, daidzein, glycitein and genistein were enzymatically released in the small intestine after dietary consumption (Bowey, Adlercreutz, & Rowland, 2003). Hydrolysis to the aglycone is needed for absorption of isoflavone glycosides (Setchell et al., 2002; Xu, Harris, Wang, Murphy, & Hendrich, 1995). The complexity of natural soy isoflavones makes the rigorous standardisation difficult to achieve, and most chromatographic methods only quantify the main isoflavone forms as total isoflavone contents (Hoeck, Fehr, Murphy, & Welke, 2000; Penalvo, Nurmi, & Aldercreutz, 2004; Wang & Murphy, 1994). In the present research, we aimed to develop a simple and practical approach, applicable for the requirements of routine quality assessment, sample authentication and assurance of the quality consistency between batches. The chromatographic fingerprint technique as a strategy for quality evaluation of herbal medicines has gained acceptance by many authorities and organisations (Li et al., 2006; Xie et al., 2008). The Computer-Aided Similarity Evaluation System (CASES) for fingerprints enables to synchronise the chromatographic peaks and to calculate the cosine values of vectorial angles among different chromatograms. The closer the cosine values, the more similar the two chromatograms. We therefore utilised this fingerprint approach

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to qualitatively characterise the isoflavone profiles of Tofu-type soybeans, and to then use this approach to compare both the compositional sameness and differences from other types of soybeans.

Tofu-type soybeans may vary from conventional soy varieties in terms of seed size, protein content, plant growth, flavour and nutritional composition. Presently, the concentration of nutritional components such as protein and oil content in soybeans are the major considerations for conventional growers. In switching to human food varieties, the isoflavone content becomes another important nutritional quality parameter that should be considered when evaluating the nutraceutical and health attributes, rather than as soy for protein production or animal feed alone. The goals of this research were to develop a chromatographic fingerprint as an approach for qualitative evaluation, as well as the development and validation of a simple, accurate and reliable method for routine quantification of total isoflavone contents. Results would provide precise quantitative information of bioavailability-related isoflavone concentrations. Total isoflavone contents as well as individual aglycone forms in soy seeds from varying varieties, locations, and sowing dates were also compared subsequently, to further establish a proper content limits for quality control needs.

2. Materials and methods

2.1. Chemicals

Standard compounds, daidzein, glycitein and genistein were purchased from Sigma Chemical Co. (St. Louis, MO). Formic acid was obtained from Acros Organics (NJ), and the internal standard formononetin was from Indofine Co. (Hillsborough, NJ). HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (MeCN), 95% ethanol (EtOH) and concentrated hydrochloric acid (HCl) were purchased from Fisher Scientific Co. (Fair Lawn, NJ). HPLC-grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA) and was used for preparing all solutions.

2.2. Seed samples

In growing season I, two Tofu-type soybean varieties (early group II maturity types named "2F11" from Iowa and "HP204" from Minnesota) were procured from NJ Plus Organics (Lincoln, NE) in 2002 and planted two times at the Rutgers Snyder Research Farm in Pittstown, NJ. Early-planted 2F11 and HP204 were planted in early June on five different locations at the Rutgers Snyder Research Farm. Both varieties 2F11 and HP204 were then directly sown later in the middle of June as late-plant. Soybeans were harvested about 190 days after sowing. In growing season II, three Tofu-type soybean varieties "Vinton81", "HP204" and "Iowa1007" were procured from NJ Plus Organics (Lincoln, NE) in 2003 and planted at seven sites in three different climatic zones in New Jersey. The southern sites were at The Rutgers Agricultural Research and Extension Center in Upper Deerfield, NJ; the central zone included the Rutgers Research Farm in Cream Ridge, NJ; and the northern sites were at the Rutgers Snyder Research Farm in Pittstown, NJ. The 2003 Tofu varieties were planted from middle to late June and harvested about 180 days after sowing. The Edamame-type soybean varieties used in this research, including "Tzuzunoko", "Emerald", "Ryokkoh", "Lucky Lion", "Early Hakucho" and "Be Sweet", were obtained from Asian Vegetable Research and Development Center (Tainan, Taiwan) and planted at the Rutgers Snyder Research Farm in Pittstown, NJ. The Edamame varieties were harvested within 120 days, when the average weights ranged from 1 to 3 g per individual bean. The purpose of the using different varieties and across location and years was only to test the strength of the analytical approach, not to define absolute differences in any particular variety per se.

2.3. Equipment

HPLC separation was performed on a Prodigy ODS (3) column, 5 μm, 150 × 4.6 mm i.d. (Phenomenex Inc., Torrace, CA). Agilent 1100 Series LC/MSD/PDA system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, quaternary pump system, diode array, multiple-wavelength detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI), and HP ChemStation software. Bruker Daltonics 4.1 and Data Analysis 4.1 software were used.

2.4. Preparation of standards for HPLC analysis

The stock solution was prepared by dissolving the appropriate amounts of ~5.0 mg each standards daidzein, glycitein and genistein in 15 ml of diluent (70% methanol) with sonication for 25 min. The final volume of each solution was then diluted to 20 ml with the diluent at room temperature. Calibration standards were prepared by diluting the stock solution with the diluent and then spiking the same amount of internal standard formononetin. The calibration curves were established on 10 data points covering a concentration range for daidzein, glycitein, and genistein in soybeans.

2.5. Sample preparation

Air dried mature soybean seeds were finely grounded with a coffee grinder. For qualitative analysis, about 1 g of powder was weighted to a 25 ml volumetric flask and ~20 ml of diluent (70% methanol) was added. The sample was sonicated at 0 °C for 20 min and allowed to warm to room temperature, and then filled to the full volume with the diluent. The extract was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 2 min to obtain a clear solution and filtered through a 0.45 μm filter. The sample (10 μl injected) was applied for LC/MS/PDA analysis right after the extraction to prevent possible decomposition in the solvent. For quantitative analysis, approximately 2 g of soybean samples were placed into a 250 ml flask along with 70 ml of 95% ethanol, and 8 ml of concentrated HCl. The mixture was refluxed for 2.5 h. The solution was allowed to cool to room temperature, and the supernatant was transferred to a 100 ml volumetric flask. The left solid residue was re-extracted three times with 70% methanol. The supernatants were combined and adjusted to the final volume of 100 ml. Each hydrolysed sample (20 μl injected), filtered through a 0.45 μm filter, was analysed by duplicate injections. The recoveries were validated by spiking with known quantities of isoflavone standards corresponding approximately to 100%, 75%, and 50% of the expected values in the soybean samples and then together extracting according to the same extraction method.

2.6. LC/MS/PDA conditions for identification

The mobile phase for chromatographic separation consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (acetonitrile) in gradient. The linear gradient elution was performed from 10% B to 35% B in 40 min at flow rate of 0.8 ml/min. Prior to the next injection, the column was equilibrated for 10 min with 10% B. The column compartment was maintained at 25 °C, and the injection volume was 10 μl. The photodiode array (PDA) detection was performed at 254 nm, and the UV spectra from 190 to 400 nm were on-line recorded for peak identification. The eluent was also monitored by electrospray ion mass spectrometer (ESI-MS) under positive ion mode, and the sample was scanned from *m/z* 100 to