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A new ultra-high pressure liquid chromatography method for the determination of total isoflavone aglycones after enzymatic hydrolysis: Application to analyze isoflavone levels in soybean cultivars

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ABSTRACT

Since only isoflavone aglycones are considered to be bioactive, the determination of total aglycones that are released from conjugated isoflavones after hydrolytic treatment may facilitate an objective alternative for quantifying isoflavone contents in soy products. Given this major benefit, a new ultra-high pressure liquid chromatography (UV-UPLC™) method was developed for the fast and reliable determination of total aglycones in soybeans (daidzein, glycitein, and genistein) after enzymatic hydrolysis applying *helix pomatia* digestive juice. Capitalizing on the enhanced performance of UPLC™, aglycones were separated within 3 min only, with a total runtime of 8 min till the next injection. Thus, especially compared to HPLC protocols, UPLC™ proved to be superior due to significantly shorter runtimes and accordingly increasing sample throughput. Additionally, regarding the performed validation (linearity, precision, recovery, selectivity, and robustness), the established method proved to be suitable for quantifying total aglycones in soybeans. Moreover, method applicability was demonstrated by analyzing 23 commercial soybean cultivars for their isoflavone contents. Cumulative aglycone levels ranged from 100 to 255 mg per 100 g, hence implying an average ratio of 52%, 41%, and 7% of total isoflavones for genistein, daidzein and glycitein, respectively. However, for some soybeans, other distinct aglycone distributions were observed as well.

1. Introduction

Belonging to the flavonoids, isoflavones comprise naturally occurring, secondary plant metabolites that are mainly found within the family of *Fabaceae*. Given the emerging attention in these phytochemicals, the medical (as well as commercial) interest in isoflavones relates to a structural similarity with 17-β-estradiol and the thereof resulting weak estrogenic activity (Benassayag, Perrot-Applanat, & Ferre, 2002). Moreover, these so-called phytoestrogens are credited for their potential protective effects against certain hormone dependent cancers, cardiovascular diseases, osteoporosis or as a natural alternative for hormone replacement therapy (Adlercreutz, 2002; Wroblewski Lissin & Cooke, 2000; Setchell & Lydeking-Olsen, 2003; Wuttke, Jarry, Westphalen, Christoffel, & Seidlová-Wuttke, 2002).

In soybeans, the three isoflavone aglycones found are genistein, daidzein and glycitein. Additionally, aglycones can be conjugated to form β-glucosides, or esterified 6-O″-malonyl-β-glucosides, or 6-O″-acetyl-β-glucosides, indicating 12 soy intrinsic isoflavone compounds in total.

For raw, unprocessed soybeans, the native isoflavone spectra predominantly consist of glucosides and esterified glucosides (especially 6-O″-malonylgenistin and 6-O″-malonyldaidzin) (Wang & Murphy, 1994). However, processing and storage may significantly impact this intrinsic isoflavone distribution, resulting in inter-conversions of conjugates or in the abstraction of free aglycones. Applying dry heat as in roasting, toasting or extrusion, primarily induces the formation of acetylglucosides; in contrast, moist heat as in cooking or baking leads mainly to the conversion to glucosides. Besides, soy foods that undergo fermentative treatment may present mostly free aglycones (Wang & Murphy, 1994; Coward, Smith, Kirk, & Barnes, 1998; Murphy, Barua, & Hauck, 2002). Moreover, a prolonged storage of soybeans implies an increase in glucosides and aglycones on expense of decreasing concentrations of malonylglucosides (Lee et al., 2003). Given the variability of possible inter-conversion between these respective isoflavone conjugates, their intrinsic bioavailability must be considered as well, suggesting the aglycone to be the bioactive form (Setchell et al., 2001, 2002).

Indeed, the quantification of all 12 isoflavone forms may add significant information about a specific product. However, stating total isoflavone contents based on the sum of the individual conjugates may hamper an objective product comparability as well (especially for the

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consumer). Including the conjugated sugar moiety pretends virtually increased isoflavone levels (factor 1.57–1.98 regarding the aglycone mass), consequently yielding discrepancies when labeled contents are reversed to bioactive aglycones amounts. As already shown for nutraceutical supplements, the labeled specification often deviated from the total (bioactive) aglycone content, hence implicating product labeling on basis of conjugated isoflavones only (Boniglia et al., 2009; Nurmi, Mazur, Heinonen, Kokkonen, & Adlercreutz 2002; Fiechter, Raba, Jungmayr, & Mayer, 2010). Eventually, the conversion of isoflavone data to aglycone-equivalents is most appropriate for a consumer-friendly labeling.

Considering the required effort in separation (12 compounds need to be separated) as well as the instability of esterified glucosides (Griffith & Collison, 2001), an isoflavone quantification on basis of a reduced number of stable analytes after hydrolytic treatments may pose a feasible alternative. In principle, conjugates are degraded towards their glucosides and/or aglycones, thus enabling drastically simplified chromatography, due to the lower number of analytes. Moreover, if hydrolyzed towards the aglycones, the thereof quantified amounts, stated as total aglycones (equal to aglycone-equivalents), are related to the actual, bioactive isoflavone level present in the product. Commonly applied hydrolytic strategies in isoflavone analyses comprise acid, basic (saponification) or enzymatic hydrolysis (Schwartz & Sontag, 2009; Shao et al., 2011) As the mildest alternative, enzymatic hydrolysis is normally performed using *beta-glucuronidase/arylsulfatase* from *helix pomatia* digestive juice, *cellulase, beta-glucosidase* or a combination of all three enzymes (Liggins, Bluck, Coward, & Bingham, 1998; Kuhnle, Dell'Aquila, Low, Kussmaul, & Bingham, 2007; Schwartz & Sontag, 2009; Shao et al., 2011).

Most commonly applied techniques for determination of isoflavones include chromatographic separations with either gas chromatography, capillary electrophoresis or HPLC, in combination with various detection strategies such as mass spectrometry, UV absorption, or electrochemical detection. However, HPLC coupled with UV or UV-DAD detection may be considered one of the most frequently used methods, mainly due to the facile detection of isoflavones based on their natural UV absorbance (aromatic ring) (Wu, Wang, & Simon, 2004; de Rijke et al., 2006).

Given the superior performance of sub-2 μm particles in LC-separations, ultra-high pressure/performance liquid chromatography (UHPLC), as commercially realized e.g., in UPLC™ (Swartz, 2005), already demonstrated high efficiency for various food dedicated applications e.g., for analyses of biogenic amines (Dadáková, Křížek, & Pelikánová, 2009; Latorre-Moratalla et al., 2009; Mayer, Fiechter, & Fischer, 2010), amino acids (Liming, Jinhui, Xiaofeng, Yi, & Jing, 2009; Fiechter & Mayer, 2011), phenolic compounds (Guillarme, Casetta, Bicchi, & Veuthey, 2010; Nováková, Spáčil, Seifrtová, Opletal, & Solich, 2010; Lu, Yuan, Zeng, & Chen, 2011) or even for ultra-fast analyses of isoflavones (Klejdus, Vacek, Lojková, Benešová, & Kubáň, 2008).

Emphasizing the intrinsic benefits of UPLC™, the major objective of this present study was to establish a fast and reliable UV-UPLC™ method for quantifying total (bioactive) isoflavone aglycones in soybeans released after enzymatic hydrolysis. Moreover, to demonstrate method applicability as well as to estimate the variability of total aglycone contents and individual aglycone distributions, 23 commercial soybean cultivars were to be analyzed regarding their isoflavone levels.

2. Experimental

2.1. Chemicals and standards

Standards for isoflavone aglycones (daidzein, glycitein, and genistein), all of >99% purity, were obtained from LC Laboratories (Woburn, MA, USA). *Helix pomatia* digestive juice as aqueous solution featuring *beta-glucuronidase* activity (EC 3.2.1.31; ≥100,000 U mL^{-1}) was purchased from Sigma-Aldrich (St. Louis, MO, USA), while all utilized supplementary chemicals and solvents exhibited either analytical or HPLC-grade and were supplied from Roth (Karlsruhe, Germany) or Fluka (Buchs, Switzerland). Ultrapure water from a SG Ultra Clear UV system (Siemens Water Technologies, Warrendale, PA, USA) was utilized for the preparation of all solutions.

Aglycone stock solutions at 200 μg mL^{-1} each were prepared by dissolving the accurately weighed portions in HPLC-grade Methanol (MeOH). Moreover, the "weighed-in" concentrations were further verified through a photometric assay (U-2000, Hitachi, Tokyo, Japan) utilizing wavelengths and extinction coefficients as summarized by Nurmi et al. (2002), and corrected throughout the following dilution steps, if necessary. An intermediate composite solution at 20 μg mL^{-1} for each aglycone was prepared from stock solutions by merging and dilution (1:10) with MeOH. From this composite solution, aglycone working standards ranging from 0.125 to 6.000 μg mL^{-1} were prepared by dilution in aqueous MeOH to yield a final solvent ratio of 30% MeOH, hence exactly matching that of chromatographic initial conditions. Depending on their concentrations, working standards were stable up to 1 week, whereas at prolonged storage, and especially for glycitein at higher concentrations, significant losses were observed.

2.2. Isoflavone extraction and enzymatic hydrolysis for the analysis of total aglycones in soybean samples

Conjugated isoflavone forms present after solvent extraction were enzymatically hydrolyzed to enable a quantitative determination of total isoflavone aglycones in soybeans. Sample preparation procedure was adapted from a previous work regarding the analysis of isoflavones in nutritional supplements (Fiechter et al., 2010) and was accordingly optimized for the applicability for soybean samples.

To obtain a homogenized sample material, approximately 200 g soybeans were ground to a 0.5 mm powder utilizing an ultra-centrifugal mill (ZM-200, Retsch, Haan, Germany). For isoflavone extraction, 300–350 mg soybean powder was weighed into a centrifugal tube and 75 mg zinc sulfate heptahydrate (250 μL of a 0.3 g mL^{-1} $ZnSO_4.7H_2O$ solution) was added to prevent cloudy extracts (Schwartz & Sontag, 2009). Twenty milliliter 80% (v/v) MeOH was added, and the mixtures extracted at room temperature for 1 h at 600 rpm using a Mini-shaker (IKA, Staufen, Germany). After centrifugation (1800×*g*, 10 °C, 15 min), the clarified extracts were drawn off and the extraction repeated once more with 20 mL fresh MeOH. The two extracts were then combined in a 50 mL volumetric flask and filled up with 80% MeOH.

For enzymatic hydrolysis, 1 mL soybean extract was transferred into a 30 mL glass tube and further diluted with 1.65 mL 0.1 M sodium acetate buffer (pH 5) to decrease the MeOH ratio down to 30% (equal to UPLC™ initial conditions). To initiate enzymatic hydrolysis, 25 μL *helix pomatia* digestive juice (~2500 U *beta-glucuronidase*) was admixed and the samples then incubated over night (>12 h) with a thermal incubator (Heidolph Instruments, Schwabach, Germany) at 37 °C and 200 rpm of shaking. Prior to chromatographic analysis, hydrolyzates were centrifuged (18,000×*g*, 10 °C, 15 min), and the obtained supernatants further filtered through a 0.20 μm regenerated cellulosic membrane (Sartorius, Goettingen, Germany). All sample preparations were performed in triplicate.

2.3. Ultra-high pressure liquid chromatography (UPLC™)

Chromatographic analysis was performed on a Waters Acquity™ ultra-performance LC (UPLC™) system (Waters, Milford, MA, USA) equipped with an Acquity™ tunable UV (TUV) detector (190–700 nm) and a Waters Acquity UPLC™ column (BEH C_8, 100 mm×2.1 mm i.d., 1.7 μm) with pre-connected 0.20 μm column in-line filter. The applied solvent system consisted of eluent (A) 0.3% formic acid in ultrapure water, and (B) HPLC-grade MeOH. Column temperature was set to 65 °C, and gradient elution was operated at a flow rate of 0.55 mL min^{-1} according to the following gradient profile: 0.0–3.5 min/30–40.5% B; 3.5–4.0 min/40.5–100% B; 4.0–5.0 min/100–100% B; 5.0–5.5 min/100–