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Analytical Methods

Effective clean-up and ultra high-performance liquid chromatography–tandem mass spectrometry for isoflavone determination in legumes

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ABSTRACT

Legumes are an excellent source of macronutrients and phytochemicals as isoflavones. The aim of this work was to develop a new analytical method for determining five isoflavone compounds, three of which are aglycons, namely daidzein, genistein, biochanin A, and two of which, daidzin and genistin, are glycosilated, in lentils and other pulses, using an effective clean-up system and UHPLC–MS/MS (triple quadrupole) method.

The recoveries obtained by spiking the lentil samples with a standard mixture of isoflavones at three levels of fortification (5, 25 and 100 μg kg^{-1}) were in the range of 54.4–111.1%, 68.6–91.1%, and 84.4–114%, respectively. The method was applied to analyse 48 lentil samples from central Italy and pulses for determining the isoflavone content, which was found to range from 1.1 to 95.6 μg kg^{-1}.

1. Introduction

Legumes are an excellent source of proteins, carbohydrates, fibre, vitamins, minerals, saponins (Caprioli et al., 2010; Messina, 1999; Salunkhe & Kadam, 1989) and other phytochemicals such as isoflavones (Antonelli, Faberi, Pastorini, Samperi, & Laganà, 2005; Delgado-Zamarreño, Perez-Martin, Bustamante-Rangel, & Carabias-Martinez, 2012; Konar, Poyrazoglu, Demir, & Artik, 2012). The richest sources of isoflavones are soybean (*Glycine max*) and derivatives, but recently there has been increased interest in isoflavones contained in other highly-consumed pulses in the Mediterranean area, such as lentils (*Lens culinaris* Medik.), chickpeas (*Cicer arietinum* L.), beans (*Phaseolus vulgaris* L.), fava beans (*Vicia faba* L.) and peas (*Pisum sativum* L.). Lentils from "Castelluccio di Norcia" are recognised by the European Union with the acronym PGI, Protected Geographical Indication (Sagratini et al., 2009). Lentils have been studied for their content of cholesterol-lowering soyasaponins (Sagratini et al., 2013; Vila-Donat et al., 2013), while less attention has been devoted to their content of phytoestrogens. Isoflavones, also described as phytoestrogen compounds, have a structure similar to that of 17β-estradiol (Skibola & Smith, 2000) and are involved in regulation of plant growth (Kuhnle et al., 2009).

In the present work, we investigated the occurrence in legumes of five isoflavones, namely daidzin, genistin, daidzein, genistein, and biochanin A, which are known to have many beneficial properties. The chemical structures of isoflavones are reported in Fig. 1. To the best of our knowledge, the first work on isoflavone quantification was done by Franke, Custer, Cerna, and Narala (1994), employing RP-HPLC-DAD to extract and quantify only "free" aglycons in foods. Rostagno, Palma, and Barroso (2005) analysed soy isoflavones using SPE coupled with HPLC-UV. Liggins et al. (2000) quantified only two isoflavones, daidzein and genistein, in various legumes using GC–MS. Delgado-Zamarreño et al. (2012) used a modified QuErChers method as a sample treatment before the determination of isoflavones in foods by UPLC–MS/MS. Recently Konar et al. (2012) employed LC separation combined with tandem mass spectrometry without previous purification to perform the quantitative analysis of phytoestrogens in legumes. In the previous works, the analytical methods developed to quantify isoflavones were rarely able to determine all target compounds, especially

Abbreviations: UHPLC–MS/MS, ultra high-performance liquid chromatography–tandem mass spectrometry; SPE, solid phase extraction; ESI, electrospray ionisation; RP-HPLC-DAD, reverse phase high-performance liquid chromatography diode array detector; HPLC-UV, high-performance liquid chromatography ultraviolet detector; GC–MS, gas chromatography–mass spectrometry.

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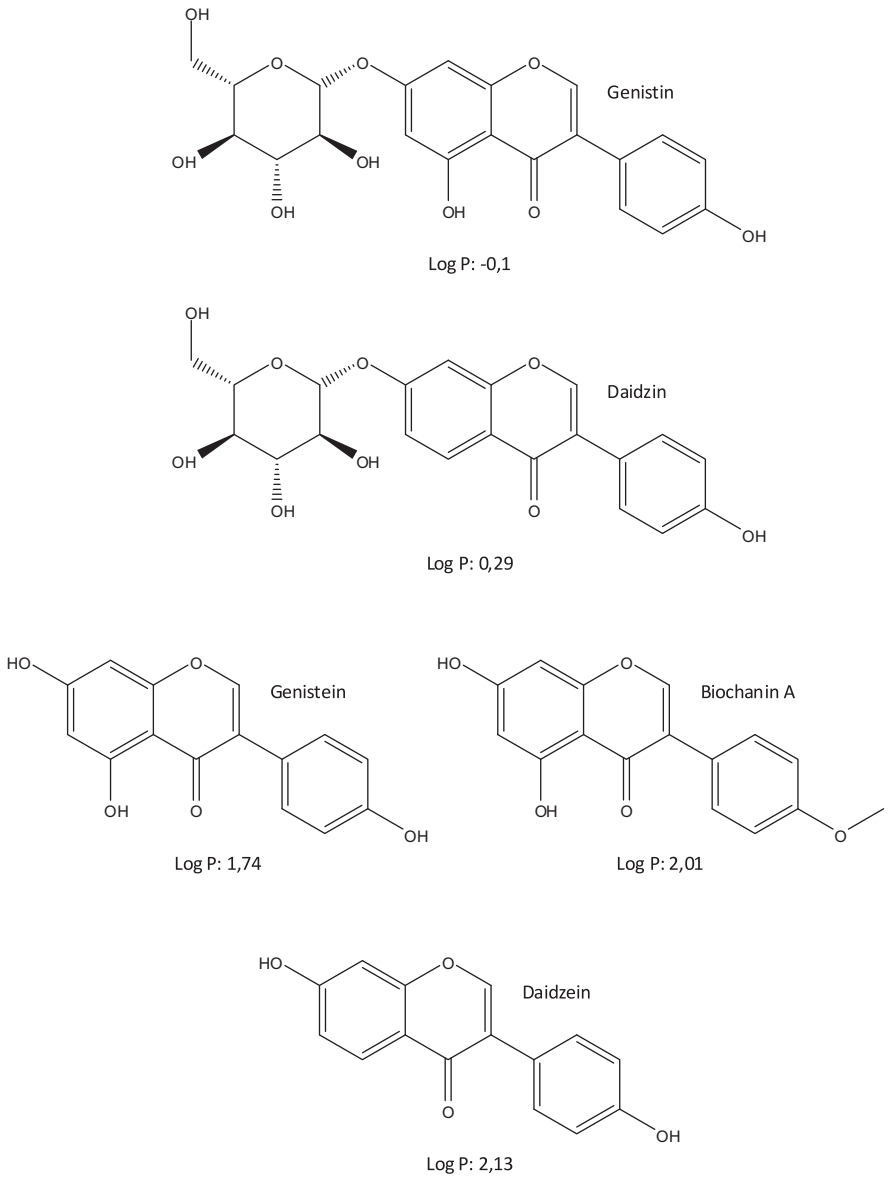

Fig. 1. Chemical structures of the five studied isoflavones.

the "free" isoflavones, due to the low content of these chemicals in the food matrix, such as lentils, or because of high signal suppression caused by the lack of clean-up steps in the extraction procedure. On the basis of these findings, the aim of this work was to establish a new analytical method for determining five isoflavone compounds, three aglycons, daidzein, genistein, and biochanin A, and two glycosilated, daidzin and genistin, in lentils and pulses. The method, which uses freezing as a clean-up step with ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS triple quadrupole) was applied to 48 lentil samples grown in fields at different altitudes and other pulses (chickpeas, beans, fava beans, peas, a mixture of beans and spelt, and a mixture of beans and lentils) from central Italy for the analysis of isoflavone content.

2. Materials and methods

2.1. Chemicals and reagents

Isoflavones, namely daidzin (>95%, $C_{21}H_{20}O_9$, molecular weight 416.38, CAS No. 552-66-9), genistin (>97.5%, $C_{21}H_{20}O_{10}$, molecular weight 432.38, CAS No. 529-59-9), daidzein ($\geqslant$98%, $C_{15}H_{10}O_4$, molecular weight 254.24, CAS No. 486-66-8), genistein (>98%, $C_{15}H_{10}O_5$ molecular weight 270.24, CAS No. 446-72-0) and biochanin A (95%, $C_{16}H_{12}O_5$, molecular weight 284.26, CAS No. 491-80-5) were purchased from Sigma Aldrich (St. Louis, MO, USA). Individual stock solutions of isoflavones at concentrations of 100 mg l^{-1} were prepared by dissolving pure standard compounds in HPLC grade methanol and storing them in glass-stoppered bottles at 4 °C. Afterwards, standard working solutions at various concentrations were prepared daily by appropriate dilution of the stock solution with methanol. HPLC-grade acetonitrile (ACN), methanol (MeOH), and ethanol (EtOH) were supplied by Sigma–Aldrich (Milano, Italy). HPLC-grade formic acid (99%) was obtained from Merck (Darmstadt, Germany) and ammonium formate was purchased from Fluka (Steinheim, Switzerland). Deionised water (<18 MΩ cm resistivity) was further purified using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents and solutions were filtered before transferring them into injection vials through a 0.2 μm nylon membrane filter from Minisart RC 4, Sartorium Stedim (Goettingen, Germany).