Determination of Phytoestrogenic Compounds of Chickpea (*Cicer Arientinum* L.) By Acid Hydrolysis and LC-MS/MS

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***Abstract*—**In this study, acidified hydrolysates of chickpea were analysed to determine their contents of 13 different phytoestrogenic compound as both free and conjugated isoflavones, lignans and coumestrol. Samples of hydrolysates were analysed by triple quadrupole LC-MS/MS. Cellulase, β-glucosidase and β-glucuronidase were used for acid hydrolysis. The highest determined phytoestrogenic compounds content of hydrolysate was secoisolariciresinol, 925.1 ± 10.9 μg/100 g. Sissotrin and glycitein which were determined as 446.8 ± 11.8 μg/100 g and 105.2 ± 9.87 respectively, were the highest determined isoflavones concentration. Daidzein, daidzin, formononetin, matairesinol, apigenin, quercetin, ruin and coumestrol were not determined in the hydrolysates.

***Keywords*—**Chickpea, Coumestrol, Isoflavone, Lignan, LC-MS/MS, Phytoestrogen.

I. Introduction

PHYTOESTROGENS are a diverse group of nonsteroidal plant compounds that mimic oestrogen and are ubiquitous in most plants, fruits and vegetables [1]. They can be found in most foodstuffs eaten by humans and animals, such as seasonings (garlic, aniseed, parsley), legumes (soy, peas, clover), grains (wheat, barley, rice, oat), vegetables (carrots, potatoes, alfalfa) and drinks (tea, coffee) [2]. Depending on their structure, phytoestrogens can be divided into flavonoid and non-flavonoid polyphenols. The main representatives of the flavonoid phytoestrogens are isoflavones [3]. Lignans and coumestans are the main non-flavonoid phytoestrogens [4].

Sample preparation procedures of plants origin products, are generally involves direct solvent extraction with aqueous methanol/ethanol for glycoside conjugate containing samples, or with ether and/or ethyl acetate for aglycone only containing samples. Enzymatic and/or acidic hydrolysis during extraction is sometimes employed depending on the study purpose, when only isoflavone and lignan are examined [3, 5].

In this study, we analysed the amounts of free and conjugated isoflavones, lignans and, coumestrol in which are the most interested phytoestrogenic compounds [6], in chickpeas (*Cicer arientinum* L.) of the Kocbasi variety samples prepared by acid hydrolysis.

II. Materials And Methods

A. Sampling and Sample Preparation

Sample of chickpea was bought in 1.0 kg amounts from the local market in Ankara (Turkey) in 2012. One kg of sample was milled (using a 0.5 mm sieve, Retsch type ZM1, Haan, Germany) and homogenised (using an Ika A11 basic grinder, IKAWerke GmbH and Co., Staufen, KG, Germany). Following homogenisation, 100 g of sample was placed in polyethylene bags and stored at 4°C prior to sample preparation.

B. Standards and Reagents

The phytoestrogen standards of ononin, genistin, daidzin, glycitin, sissotrin, biochanin A, glycitein genistein, formononetin, daidzein, matairesinol, secoisolariciresinol, and coumestrol were purchased from Sigma (St. Louis, MO, USA). 100 μg/mL of standard stock solutions were prepared using methanol. All reagents, solvents and chemicals were of analytical or HPLC grade and were obtained from Sigma or Merck (Darmstadt, Germany).

C. Apparatus

Ultrapure water was prepared using a Milli-Q System (Millipore S.A., Molsheim, France). Additionally, during sample preparation, the following equipment was used: ultra-centrifuge (Universal 320R, Hettich, Tufflingen, Germany), ultrasonic water bath (LBS2, Falc Instrument, Treviglio, Italy), sample concentrator under nitrogen (EVA-EC1-L 24-16, VLM, Germany) and general laboratory equipment.

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D. Acid Hydrolysis

As acid hydrolysis, the method used by Konar et al. [7] was performed. Hydrolisation was performed by adding 20 mL of 80% MeOH, 20 mL of 3.4 N HCL and 500 µL of 1% BHT (Butylated hydroxytoluene) solution (MeOH:BHT, 99:1) to a 2 g sample and mixing at 75°C for 150 minutes. After the hydrolysis period, 10 mL of hydrolysate and 1.2 mL of 10 M NaOH solution were mixed in a vortex. Then, the mixture was centrifuged at 9000 x *g* for 5 minutes, and the obtained supernatant was concentrated under nitrogen to dryness and stored at +4°C until the LC-MS/MS analysis. The samples underwent chromatographic analyses on the same day, or they were kept at 4°C in packaged form for protection against light. They were reconstituted in 1 mL 80% (v/v) aqueous MeOH and filtered through 0.45 µm membrane filters prior to injection into the LC-MS/MS system.

E. LC-MS/MS Conditions

The method of LC–MS/MS applied by Konar et al. [7] was used. An 6410A Triple Quadrupole LC–MS/MS from Agilent Technologies (Waldbronn, Germany) equipped with the Agilent LC 1200 series autosampler, a binary pump, and a thermostatted column compartment was used. The LC separation was performed on a 50 mm × 2.1 mm ID 3 µm Zorbax Eclipse XDB C18 column (Waters, Millford, MA, USA) at 35 °C. Through the application of this method, the total ion chromatograms (TICs) of the samples were obtained. All samples were injected into the LC/MS-MS system in triplicate. MS/MS acquisition parameters (MRM mode) used for identification of the target phytoestrogens are provided in Table 1.

F. Statistical Analysis

The LC-MS/MS experiments were performed in triplicate. The results were analysed to determine standard deviations (SD) using MS Excel 2007 (Microsoft Corporation, USA).

III. Results And Discussion

There have been some studies describing the amounts of phytoestrogenic compounds, especially isoflavones in chickpea [3, 7, 8, 9, 10].

Konar et al. [7] used LC-MS/MS on sample of conventionally extracted chickpea to determine that the total free isoflavone (total of formononetin, genistein, glycitein, daidzein and glycitein) and the total conjugated isoflavone (total of ononin, sissotrin, glycitin, daidzin, and genistin) were 1722 µg/kg and 1356 µg/kg (wet weight), respectively. Also non-isoflavone phytoestrogenic compounds, extracted by conventional extraction, were determined by Konar [3]. In the study conducted by Kuhnle et al. [8], the amounts of total isoflavone (total of glycitein, formononetin, biochanin A, daidzein and genistein), coumestrol, matairesinol and secoisolariciresinol in enzymatically hydrolyzed sample of

TABLE I
MS/MS ACQUISITION PARAMETERS (MULTIPLE REACTION MONITORING, MRM, MODE) USED FOR THE IDENTIFICATION OF THE TARGET PHYTOESTROGENS

Compound	Precursor ion	Product ion	Fragmentor	Collision energy	Polarity
Daidzein	252.6	223.1, 207.7	130	26	Negative
Coumestrol	266.6	238.6, 210.6	130	18, 22	Negative
Formononetin	266.6	251.6, 222.7	112	10, 26	Negative
Genistein	268.6	158.6, 132.8	130	26	Negative
Biochanin A	282.6	267.5, 238.6	112	14, 26	Negative
Glycitein	282.6	267.6, 239.6	112	10, 18	Negative
Matairesinol	356.5	203.0, 82.9	112	22, 18	Negative
Secoiso-lariciresinol	360.4	164.2	90	20	Negative
Daidzin	417.3	255.1, 199.0	90	10, 30	Positive
Ononin	431.3	270.3, 269.1	90	10	Positive
Genistin	433.3	271.1	90	10	Positive
Glycitin	447.3	284.9, 269.8	90	18, 30	Positive
Sissotrin	447.3	285.1	90	10	Positive

chickpea were 6070 µg/kg, <10 µg/kg, 20 µg/kg and <10 µg/kg, respectively. Mazur et al. [10] used GC-MS on sample of acid-hydrolysed chickpea to determine that the total isoflavone amount (total of formononetin, biochanin A, daidzein and genistein) was 11,477 µg/kg. They also identified coumestrol (50.0 µg/kg), matairesinol (0.00 µg/kg), and secoisolariciresinol (84.0 µg/kg) content of the sample. Using the GC-MS technique, Liggins et al. [9] tested the amounts of daidzein and genistein in various legumes, and they reported that chickpea contain 1241 µg/kg (wet weight) of these isoflavones (total). As evidenced by the variation in previous studies, different sample preparation methods (conventional extraction, acid hydrolysis and enzymatic hydrolysis) result in the detection of different phytoestrogen levels in chickpea samples.

The quantitative results derived from LC-MS/MS analysis, performed on acidified and non-acidified enzymatic hydrolysates of sample of chickpea are shown in Table 2. In our study, we used acid hydrolysis by HCl for preparation of samples of chickpea. In chickpea samples, prepared with acid hydrolysis, biochanin A (2.86 ± 0.09 µg/100 g), , genistein (0.50 ± 0.07 µg/100 g), genistin (3.86 ± 1.01 µg/100 g), glycitein (105.2 ± 9.87 µg/100 g), glycitin (55.5 ± 4.98 µg/100 g), ononin (7.95 ± 0.87 µg/100 g) sissotrin (446.8 ± 11.8 µg/100 g) and secoisolariciresionol (925.1 ± 10.9 µg/100 g) were identified at varying levels, whereas daidzein, daidzin, formonetin, apigenin, quercetin, rutin, coumestrol and matairesinol could not be identified in chickpea samples. The obtained data showed that types and levels of the identified compounds varied according to the sample preparation method. So we recommend that, to determine total phytoestrogenic compound in chickpea must be choosen as

enzymatic and enazymatic and acid hydrolysis by taking into consideration the especially for free isoflavone levels of sample prepared by these sample preparation methods.

TABLE II
PHYTOESTROGENIC COMPOUND CONTENT OF CHICKPEA SAMPLES

Compound	Concentration
BiochaninA	2.86 ± 0.09
Daidzein	nd
Daidzin	nd
Formononetin	nd
Genistein	0.50 ± 0.07
Genistin	3.86 ± 1.01
Glycitein	105.2 ± 9.87
Glycitin	55.5 ± 4.98
Ononin	7.95 ± 0.87
Sissotrin	446.8 ± 11.8
Matairesinol	nd
Secoisolariciresinol	925.1 ± 10.9
Apigenin	nd
Quercetin	nd
Rutin	nd
Coumestrol	nd

(mean, SD: Standard deviation, CV: Coefficient of variation, n=3, µg/100 g, wet weight)

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