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Extraction Time for Soybean Isoflavone Determination

Mercedes C. Carrão-Panizzi[1*], Silvana Pedroso de Goés Favoni[2] and Akio Kikuchi[3]
[1] Centro Nacional de Pesquisa da Soja – Embrapa Soja; C. P. 231; 86001-970; Londrina - PR - Brazil. [2] Estagiária Embrapa Soja. [3] National Agricultural Research Center for Western Region; Kagawa - Japan

ABSTRACT

Studies were carried out on the optimization of the extraction procedures for isoflavones determination in soybean by HPLC. Isoflavones were extracted from 100 mg samples of full fat soybean flour "Kinako" (milled toasted grains). Recovery of average total isoflavones was higher when extraction was performed with agitation(217.2 mg/100g), than without agitation(191.0 mg/100g). Isoflavone extraction without agitation for 1,4, 20 and 24 hours, were equally efficient for total and individual compounds. These results suggested that an efficient isoflavone recovery could be achieved with extraction for one hour with agitation.

Key words: Soybeans, isoflavones, glucosides, aglucones, extraction, ethanol, HPLC

INTRODUCTION

Isoflavones are phenolic compounds used to prevent and treat of the chronic diseases (Barnes et al., 1999). Since isoflavones are present in soybean, they are becoming important components of the human diet. Several studies indicated variability for isoflavone content among soybean cultivars (Wang and Murphy, 1994a; Tsukamoto et al., 1995; Carrão-Panizzi et al., 1998), and within soybean food products, as a consequence of the different processing techniques (Wang and Murphy, 1994b; Coward et al., 1998).

In soybeans, isoflavones are found mostly as β-glucoside conjugate forms, which include, daidzin, genistin, glycitin and their malonil and acethil derivatives (Kudou et al., 1991). Isoflavone glucosides are hydrolised by the action of β-glucosidase, to the aglucone forms daidzein, genistein and glycitein (Matsuura et al., 1989). Soaking in water or fermentation are processing methodologies that enhance this hydrolysis (Coward et al., 1998).

Different methods are used for isoflavone determination although the High Performance Liquid Chromatography (HPLC) is the most common procedure, different research groups use different methodologies for isoflavone extraction. This includes solvents such as methanol (Coward et al., 1998) or ethanol, temperatures and length of time for extraction (Kudou et al.,1991; Kitamura et al., 1991; Tsukamoto et al., 1995). In the Breeding Laboratory of Embrapa´s National Soybean Research Center, isoflavones are determined by using the methodology of Kudou et al. (1991), with some modifications. This work was carried out to define an optimum isoflavone extraction time with or without agitation. The shortest extraction time is important when a large number of samples are analysed, as is commom practice in breeding programs.

* Author for correspondence

MATERIALS AND METHODS

Extraction: Isoflavones were extracted from 100 mg samples of full fat soybean flour Kinako (milled toasted grains). Samples were placed in test tubes with 4.0 ml of 70% aqueous ethanol containing 0.1% acetic acid, at room temperature. The samples were extracted for different periods of times (1, 4, 8, 12, 16, 20, and 24 hours), with and without constant agitation (shaker at 250 rpm – Orbital Tecnal Mod. TE140). Each procedure was replicated two times. After extraction, 1.5 ml of the extract was transferred to an "Eppendorff" tube and stored at 5 °C.

HPLC Analysis: The samples were centrifuged for 10 minutes at 13.500 rpm at 10°C temperature (Centrifuge Eppendorf mod. 5417R). After centrifugation, 100 µl of the supernatant was transferred to an autosampler (10 µl was the volume injected). Isoflavone analysis were performed on ODS c-18 column (YMC-Pack ODS-AM), S-5 mm, 120 A (250 x 4.6 mm I.D.). The mobile phase (solvent A) was a solution of acetonitrile and 0.1% acetic acid, while solvent B was a solution of water and 0.1% acetic acid. Initial gradient was 20% for the solvent A in the first 20 minutes, passing to 100% for 5 minutes and 20% for the last 15 minutes. The effluent was monitored at 260 nm. The complete elution of each sample was performed in 40 minutes. Standard solutions daidzin, daidzein, genistin and genistein (SIGMA) were 0.0125 mg/ml.

Statistical Analysis: Treatments were evaluated in a factorial experiment in a randomized complete design. Before testing treatments by ANOVA, the data were tested for normal distribution (Shapiro and Wilk, 1965), homogenety of variance (Hartley, 1940; Burr and Foster, 1972), and model additivity (Tukey, 1949). Differences among mean values were determined by using Tukey's test at $P \leq 0.05$ (Cochran and Cox, 1957). Statistical Analysis System (SAS, 1995) was used to analyse the data.

Table 1 - Average (± SD) concentration of total isoflavones (mg/100g) in soybean flour (Kinako), extracted with and without agitation (CV=6.8%)[1].

Extraction time (hours)	Constant agitation	Without agitation	Mean
1	205.4 aA (±13.3)	228.4 aA (±10.8)	216.9 a
4	218.2 aA (±6.2)	198.9 abA (±11.6)	208.9 ab
8	223.0 aA (±1.3)	164.6 bcB (±6.8)	193.8 ab
12	225.4 aA (±9.8)	135.6 cB (±18.9)	180.5 b
16	229.9 aA (±17.1)	183.3 abB (±37.6)	206.6 ab
20	229.6 aA (±5.5)	208.1 abA (±2.5)	218.8 a
24	189.0 aA (±4.9)	218.7 aA (±7.7)	203.6 ab

[1]Mean values followed by the same capital letter in the lines and the same small letter in the columns are not significantly different (Tukey P≤0.05).

Table 2 - Average (± SD) concentration of isoflavones compounds (mg/100g) in soybean flour (Kinako), extracted with agitation[1].

Time (hours)	Daidzin	Genistin	Malonyl Daidzin	Malonyl Genistin	Daidzein	Genistein
1	38.5 a (±0.95)	61.1 a (±4.13)	36.1 a (±3.35)	59.6 a (±3.96)	3.9 a (±0.21)	6.2 a (±0.39)
4	38.4 a (±1.48)	66.4 a (±1.53)	38.3 a (±1.71)	64.5 a (±1.76)	4.1 a (±0.16)	6.6 a (±0.15)
8	39.0 a (±0.72)	67.6 a (±1.22)	39.5 a (±1.61)	65.9 a (±1.25)	4.2 a (±0.10)	6.7 a (±0.18)
12	39.0 a (±1.74)	70.0 a (±2.73)	37.3 a (±1.34)	67.8 a (±2.59)	4.2 a (±0.23)	7.0 a (±0.32)
16	40.5 a (±4.23)	70.6 a (±2.24)	38.9 a (±4.72)	68.6 a (±2.04)	4.3 a (±0.26)	7.0 a (±0.16)
20	40.7 a (±1.04)	70.3 a (±1.52)	39.3 a (±1.73)	68.0 a (±1.49)	4.5 a (±0.12)	6.8 a (±0.14)
24	32.7 a (±1.05)	58.2 a (±1.63)	31.7 a (±0.92)	56.4 a (±1.55)	3.9 a (±0.21)	6.1 a (±0.14)

[1]Mean values followed by the same letter in the columns are not significantly different (Tukey P≤0.05; CV(%): daidzin = 7.5; genistin = 6.7;malonyl daidzin = 8.6; malonyl genistin = 8.5; daidzein = 7.0; genistein = 6.8).

RESULTS AND DISCUSSION

Isoflavone extractions of the soybean flour under constant agitation were the same among the different extraction times for total isoflavone (Table 1), as well as for each isoflavone compound (Table 2). Isoflavone extraction without agitation for 1, 4, 20 and 24 hours were equally efficient for total isoflavones and for the individual compounds (Tables 1 and 3).

Comparing both extraction conditions, there were some statistical differences for total isoflavones at 8 and 12 hours of extraction time (Table 1), with lower recovery of the isoflavones without agitation. The same tendency was observed for individual isoflavone compounds without agitation (Table 3). Looking at the concentrations of each isoflavone compounds, no differences in absolute values were observed in the extractions for one hour and 24 hours (Table 3). Comparing the average of total isoflavones recovery in the two extraction methodologies, data indicated higher isoflavone concentration when extraction was performed with agitation (217.2 mg/100g). Without agitation conditions, average total isoflavone concentration was 191.0 mg/100g. These results suggested that an efficient isoflavone extraction could be achieved for one hour under constant agitation, as already observed by Kitamura et al. (1991) and Carrão-Panizzi et al. (1996). Cole and Cousin (1994) tested soybean isoflavone extraction with a solution of ethanol 80%, under refluxe at room temperature. These authors suggested

that extraction at room temperature should be more efficient, because isoflavone malonyl forms were unstable in high temperatures, which promoted inter-conversion of the 6"O-malonyl-glucosides to the β-glucoside forms. Similar methodology was used by Carrão-Panizzi (1996), with an efficient recovery of isoflavones using extraction solution of ethanol 70%, at constant agitation, at room temperature.

Kudou et al. (1991) extracted isoflavones with a solution of 70% ethanol for 24 hours at room temperature and for 15 hours at 80 °C. In the extraction at room temperature, manolylated isoflavone glucosides were the

major constituents. When the extraction was carried out at 80°C, lower concentrations of these compounds were observed. Tsukamoto et al. (1995) used the same method of Kudou et al. (1991), extracting isoflavones from whole seeds and embrionic axis for 24 hours.

Table 3 - Average (± SD) concentration of isoflavone compounds (mg/100g) in soybean flour (Kinako), extracted without agitation[1].

Time (hours)	Daidzin	Genistin	Malonyl Daidzin	Malonyl Genistin	Daidzein	Genistein
1	39.7 a (±2.41)	69.1 a (±1.36)	39.9 a (±2.31)	66.7 a (±1.24)	4.3 ab (±0.36)	6.9 a (±0.15)
4	32.7 ab (±1.07)	61.4 ab (±3.89)	35.1 ab (±2.60)	59.5 ab (±3.57)	3.9 ab (±0.29)	6.2 ab (±0.35)
8	28.3 bc (±1.03)	51.3 bc (±1.33)	27.8 bc (±1.11)	50.1 bc (±1.20)	3.3 bc (±0.08)	5.3 bc (±0.08)
12	22.8 c (±2.59)	42.0 c (±4.63)	22.3 c (±2.88)	41.2 c (±4.65)	2.7 c (±0.37)	4.5 c (±0.49)
16	31.8 abc (±5.54)	56.3 abc (±9.69)	31.0 abc (±4.90)	55.0 abc (±9.28)	3.6 abc (±0.60)	5.7 abc (±0.95)
20	35.9 ab (±0.82)	63.7 ab (±1.50)	36.1 ab (±0.73)	62.1 ab (±1.27)	3.9 ab (±0.22)	6.3 ab (±0.22)
24	37.6 a (±0.92)	66.8 a (±2.45)	37.2 ab (±0.91)	65.2 a (±2.11)	4.5 a (±0.60)	6.9 a (±0.54)

[1]Mean values followed by the same letter in the columns are not significantly different (Tukey P≤0.05; CV(%): daidzin = 7.5; genistin = 6.7;malonyl daidzin = 8.6; malonyl genistin = 8.5; daidzein = 7.0; genistein = 6.8).

According to the results, isoflavone extraction from soybean flours could be efficiently achieved by using ethanol 70% as solvent for one hour with agitation at room temperature. The constant shake allow better contact among the soybean particules and the solution. Extraction for 24 hours without agitation was also efficient. However, when a large number of samples have to be analysed, analysis proceedures that include extractions for one hour, are an important factor to save time.

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RESUMO

A soja apresenta isoflavonas que são compostos relacionados com a saúde humana. A otimização dos procedimentos de extração para determinação de isoflavonas por HPLC, são importantes quando muitas amostras são analisadas. As isoflavonas foram extraídas a partir de 100 mg de amostras de farinha integral de soja (Kinako) (grãos tostados e moídos), com solução aquosa etanol (70%), adicionado de 0,1% de ácido acético, à temperatura ambiente, por 1, 4, 8, 12, 16, 20, e 24 horas, com e sem agitação constante. A recuperação das isoflavonas totais médias foi mais alta quando a extração foi conduzida sob agitação (217.2 mg/100g) do sem agitação (191 mg/100g). A extração de isoflavonas por 1, 4, 20 e 24 horas, sem agitação, foi igualmente eficiente para o total de isoflavonas e para os compostos individualmente. Com agitação constante, não houve differenças na recuperação de isoflavonas para todas as condições de extração. Estes resultados sugerem que uma recuperação eficiente de isoflavonas pode ser obtida quando a extração é realizada por uma hora e sob agitação constante.

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