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Hydrothermal Treatments in the Development of Isoflavone Aglycones in Soybean (*Glycine max* (L.) Merrill) Grains

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ABSTRACT

Studies were carried out to enhance the development of isoflavone aglycones in soybean. Grains of two soybean cultivars, BR 36 and IAS 5, 115 and 278 mg/100g of total isoflavone, respectively, were treated hydrothermalyl at 45, 60 and 85°C for 5, 30 and 60 minutes. Pre-treatments of grains at 60°C for 60 minutes allowed a considerable increase of the isoflavone aglycones. Non-treated grains of BR 36 and IAS 5 showed 1.2 mg/100g of genistein, after hydrothermal treatments,which increased to 12 and 53 mg/100g, in each variety, respectively. At higher temperature (85 °C) there was a decrease of the aglycones due to inactivation of β-glycosidases. Malonyl compounds were also reduced at higher temperatures. In processing functional soybean foods, hydrothermal treatments of the soybean grains, as well as high isoflavone content soybean cultivars will enhance development of aglycone forms.

***Key words**: Soybean, isoflavones, glycosides, aglycones, temperature*

INTRODUCTION

Isoflavones are chemical compounds with biological activity in the human body, preventing chronic diseases (Barnes et al., 1999). Because of the high amounts of these compounds present in soybean, it became a potential ingredient for functional food products. Three types of isoflavones (daidzin, genistin and glycitin), in four chemical forms are found in soybean: conjugate glycosides, malonyl glycosides, acetyl glycosides and aglycones (daizein, genistein and glycitein) (Wang and Murphy, 1994a). In soybean grains, a large variability for isoflavone content among varieties has been observed (Eldridge and Kwolek, 1983, Wang and Murphy, 1994b, Tsukamoto et al., 1995, Carrão-Panizzi et al., 1995, 1998), which are due to genetic and environmental effects (Kitamura et al., 1991, Tsukamoto et al., 1995, Carrão-Panizzi et al., 1999).

As a consequence of processing conditions different concentrations of the isoflavone forms are found in food products. Isoflavone glycosides are the major forms found in the soybean grains and in non- fermented foods (Coward et al., 1998). The aglycone forms are present in higher concentrations in fermented soybean foods. The isoflavone glycosides are hydrolysed by the action of the β-glycosidases (Matsuura et al., 1989) and by intestinal enzymes to develop the aglycone forms, daidzein, genistein and glycitein (Tam et al., 1998). Genistein is effective in the prevention

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of cancers related to hormones, as breast and prostate (Peterson and Barnes 1991, 1993).
In order to enhance formation of the isoflavone aglycones this study was carried out to determine the effects of heat tratments on the hydrolyzation of isoflavone glycosides, forming isoflavone aglycones.

MATERIAL AND METHODS

Sample preparation

Samples of soybean grains (10g) of cultivars IAS 5 and BR 36, harvested in Ponta Grossa, State of Paraná, Brazil, (1998/99 soybean season), were treated at 45, 60 and 85ºC by soaking in distilled and deionized water for 5, 30 and 60 minutes. After the hydrothermal treatments, samples were washed with cold water and dried in a forced air oven. After drying grains were ground in domestic mill "Braun model KSM 2B. Each treatment was replicated 4 times. Samples from cultivar IAS 5 were also tested at different other hydrothermal treatments, 50°C for 1, 2, 4, 5, 6 and 16 hours, and 60°C for 0, 40, 45 and 50 minutes.

Isoflavone analyses

Isoflavones were extracted from 100 mg samples (milled soybean grains). Samples were placed in test tubes with 4.0 ml of 70% aqueous ethanol containing 0.1% acetic acid, at room temperature for 5 h (tubes were shaken at 15 minutes intervals). After extraction, 1.5 ml of the extracts were transferred to Eppendorff tubes and stored in a freezer, before the HPLC analysis.
For the HPLC analysis, the samples were centrifuged for 10 minutes at 13,500 rpm at 10°C temperature (Centrifuge Eppendorf mod. 5417R). After centrifugation, 80 µl of the supernadant was transferred to the micro tubes of the HPLC apparatus and placed in the auto sampler racks and 10 µl were 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. The initial gradient was 20% for solvent A and 80% for solvent B at a flow rate of 1.0 ml per minute. A linear gradient of 50% in solvent A was developed in a period of 20 minutes, reaching 100% gradient in other 5 minutes. For the last 15 minutes, it returned to the initial 20%. UV absorption was measured at 260 nm. The complete elution of each sample was performed in 40 minutes. Standard solutions of daidzin, daidzein, genistin and genistein (SIGMA) were 0.0125 mg/ml.

Activity of β-Glycosidase

β-glycosidase activity was analysed according to the methodology described by Matsuura et al. (1989), based on the measure of the absorbance of *para*-nitrophenol (p-NP), liberated by the action of the enzyme into the substract, *para*-nitrophenyl β-D-glucopiranosideo (p-NPG). Samples of 100 mg were placed in test tubes with lids and 1.5 ml citrate buffer (0.05M, pH 4.5 with NaCl 0.1M), was added. The enzyme extraction was carried out for 1 h at room temperature under shaking conditions at every 15 minutes in an authomatic test tubes agitator (Marconi MA 162). Samples were centrifuged in Eppendorf 5415 C microcentrifuge at 15,000 rpm for 3 minutes. The supernatant was utilized for the analyse.
The substrate of the β-glycosidase was p-NPG 1mM in sodium phosphate (Na_3PO_4) 0.1 M buffer, pH 6.7.
For the enzymatic reaction, 200 µl of the substrate and 200 µl of the enzyme solution were placed in test tubes and kept in water bath at 40^0C for 2:30 h to liberate p-nitrophenol. The reaction was stopped with the addition of 2 ml of sodium carbonate solution (0.25 M, pH 9.0). The amount of p-nitrophenol liberated by the action of β-glycosidases was determined in spectrophotometer (Cecil 3000) at 420 nm against the blank. The UA (Unit Activity) was defined as the amount of enzyme that liberated 1 µmol of p-NP from p-NPG per ml per minute.

Statistical Analysis

Treatments were evaluated in a factorial experiment in a complete randomized 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.

RESULTS AND DISCUSSION

The concentration of daidzein and genistein compounds increased considerably when non-treated soybean grains were compared with the hydrothermally treated grains. In the cultivars IAS 5 and BR 36, the aglycone concentration in non-treated grains were respectively, zero and 1.1 mg/100g for daidzein, and 1.2 mg/100g for genistein, in both cultivars. High amounts of daidzein were observed in grains of IAS 5 treated hydrothermally at 60°C for 60 minutes, as well as in the grains of BR 36 (Table 1). The genistein content followed the same trend observed for the daidzein. For IAS 5, there was an increase of 44 times for in the genistein levels at 60 ºC for 60 minutes (Table 2), as compared to non-treated grains (1.16 mg/100g). BR 36 cultivar presented lower genistein content than IAS 5, although showed increased genistein concentration 10 times. The soybean cultivar IAS 5 was recognized as a high isoflavone content genotype (277.8 mg/100g for total isoflavone), whereas BR 36 cultivar was a low isoflavone content genotype (114.7 mg/100g) (Carrão-Panizzi et al. 1998, 1999). Data from the experiments carried out in this study indicated that genetic differences were maintained even after heat treatments.

There were no differences in daidzein and genistein content when grains of BR 36 cultivar were treated for 30 and 60 minutes at 60 °C (Tables 1 and 2). Lower amount of the glycoside compounds in this cultivar could limit the development of aglycones.

Table 1 - Daidzein isoflavone concentration (mg/100g), in soybean grains of cultivars IAS 5 and BR 36, submitted to hydrotermic treatments, as a function of time (minutes) and temperature (ºC).

	IAS 5			BR 36		
Temperature	**5min**	**30min**	**60min**	**5min**	**30min**	**60min**
45	3.1 aB	4.6 bAB	9.9 bA	2.0 aB	4.2 bA	5.0 bA
	(± 0.46)	(± 0.53)	(± 0.62)	(± 0.17)	(± 0.82)	(± 0.38)
60	3.2 aB	7.2 abB	30.2 aA	2.4 aB	6.8 aA	7.6 aA
	(± 0.36)	(± 2.08)	(± 7.45)	(± 0.27)	(± 0.23)	(± 0.47)
85	5.2 aAB	10.9 aA	4.4 cB	2.8 aA	2.7 cA	2.5 cA
	(± 1.17)	(± 0.75)	(± 0.20)	(± 0.17)	(± 0.71)	(± 0.27)

Mean (±SD) values followed by the same lower case letters in columns and capital letters in lines are non significantly different by Tukey test (P≤0.05).

Table 2 - Genistein isoflavone concentration (mg/100g) in soybean grains of cultivars IAS 5 and BR 36, submitted to hydrothermic treatments, as a function of time (minutes) and temperature (ºC).

	IAS 5			BR 36		
Temperature	**5min**	**30min**	**60min**	**5min**	**30min**	**60min**
45	2.7 bC	9.7 bB	20.9 bA	2.7 bC	8.0 bB	10.2 bA
	(± 0.61)	(± 0.53)	(± 1.14)	(± 0.17)	(± 0.82)	(± 0.38)
60	3.4 bC	15.0 bB	52.7 aA	3.4 abB	11.1 aA	11.9 aA
	(± 0.61)	(± 5.81)	(± 5.53)	(± 0.27)	(± 0.23)	(± 0.47)
85	7.4 aB	18.9 aA	9.7 cB	4.0 aB	6.1 cA	4.6 cB
	(± 2.83)	(± 1.71)	(± 1.00)	(± 0.17)	(± 0.83)	(± 0.27)

Mean (± SD) values followed by the same lower case letters in columns and capital letters in lines are non significantly different by Tukey test (P≤0.05).

Formation of the aglycones was time and temperature dependent, since the glycoside hydrolysis occured by the action of the β-glycosidase enzyme (Matsuura et al., 1989, 1993). Depending upon the soaking temperatures from 10 to 80°C, the amount of daidzein and genistein changed markedly, reaching the highest amount at 50°C (Matsuura et al., 1989). These results were different from those observed in this experiment, where at 60°C aglycones were formed.

Matsuura et al. (1993) reported inactivation of the β-glycosidases at 60°C and an optimum temperature for the enzyme at 45°C. Of the original activity, 80 % remained at 55°C.

A reduction of the aglycones was observed for both cultivars at higher temperature (85°C) and longer period (60 minutes) (Tables 1 and 2), which could be a result of the inactivation of β-glycosidase enzymes (Figs.1 and 2), as observed by Ha et al. (1992).

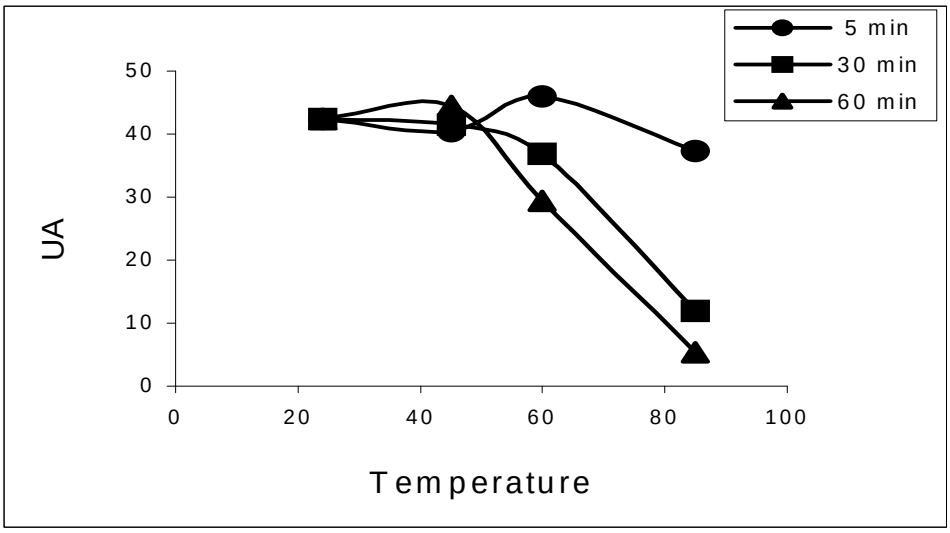

Figure 1 - Activity of β-glycosidases in grains of the soybean cultivar IAS 5, submitted to different hydrothermic treatments (45, 60 and 85^{0}C for 5, 30 and 60 minutes).

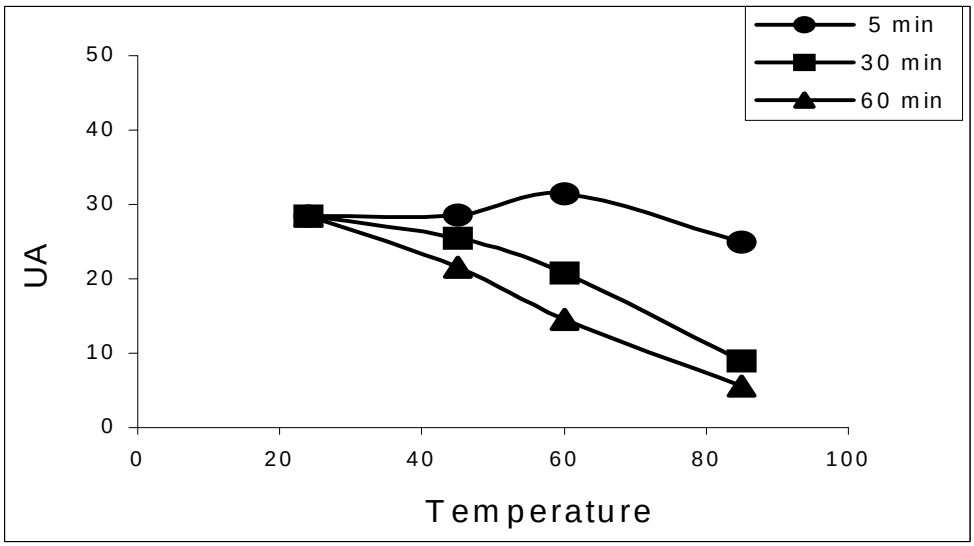

Figure 2 - Activity of β-glycosidases in grains of the soybean cultivar BR 36, submitted to different hydrothermic treatments (45, 60 and 85°C for 5, 30 and 60 minutes).

Additional data from two other experiments carried out with IAS 5 cultivar indicated that at 50°C genistein and daidzein increased over time, while total isoflavone decreased (Table 3). The same trend was observed at 60°C. Higher concentration of these aglycone compounds, however, were observed at 50°C, considering longer treatment.

Aqueous heating treatment caused conversion of the malonyl forms to glycosides forms (Kudou et al., 1991, Coward et al.,1998). Results from this experiment also showed that longer period of heating treatment decreased the malonyl compounds in both cultivars (Figs.3, 4, 5).

Table 3 - Isoflavone contents in soybean grains of cultivar IAS 5, submitted to different hydration times at a constant temperature of 50^0C.

Time (hours)	Daidzin	Genistin	Malonyl-Daidzin	Malonyl-Genistin	Daidzein	Genistein	Total
0	21.8 a (± 0.28)	28.9 a (± 0.85)	79.6 a (± 1.23)	146.3 a (± 5.70)	0	1.2 e (± 0.05)	277.8 a (± 7.41)
1	13.9 b (± 0.85)	20.0 b (± 1.34)	58.6 c (± 0.42)	96.7 b (± 3.22)	5.2 d (± 0.18)	12.0 d (± 0.64)	206.4 b (± 5.80)
2	13.3 b (± 1.80)	17.3 bc (± 0.74)	61.9 b (± 9.44)	97.7 b (± 4.60)	7.0 d (± 1.03)	15.5 d (± 1.34)	212.6 b (± 8.95)
4	19.2 a (± 11.21)	14.8 c (± 2.09)	59.7 bc (± 8.52)	81.9 c (± 9.68)	12.6 c (± 0.35)	24.7 b (± 0.35)	212.9 b (± 1.40)
5	9.3 c (± 1.24)	12.9 cd (± 0.81)	40.9 d (± 6.26)	65.9 d (± 6.01)	15.8 bc (± 0.11)	33.0 b (± 0.74)	177.7 c (± 13.68)
6	11.5 bc (± 0.78)	19.4 b (± 3.41)	37,83 d (± 0.62)	66.2 d (± 9.12)	17.1 b (± 1.80)	33.0 b (± 0.77)	185.1c (± 1.50)
16	4.5 d (± 0.39)	12.1 c (± 0.88)	11,00 e (± 0.35)	29.4 e (± 1.38)	30.5 a (± 0.18)	53.9 a (± 1.34)	141.4 d (± 1.63)

* Mean values followed by the same letter in the columns are not significantly different (Tukey (P≤ 0.05).

Table 4 - Isoflavone contents in soybean grains of cultivar IAS 5, submitted to different hydration times at a constant temperature of 60^0C.

Time (minutes)	Daidzin	Genistin	Malonyl-Daidzin	Malonyl-Genistin	Daidzein	Genistein	Total
0	21.8 a (± 0.28)	28.9 a (± 0.85)	79.6 a (±1.23)	146.3 a (±5.70)	0	1.20 c (±0.05)	277.8 a (±7.41)
40	10.7 b (±1.14)	17.3 b (± 0.97)	44.8 bc (±2.29)	93.6 c (± 2.40)	11.8 b (± 0.31)	22.3 b (± 0.27)	200.7 c (± 3.28)
45	14.2 b (± 0.61)	20.0 ab (± 0.20)	56.8 b (± 3.49)	104.4 b (± 2.20)	14.6 a (± 0.90)	25.0 a (± 0.41)	235.0 b (± 1.15)
50	10.5 b (± 0.25)	16.5 b (± 0.40)	44.2 b (± 0.31)	92.5 c (± 2.01)	13.5 ab (± 0.23)	25.8 a (±0.06)	203.1 c (± 3.27)

* Mean values followed by the same letter in the columns are not significantly different (Tukey test P≤0.05)

Concentrations and presence of different isoflavone compounds in foods should be taken into consideration, mainly when soyfoods are being used to preventing of chronic diseases. Isoflavone glycosides are absorbed after metabolized to the biologically active isoflavones genistein and daidzein (Wiita and Setchell, 2001), so they are not absorbed intact and their biovailability requires initial hydrolysis of the sugar moiety by intestinal β-glycosidases for uptake to the peripheral circulation (Setchell et al., 2001). However, the presence of bioavailable isoflavone forms are important in the processing of functional soybean foods that can be used for consumer that aim to have health benefits as well as for clinical studies.

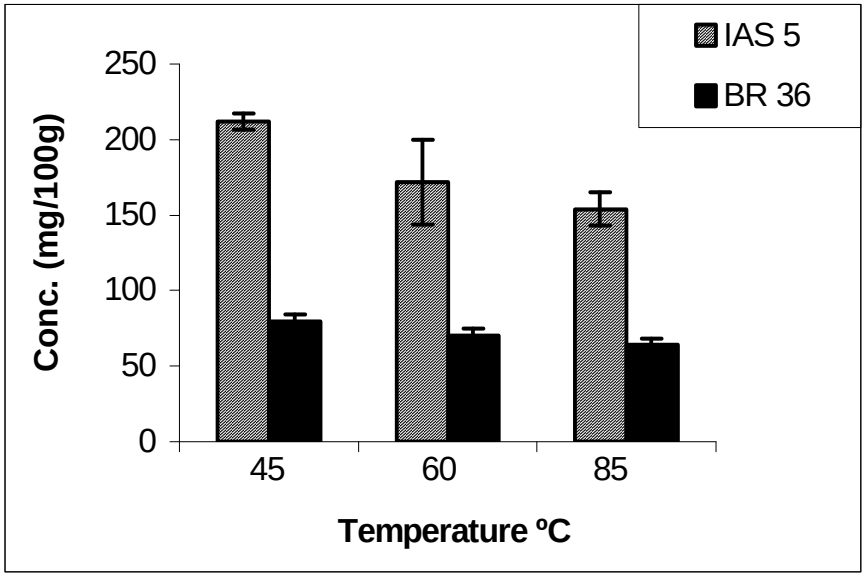

Figure 3 - Total malonyl isoflavones (mg/100g) in soybean grains of cultivars IAS 5 and BR 36, submitted to hydrothermic treatments at different temperatures (ºC) for 5 minutes (C).

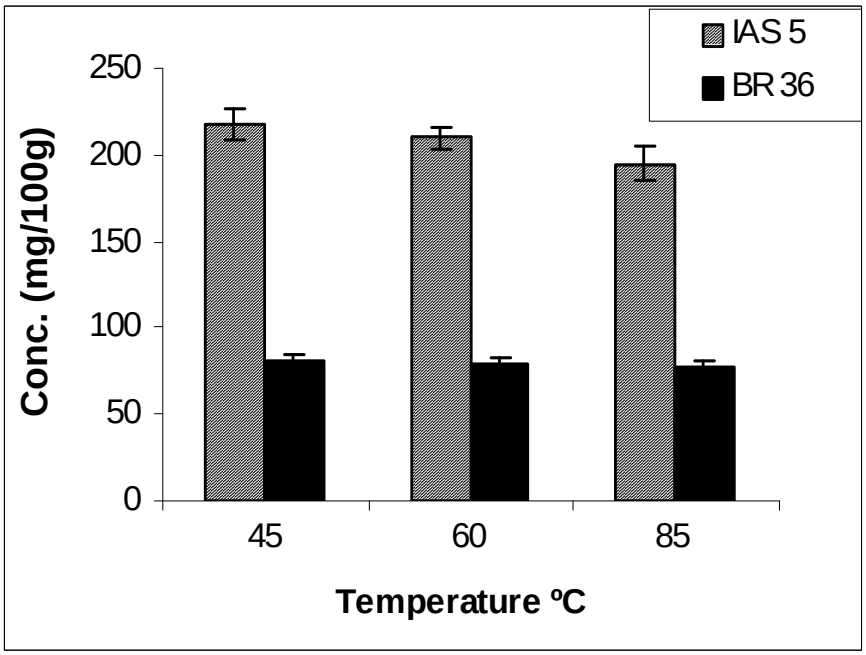

Figure 4 - Total malonyl isoflavones (mg/100g) in soybean grains of cultivars IAS 5 and BR 36, submitted to hydrothermic treatments at different temperatures (ºC) for 30 minutes (C).

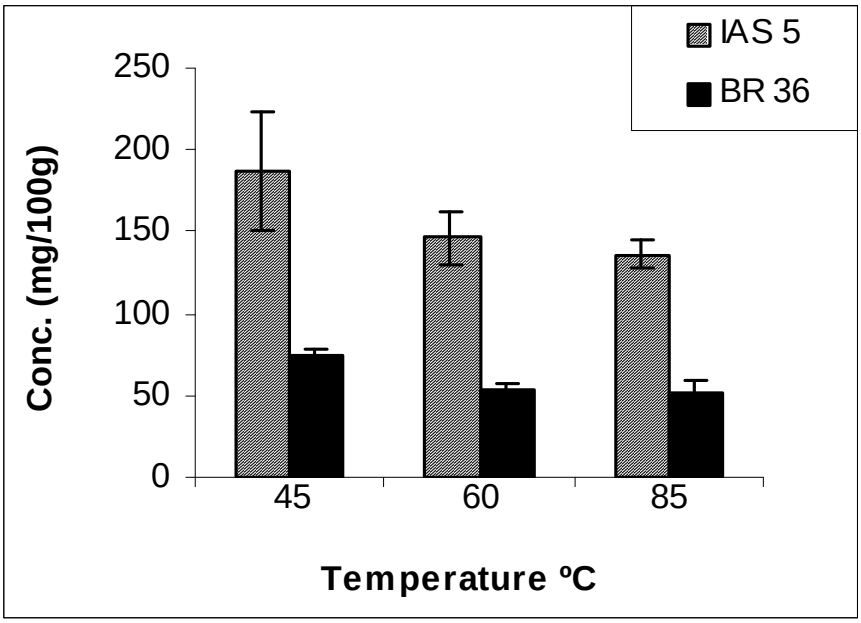

Figure 5 - Total malonyl isoflavones (mg/100g) in soybean grains of cultivars IAS 5 and BR 36, submitted to hydrothermic treatments at different temperatures (ºC) for 60 minutes (C).

To optimize processing technology of functional soybean foods, time and temperature are important factors to activate the development of isoflavone aglycones, as observed in the presented studies. Therefore, it is suggested that a pre-heat treatments of the soybean grains before processing would enhance the aglycones forms, which are readily available compounds. Soybean cultivars with high levels of isoflavones should also be considered in processing for functional soybean foods.

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RESUMO

Estudos foram conduzidos para aumentar isoflavonas agliconas (compostos mais biodisponíveis e mais efetivos na prevenção de doenças crônicas) em grãos de soja. Pré-tratamentos hidrotérmicos dos grãos foram conduzidos a 45, 60 e 85 ºC por 5, 30, e 60 minutos. Duas cultivares de soja BR 36 e IAS 5 (115, e 278 mg/100g de isoflavonas totais, respectivamente), foram usadas nos experimentos. Pré-tratamentos dos grãos a 60 ºC por 60 minutos permitiram um considerável aumento das isoflavonas agliconas. Grãos não tratados de BR 36 e IAS 5 apresentaram 1,2 mg/100g de genisteína. Depois dos tratamentos hidrotérmicos, este composto aumentou para 12 e 53 mg/100g, em cada variedade, respectivamente. Em altas temperaturas (85 ºC) houve diminuição das agliconas devido a inativação das enzimas β-glicosidases. Os compostos malonil (térmicamente instáveis), também foram reduzidos sob altas temperaturas. No processamento de alimentos funcionais de soja, pré-tratamentos hidrotérmicos dos grãos, bem como a utilização de cultivares com alto teor de isoflavonas permitirão maior desenvolvimento das formas agliconas.

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