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Thermal behavior of malonylglucoside isoflavones in soybean flour analyzed by RPHPLC/DAD and eletrospray ionization mass spectrometry

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ABSTRACT

Soybean (*Glycine max* (Merrill) L.) contains high content of aglycone isoflavones, as well as glucoside and malonylconjugates. In this work, the content of isoflavones in defatted soy flour was determined by reversed-phase high-performance liquid chromatography (RPHPLC) after alcoholic extraction in methanol/water mixture in the ratio 80:20 (v/v). It was observed that the heating treatment transformed the malonylglucosides into glucoside isoflavones. After heat treatment at 121 °C for 30 min, nearly all malonylisoflavones were converted into glucoside, but acetylisoflavones were not detected via RPHPLC analysis. Electrospray ionization mass spectrometry confirmed the presence of malonylisoflavones in heat-treated defatted soy flour by direct infusion analysis.

1. Introduction

Many vegetables are source of several chemical compounds with high importance to folk and modern medicine. The consumption of such foods (Kurzer & Xu, 1997) has been increasing steadily, and the food industries are concentrating more and more their attention to functional food types. U.S. market for functional foods, as estimated by the Nutrition Business Journal, may reach US$ 60 billion by 2010 (Henry, 1999).

Soybeans [*Glycine max* (Merrill) L.] and soy-based foods have long been consumed mainly by Asians, and have become very popular due to their good quality protein and oil content (Wang & Murphy, 1994). Soybean is an important food crop, and Brazil is a major producer of the soybean-complex (protein–oil–flour) (CONAB, 2003). The benefits of soybean to human health have long been known and are widely recognized around the world. Soybean provides potential benefits for several human diseases due to positive effects of several of its chemical components, mainly isoflavones and proteins. These natural constituents of soybeans display important biological activities, such as anticarcinogens, blood glucose lowering, and antioxidant (Lee et al., 2003).

More recently, attention has been paid to the isoflavone analysis of soy-based products (Fig. 1) and to the behavior of isoflavones during the variety of food processing technologies. During soybean protein processes, the malonylglucoside isoflavones are transformed to glucoside forms, and after the enzyme treatment it may be converted into aglycones (Park, Aguiar, Alencar, Mascarenhas, & Scamparini, 2002; Park, Aguiar, Alencar, Mascarenhas, & Scamparini, 2001; Park, Alencar, Nery, Aguiar, & Sato, 2001). There are indications that the aglycone forms might be more bioactive (Grün et al., 2001) than their parent molecules. However, isoflavone profiles should greatly depend on the extent and level of heating during soy processing.

The objective of this study was therefore to investigate the effect of heating on isoflavone profiles during the soybean flour production, and to characterize the heating products via high-resolution electrospray ionization mass (and tandem) mass spectrometry: ESI-MS(/MS).

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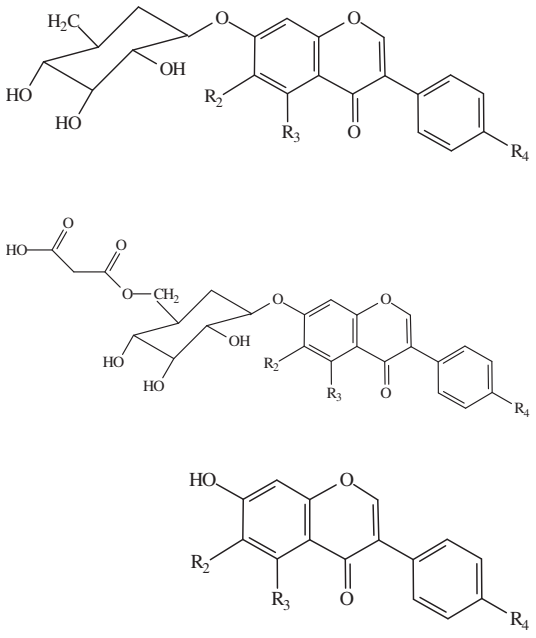

(1) daidzin, MW 416.38
(2) glycitin, MW 446.41
(3) genistin, MW 432.38
(4) malonyldaidzin, MW 502.43
(5) malonylglycitin, MW 532.46
(6) malonylgenistin, MW 518.43
(7) daidzein, MW 253.23
(8) glycitein, MW 283.12
(9) genistein, MW 269.23

Fig. 1. Chemical structures and molecular weight (MW) of the soy isoflavones.

2. Material and methods

2.1. Soybean sample and extract preparations

Soybean cv. IAC 15-1 was supplied by the Instituto Agronômico (Campinas/SP, Brazil). To extract the soybean oil, 50 g of soybean grains were finely milled, mixed with 500 mL of hexane (Synth Co., São Paulo, Brazil) and stirred for 1 h at room temperature, and then centrifuged (3000*g* for 10 min). The precipitates were kept under a hood to remove residual hexane. One-gram portion of defatted soybean flour was placed into screw-top test tubes containing 5 mL of deionized water and slightly stirred to mix. Then a set of tubes containing the mix were autoclaved at 121 °C (1 kgf/cm^2) for 20, 40, and 60 min, and the other set of tubes incubated in water bath at 100 °C for 20, 40 and 60 min. A third set of tube samples were held at room temperature (25 °C) as control (without heating). After these treatments, all tubes were freeze-dried and the dried material was dissolved (proportion, 1:10, w/v) in methanol/water mixture in the ratio 80:20 (Merck, São Paulo, Brazil) and placed in a shaker for 1 h at room temperature. The insoluble residue was separated by centrifugation and the supernatant was used for the analyses of isomeric isoflavones by reversed-phase HPLC and ESI-MS(/MS).

2.2. Chromatographic analysis

The analyses of isoflavones from soybean flour were performed by reversed-phase high-performance liquid chromatography (RPHPLC) with a chromatographer equipped with YMC Pack ODS-A column and diode array detector (SPD-M10Avp, Shimadzu Co., Kyoto, Japan). Elution was carried out at a flow rate of 0.5 mL min^{-1} using a solvent gradient consisting of a linear increase of the proportion of methanol from 20 to 80 parts into water (Merck Co., São Paulo, Brazil) in 19 parts distilled water and 1 part acetic acid (Synth Co., São Paulo, Brazil). Eluted isoflavones were detected by their absorbance at 254 nm. Quantitative data for daidzin (**1**), glycitin (**2**), genistin (**3**) and their malonylconjugates (**4**–**6**) and aglycone (**7**–**9**) forms (Fig. 1) were obtained by comparison to known standards (Sigma Co., Saint Louis, USA and Funakoshi Co., Tokyo, Japan).

2.3. ESI-MS(/MS) analysis

ESI-MS(/MS) experiments were performed on an orthogonal acceleration quadrupole–time-of-flight mass spectrometer (Q-TOF-MS) equipped with an ESI ionization with a Z-spray configuration (Micromass, Manchester, UK) and main operation conditions as described elsewhere (Aguiar, 2004; Aguiar, Baptista, Alencar, Haddad, & Eberlin, 2007). The following typical operating conditions were used: 3.3 kV capillary voltage, 35 V cone voltage, and 100 °C desolvation gas temperature. Tandem ESI-MS/MS experiments were performed via 15 eV collision-induced dissociation of selected ions with argon. Mass selection was performed by Q1 using a unitary *m*/*z* window, and collisions were performed in the hexapole collision cell, followed by mass analysis of product ions by the high-resolution orthogonal-reflectron time-of-flight analyzer.

2.4. Statistical analysis

To determine the significance of differences between the mean values, data were subject to randomized block design and were evaluated by analysis of variance and the Tukey test ($P < 0.05$) using the Statistica for Windows Release 5.0 (1995) computer program (Statsoft Inc., Tulsa, OK, USA). All values were the mean of three repetitions, and are presented as the mean ± standard deviation.

3. Results and discussion

As Table 1 show, the heat treatment of the soybean flour was found to promote the conversion of malonylglucoside to glucoside isoflavones. Increases in the glucoside isoflavone contents during heating were observed in six samples of defatted soybean flour analyzed when those samples were compared to control sample (without heating). Extraction of isoflavones from defatted soybean flour at room temperature gave the highest amounts of malonylglucoside isoflavones, with low quantity of daidzin, glycitin, and genistin (glucoside forms). Nevertheless, the defatted soybean flour treated at 121 °C for 40 min showed higher concentrations of daidzin, glycitin and genistin than their malonylconjugates. At 25 °C, the cv. IAC Foscarin-31 (Brazilian soybean cultivar) exhibited

Table 1
Isoflavone contents in defatted soy flour after heating (μg/g).[a]

Isoflavones	100 °C			121 °C		
	20 min	40 min	60 min	20 min	40 min	60 min
Daidzin	910.1 ± 5.1^{A}	804.2 ± 9.6^{B}	757.9 ± 6.6^{C}	1017.4 ± 17.7^{A}	870.1 ± 9.2^{B}	737.9 ± 11.2^{C}
Glycitin	102.5 ± 2.5^{A}	89.0 ± 3.0^{B}	78.4 ± 1.5^{C}	102.2 ± 1.0^{A}	87.9 ± 0.9^{B}	78.3 ± 0.5^{C}
Genistin	880.0 ± 2.0^{A}	799.2 ± 4.6^{B}	764.8 ± 8.1^{C}	978.5 ± 6.1^{A}	712.6 ± 6.6^{B}	701.8 ± 3.6^{C}
Malonyl daidzin	75.7 ± 0.4^{A}	54.5 ± 1.5^{B}	41.7 ± 2.0^{C}	6.1 ± 0.3	n.d.[b]	n.d.[b]
Malonyl glycitin	7.3 ± 0.3^{A}	4.9 ± 0.2^{B}	4.1 ± 0.9^{B}	n.d.[b]	n.d.[b]	n.d.[b]
Malonyl genistin	81.5 ± 0.3^{A}	59.0 ± 0.5^{B}	46.4 ± 1.2^{C}	6.2 ± 0.2	n.d.[b]	n.d.[b]
Daidzein	233.5 ± 3.5^{A}	210.7 ± 2.5^{B}	197.4 ± 2.5^{C}	134.3 ± 5.6^{A}	221.2 ± 2.6^{B}	274.3 ± 4.1^{C}
Glycitein	23.3 ± 0.3^{B}	24.6 ± 0.7^{A}	24.5 ± 1.7^{A}	61.5 ± 0.5^{A}	60.8 ± 0.9^{A}	55.2 ± 1.5^{B}
Genistein	234.1 ± 3.1^{A}	208.8 ± 3.5^{B}	198.2 ± 2.5^{C}	127.3 ± 2.5^{A}	272.5 ± 3.0^{B}	295.2 ± 3.0^{C}
Total	*2548.0*	*2254.1*	*2111.5*	*2433.4*	*2224.2*	*2142.8*

[a] Results are expressed as mean ± standard deviation ($n = 3$). Means with same row with common letters are not significantly different ($P < 0.05$).
[b] n.d. = Not detected.

1.4 mg g^{-1} as mean concentration of isoflavones, whereas cv. IAC 15-1 (other Brazilian soybean cultivar) showed about 3.0 mg g^{-1} of defatted soy flour. Heating at 121 °C for 40 min promoted a reduction of up to 17.5 times in the malonylcojugate isoflavones and an increase of approximately 2.5 times in the concentration of glucoside isoflavones (Table 1 and Fig. 2). According to Coward, Barnes, Setchell, and Barnes (1993), this reduction is due to the easy decarboxylation of malonylglucoside isoflavones to their corresponding glucoside derivatives, which explains the high content of daidzin, glycitin and genistin (glucoside forms) in soy flour treated by heating. Soybeans and defatted soy flour, with minimum heating, contained mainly malonylglucoside forms, in opposite to β-glucosides and acetylglucoside forms with a few quantities (Barnes, Kirk, & Coward, 1994). In our study, however, soy flours heated to 100 °C are found to contain mainly glucoside isoflavones (Fig. 2).

We observed, however, that the conversion of malonylconjugates to glucoside forms during the heat treatment occurred without formation of acetylconjugate isoflavones, and the soy samples treated at 121 °C for 40 min showed that almost all malonylconjugates were transformed into isoflavone glucosides (Table 1 and Fig. 2). After the heat treatment, any of the acetyl isoflavone forms were not detected by RPHPLC analysis. For all samples, the extraction after heating showed an increase in the glucoside forms when compared with those samples obtained from extraction at room temperature. According to Coward et al. (1993), malonylconjugates are instable and sensible to heating, and they are converted to glucoside isoflavones.

In general, flavonoids after food processing are about a half contents than in fresh foods (Andlauer & Fürst, 1998) and, according to Arditi, Meredith, and Flowerman (2000), some soyfood contain no isoflavones, because they are removed during the alcoholic extraction to produce soy concentrate protein. Aguiar, Alencar, Pacheco, and Park (2001) observed that isoflavone losses occur during industrial processes of soyfoods, such as soymilk, soy concentrate protein or soy isolate protein.

Isoflavone loss was also observed by Mahungu et al. (1999) when investigating the influence of the extrusion processing of corn/soy mixture on the stability of isoflavones. They reported that extrusion barrel temperature influence the most the isoflavone profile, especially the decarboxylation of malonylglucoside, and found that the amount of extractable isoflavones decreased after extrusion. According to Wu et al. (1992), baking degrades isoflavones and cleaves malonyl groups, acetyl groups, and glycosidic bonds due to heating.

However, the profile of malonylglucoside isoflavones should greatly depend on the level of heating (the temperature) utilized in the soy processing such as the degreasing of soy oil or soy protein concentration and isolation. In this work, malonylglucoside

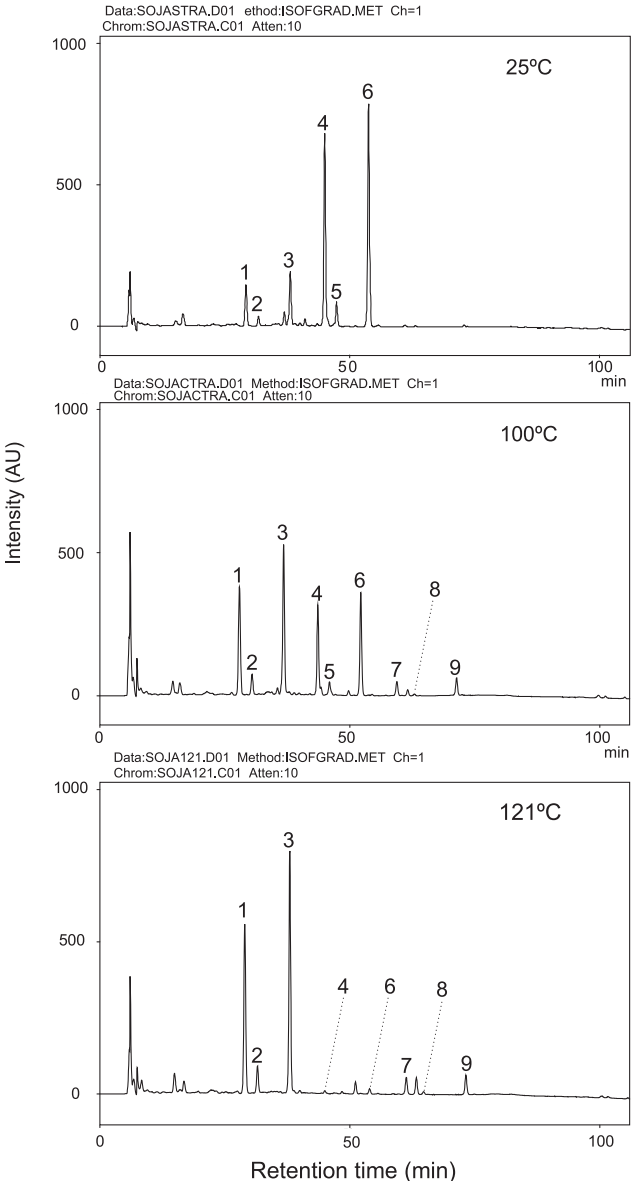

Fig. 2. RPHPLC chromatograms of methanolic extract of defatted soy flour treated for 30 min. marked peaks are due to: daidzin (**1**), glycitin (**2**), genistin (**3**), malonyl daidzin (**4**), malonyl glycitin (**5**), malonyl genistin (**6**), daidzein (**7**), glycitein (**8**) and genistein (**9**). *Y*-axis has intensity; *X*-axis has retention time in minutes.

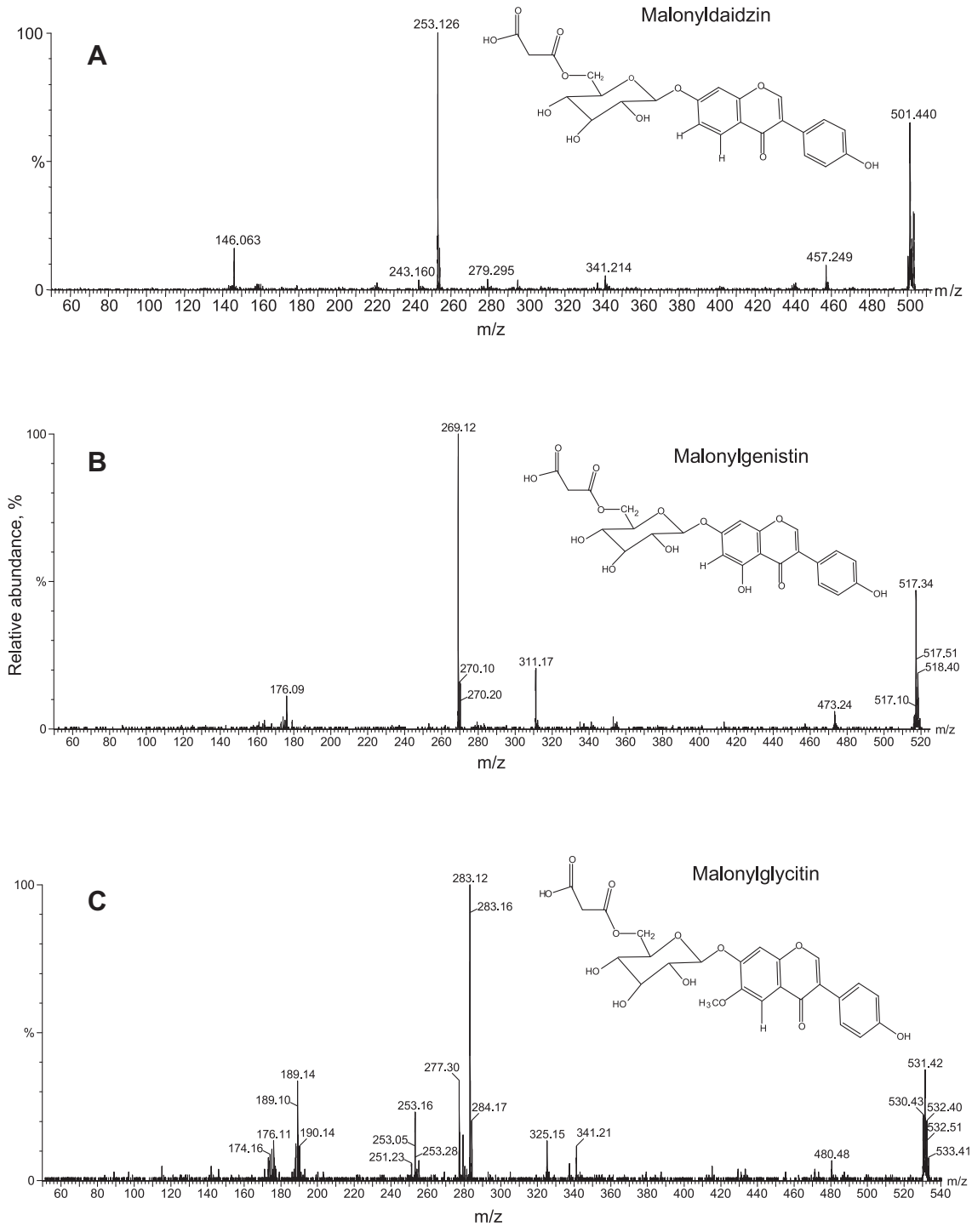

Fig. 3. ESI-MS/MS of the de-protonated malonylglucoside isoflavones extracted from defatted soy flour. A: malonyl daidzin (*m*/*z* 501); B: malonyl genistin (*m*/*z* 517); and C: malonyl glycitin (*m*/*z* 531). *Y*-axis has relative abundance in %; *X*-axis has *m*/*z* (mass to charge ratio).

isoflavones were found to be converted into glucoside forms by heating, and the increasing (+) or decreasing (−) in isoflavone percentages were: daidzin (+377.8%); glycitin (+250.8%); genistin (+382.6%); malonyl daidzin (−20.8%); malonyl glycitin (−21.8%); and malonyl genistin (−20.4%).

Fig. 2 shows typical RPHPLC chromatograms of isoflavones extracted from defatted soy flour treated at 25 °C, 100 °C and 121 °C for 30 min. It is observed that the isoflavone profiles changed as a function of temperature. The malonyl forms are decarboxylated to form glucoside isoflavones at 100 °C; and at 121 °C, practically all malonyl groups are decarboxylated.

Furthermore, boiling, blanching, freezing, and freeze-drying could be responsible for significant reduction in total isoflavone contents (Simonne et al., 2000). For example, freezing kept 53% of the initial total isoflavones, boiling 46%, and freezing-drying 40%. The authors reported that freeze-drying resulted in the greatest

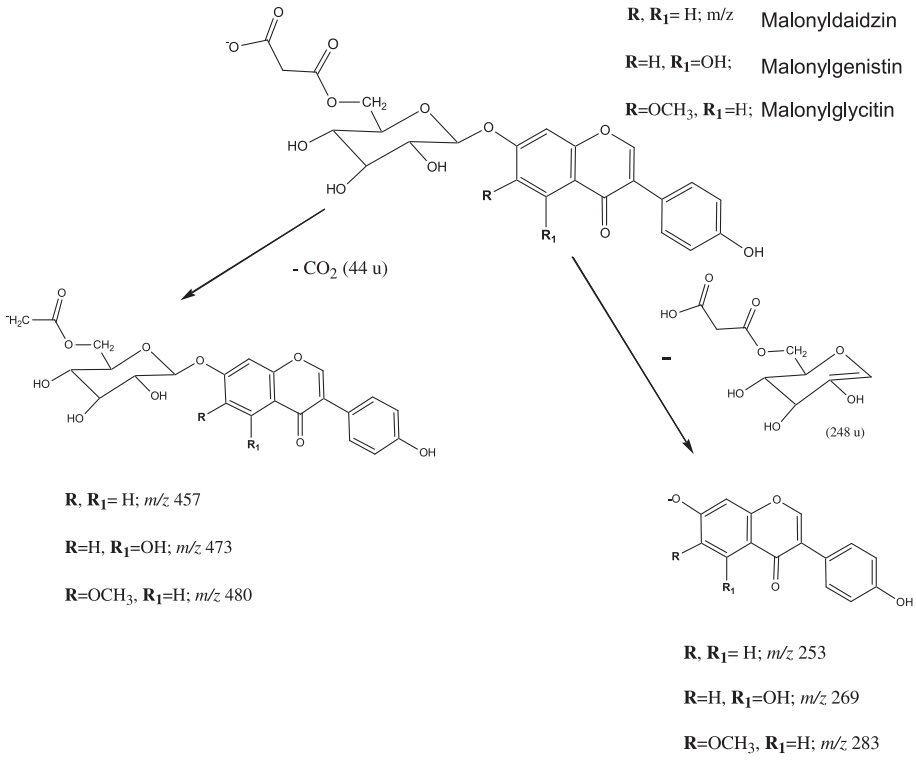

Fig. 4. General fragmentation routes for the de-protonated malonylconjugate isoflavones.

loss (around 60%) of total isoflavones, with the initial loss (56%) caused however by blanching, and that only 4% were due to the freeze-drying process.

The study of the ubiquitous class of phytochemicals known as the flavonoids has been confined largely to their distribution in the plant kingdom, the elucidation of their structures, and the pathways by which they are synthesized (Heinonen, Wahala, & Adlercreutz, 1999; Hughes, Croley, Metcalfe, & March, 2001; Moraes & Lago, 2003). The advent of fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) combined with tandem mass spectrometry (MS/MS) has allowed a ready study of the flavonoids, their characterization and the determination of flavonoids at low concentrations (Fabre, Rustan, Hoffmann, & Quetin-Leclercq, 2001; Hughes et al., 2001). Furthermore, electrospray ionization (ESI) is a very soft technique that generates mainly intact protonated molecules for a large variety of plant metabolites (Abreu, Mazzafera, Eberlin, Zullo, & Sawaya, 2007; Waridel et al., 2001).

Identification of isoflavones was therefore performed by high-resolution mass (and tandem mass) spectrometry in negative ion mode: ESI-MS(/MS). For ESI-MS/MS, collisions with argon at 15–30 eV were performed, and the fragmentation patterns observed for the malonylglucoside isoflavones were used for their identification (Fig. 3A: malonyl daidzin, 3B: malonyl genistin, and 3C: malonyl glycitin). Fig. 4 displays fragmentation routes for these de-protonated molecules. Two typical fragmentations are observed: the neutral loss of glucosidic group of 248 Da and CO_2 of 44 Da. It was also observed that C-7′ glucoside forms of isoflavones tend to undergo losses of the glucosidic group as a neutral molecule of 164 Da (Fig. 5).

In the ESI-MS of genistein, an ion of m/z 107 was always present in all samples analyzed (data not shown). According to Hughes et al. (2001), this ion may be due to HO–(C_6H_2)–O^- and is derived from m/z 151 by the loss of CO_2.

In a previous study, Aguiar et al. (2007) detected the presence of genistein in chickpea and soybean. ESI-MS/MS showed characteristic fragment ions of m/z 91, 107, 133, 159, 224 and 269 for both of the sample and for a genistein authentic standard.

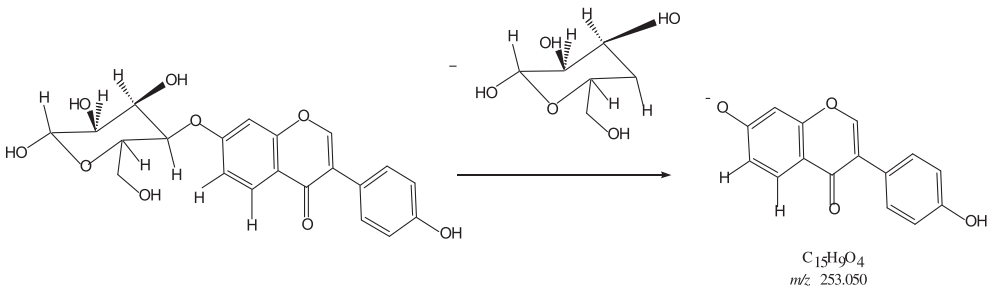

Fig. 5. Fragmentation of the glucoside isoflavone by the loss of glucosidic group as a neutral molecule.

4. Conclusions

In conclusion, our study demonstrated that heat treatment of soybean flour increases the amount of glucoside isoflavones due to decarboxylation of the corresponding malonylconjugate forms. After heat treatment at 121 °C for 30 min, nearly all malonylisoflavones were converted into glucoside isoflavones, but RPHPLC analyses showed absence of acetylisoflavones. ESI-MS(/MS) analyses confirmed the presence of malonylisoflavones in the defatted soy flour after heating.

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