Profiling of Different Bioactive Compounds in Soy Milk-sea Buckthorn Beverage

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The growing interest in new functional foods with special characteristics and health properties has led to the development of new beverages based on soy milk-sea buckthorn syrup. The effect of time, temperature of fermentation and storage on the content of daidzin isoflavone, total polyphenols compounds as well as on the antioxidant activity of soy milk and sea buckthorn beverage was studied. Final concentrations of daidzin in new beverages fluctuated for all samples. Significantly concentrations of daidzin (6-11 mg of daidzin/100 mL) were found in the samples fermented at 30°C. The DPPH radical scavenging activity was highest in beverage (73% and 98%, respectively) for samples fermented at 30°C. The total phenolic content values were highest in beverage: 34.6 - 40.6 mg gallic acid /mL from the samples fermented at 30°C. Results suggest that most of these compounds could contribute to health benefits.

Keywords: bioactive compounds, soy milk, sea buckthorn syrup, lactic fermentation,
soy milk and the following proportions of buckthorn syrup: 5.0% (v/v), 10.0% (v/v), 13.0% (v/v) and 20.0% (v/v). Microorganism was cultivated in 100 mL of sterilized soy milk with different amounts of sea buckthorn syrup at 30°C and 37°C for 12 h. After 12 h of fermentation, the fermented samples were stored at 4°C ± 1°C for 14 days and total phenolic content, antioxidant capacity and isoflavone (daidzin) were measured.

Isoflavone profile
Isoflavone (daidzin) was extracted and then analyzed by high-performance liquid chromatography (HPLC). To perform the isoflavone extraction the beverages were freeze-dried and stored at room temperature until analysis. A portion of 100 mg of freeze-dried beverage was placed in test tubes with 4.0 mL of 70% aqueous ethanol containing 0.1% acetic acid at room temperature. Mixtures obtained were centrifuged during 1 h at 5.433 x g (Hettich Zentrifugen, Germany) at 20°C. After stirring the test tubes were subject to sonication in ultrasonic bath (JP Selecta, Barcelona, Spain) for 15 min.

Determination of daidzin by HPLC
Analysis of isoflavone from all extraction was carried out using an Agilent 1200 HPLC equipped with quaternary pump, auto-sampler and UV detector was used with a stainless steel reverse phase 150 × 4.6 mm, 5 μm particle size, Hypersil C18 HPLC column (Thermo Scientific). The HPLC gradient and conditions are presented below.

Solvent A consisting of acetonitrile and solvent B was water and 0.25% acetic acid. Flow rate was set to 0.45 mL/min. The gradient was follows: 0-3 min: 100% solvent B, 3-50 min: 100% solvent A, 50.1-52 min: 100% solvent B. Daidzin identification was performed by comparison of the retention time of separated compound with retention time of the daidzin standard used in experiment.

Isoflavone standard was purchased from Sigma Aldrich (St. Louis, USA). The stock standard solution at 10 μg/mL was prepared in aqueous methanol (1:1, v/v) and stored in darkness at 4°C.

Total phenolic content
Total phenolic compounds were determined by the colorimetric method described by [9] using the Folin-Ciocalteu reagent. An aliquot of 0.5 mL of the beverage was mixed with 0.5 mL of Folin-Ciocalteu reagent and 10 mL of saturated Na₂CO₃ solution. Samples were kept at room temperature for 1 h. After this time, absorbance at 725 nm was measured using a UV-Vis Jenway 6506 spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK). Concentrations were determined by comparing the absorbance of the samples with a calibration curve built with 0, 100, 250, 500 and 1000 mg gallic acid/100 mL (Scharlau Chemie, SA, Barcelona, Spain). Results were expressed as mg of gallic acid per 100 mL of beverage.

Antioxidant capacity
The antioxidant capacity was studied by the evaluation of free radical-scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [10]. Aliquots of 0.1 mL beverage were mixed with 3.9 mL of methanolic DPPH (0.025 g/L) and 0.090 mL of distilled water. The homogenate was shaken vigorously and kept in darkness for 30 min. Absorption of the samples was measured using a UV-Vis Jenway 6506 spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK) at 515 nm against a blank of methanol without DPPH. Results were expressed as percentage of inhibition of the radical DPPH; which can be related to the decrease in absorbance with respect to the control value (DPPH initial absorption value).

Statistical analysis
Statistical analysis was performed using Statgraphics plus v.3.1 package (Manugistics Inc., Rockville, MA, USA). Data were analysed by multifactor analysis of variance and a Duncan multiple-range test was applied to determine differences among means, with a significance level of 0.05. All experimental analyses were carried out in triplicate.

Results and discussions
Determination of daidzin by HPLC
Figures 1 and 2 show the chromatograms obtained for standard solution of daidzin, for soy milk and 20% sea buckthorn fermented at 30°C and for soy milk and 20% sea buckthorn fermented at 37°C. The highest values for daidzin in all samples analyzed in the present study were presented by beverages with 20% sea buckthorn syrup. The values were in the range 7-10 mg of daidzin/100 mL beverage for fermented samples at 30°C. However, for samples fermented at 37°C presented lower daidzin content ranging from 1 to 7 mg/mL. All samples were significantly different (p < 0.05). Soy milk is sources of isoflavones, but their contents can be highly variable and thus appear to be partially responsible for the large variability of isoflavone contents in soy-based beverages [11]. In terms of values for daidzin they are comparable to the values reported by another reserchers. The values of daidzin present in soy-based beverages reported by [11]
ranged from 6 to 11 mg of daidzin/100 mL and between 4-10 mg of daidzin/100 mL.

From a nutritional point of view, the chemical form in which isoflavones occur in soy based foods may influence the biological activity, the bioavailability, and therefore the physiological effects of these dietary constituents. Only the aglycone forms are absorbed by the intestinal tract and are therefore biological active. But, glucosides, once ingested, are hydrolyzed by bacterial β-glucosidases in the intestinal wall, resulting in the conversion to their corresponding aglycones.

Other studies carried out have indicated that a range between 23 and 50 mg/d of isoflavones is sufficient to have significant endocrine effects [12].

Taking into account the results obtained in our study, we can point out that the fermented beverages may be a good way to incorporate a significant quantity of isoflavones in the diet, increasing the daily intake of these bioactive compounds. A regular portion (400 mL) of the beverage could contribute with 24-40 mg of daidzin, approximately.

In general, it has been reported that soy isoflavones are stable compounds during cold storage. No degradation of total isoflavones in soy food has been observed throughout storage time; nevertheless, they can be subject to several inter-conversions. Decarboxilation of malonate to acetate, de-esterification of malonate to underivatized glucoside as well as generation of aglycones are some reactions that could take place depending on processing, storage conditions, and molecular configuration of the compound [12].

Lower values obtained in our experiment are probably due to the microorganism (Bb-12®) used in lactic acid fermentation. It is known that lactic acid bacteria require carbon source for a good development. Also, the different concentrations present make it difficult to carry out an accurate determination of the concentration changes and some of the differences observed could be due to the low sensitivity of the analytical method.

**Total phenolic content**

The concentration of total phenolic compounds found in the beverages analyzed varied widely between samples (fig. 4). Analyzing the results presented in figure 4, one can see that the concentration of total phenolic compounds ranged between 34.6 - 40.6 mg gallic acid /mL from the samples fermented at 30°C, while for fermentation at 37°C values increased from 29.4 to 41.5 gallic acid /mL. The results obtained were significantly different (p < 0.05) for both fermentation.

Total phenolic content increased with increasing concentration of sea buckthorn syrup. The values reported for the beverages analyzed in this study show that the extraction protocol used in the experiment has allowed us to obtain extracts enriched in bioactive compounds, which may allow us to found beverages with physiologically active concentrations of those compounds after 14 day of storage.

In terms of values for total phenolic content, those obtained in this work are comparable to the ones previously obtained by other researchers. Values between 31.1 and 32.8 mg/100 g in tomatoes juice was reported by [10]. Also, [13] reported for fruit juice soy-milk values ranged from 79.88 to 83.09 mg of gallic acid/100 mL. Instead, [14] found higher values of total phenolic content: 684.27 ± 1.0 for orange juice compared with our beverage.

Furthermore, different phenolics can present different answers with the Foline Ciocalteu’s Reagent, presenting lower absorption which leads to an underestimation of various compounds [15].

Concerning the evolution of beverages over time, the total phenolic content remained quite stable for all the beverages, and it is possible a precipitation of flavanones after 14 days of storage.

**Antioxidant capacity**

The antioxidant capacity of beverages evaluated after 14 days of storage ranged from 73% (sample with 5% sea buckthorn syrup) to 98% (sample with 20% sea buckthorn syrup fermented at 30°C) of DPPH inhibition with significant differences among samples (fig. 5). Also, in figure 5 can be observed that for samples fermented at 37°C are obtained values which varied between 57-78%.

The changes observed in the antioxidant capacity of the beverages are explained by the relative percentages of sea buckthorn syrup and temperature of fermentation.
used, to obtained different levels in all beverages. Lower values were reported by [13] from fruit juice-soy milk beverage. The values found of researchers ranged between 27.26% - 29.61%.

The percentage of DPPH inhibition in beverages increased gradually after 14 days of storage, and it was significantly lower in those beverages fermented at 37°C.

The antioxidant capacity of fruit juices is related to the composition and concentration of bioactive compounds such as vitamins, phenols, carotenoids or flavonoids. Vitamin C and phenols are reported to be the major antioxidant components in fruit and vegetable juices [13].

The results of the present study suggest that the antioxidant activity of the functional soy milk and sea buckthorn beverages under investigation can be attributed to their phenolic content.

Determination of the antioxidant capacities of beverages can be quite important from a nutritional point of view, since it has been established that they are the main contributors to the total antioxidant capacity of a whole diet such as the Mediterranean diet. Moreover, the antioxidant capacity present in beverages may be more bioaccessible than the capacity associated with solid plant foods, where enzymatic action is necessary to release antioxidant compounds [16].

Conclusions

The differences in quantitative determinations of daidzin isoflavone, total phenolic content and antioxidant capacity in tested beverages were observed, indicating that sample preparation may affect the results. The results obtained indicate that, from among all compounds analysed, daidzin isoflavone was most affected by time and temperature of fermentation and storage. The total phenolic content and antioxidant capacity of the samples analysed was higher in the samples after 14 days of storage. The results found in this research suggest that soy milk and sea buckthorn beverage might be considered as a rich source of available bioactive compounds. Further research is needed to investigate the bioavailability and biological effect of beverage bioactive compounds in humans.

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