Extraction, characterization and microencapsulation of isoflavones from soybean molasses

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ABSTRACT: *This study aimed to extract isoflavones from soybean molasses with different solvents, furthermore, the selected extract, which showed overall desirable characteristics was selected to evaluate the potentials of different encapsulating agents. The encapsulating agents employed for the study included 18% Maltodextrin DE20 (T1), 18% Hi-maize (T2), and a mixture of equal proportions of 9% Maltodextrin DE20 and 9% of Hi-maize (T3). Solvents such as 80% ethanol and methanol, and grain alcohol in varying different concentrations of 50 and 80% were used for the studies. The best solvent for the extraction of phenolics and total isoflavones was 50% cereal alcohol, this extract also presented higher antioxidant activity. Evaluation of the encapsulating agents revealed that 18% Hi-maize with inlet air of 130 °C was best suited for the encapsulation of isoflavones. The ORAC method showed that microcapsules with the 18% Hi-maize encapsulating agent also had higher antioxidant activity.*
Key words: *Spray-drying, antioxidant, phenolics, isoflavones.*

Extração, caracterização e microencapsulação de isoflavonas do melaço de soja

RESUMO: *Este estudo teve como objetivo extrair isoflavonas do melaço de soja com diferentes solventes, além disso, o extrato selecionado com as características desejáveis em geral foi selecionado para avaliar os potenciais de diferentes agentes encapsulantes. Os agentes encapsulantes foram: 18% de maltodextrina DE20 (T1), 18% Hi-maize (T2) e uma mistura em proporções iguais de 9 % de maltodextrina DE20 e 9% de Hi-maize (T3). Foram testados os solventes, etanol e metanol a 80% e álcool de cereais a 50 e 80%. O melhor solvente para a extração de fenólicos e isoflavonas totais foi o álcool de cereais a 50%, sendo que este extrato também apresentou maior atividade antioxidante. Entre os agentes encapsulantes testados, 18% Hi-maize com ar de entrada de 130 °C mostrou ser o melhor para encapsular as isoflavonas. O método ORAC mostrou que as microcápsulas com o agente encapsulante Hi-maize a 18% também apresentaram maior atividade antioxidante.*
Palavras-chave: *Spray-drying, antioxidante, fenólicos, isoflavonas.*

INTRODUCTION

Soy is one of the most important products in the Brazilian economy, holding a prominent place in the export agenda of the country production was estimated at 116.996 34 million tons in the 2017/2018 harvest, placing Brazil as a the second biggest grain production (CONAB, 2018), which on the other hand, resulted in a significant waste generation that pose as potential environmental degrading agent.

The soybean grain has high isoflavone content, varying from 12 to 461 mg/100 g, making them the main phenolic found in this legume (CARRÃO-PANIZZI et al., 2009).

Due to the environmental impacts associated with by-products generation during soy-based food production, there is a dire need to reuse this waste in order to minimize production costs and enhance total utilization of food (CANTERI et al., 2008). Soy molasses, a dark brown syrup rich in carbohydrates and isoflavones, is usually produced concurrently with the soybean protein concentrate. In addition, this waste product has been used as raw material for fermentation (SIQUEIRA et al., 2008) predominately in bioethanol

production (SIQUEIRA et al., 2008; LONG & GIBBONS, 2013). Since soy molasses is an agro-industrial waste with a high generation volume, and a material of low commercial cost, this comparative advantages makes this byproduct a potential source for the extraction of bioactive compounds such as isoflavones for commercial purposes.

The use of bioactive compounds alone in food is a challenge for the food industry due to its instability. Microencapsulation process is intended to protect the encapsulated compounds against environmental factors and the components of the food itself, as well as to mask undesirable flavor and aroma characteristics that these compounds can confer on food (CHAMPAGNE & FUSTIER, 2007; FAVARO et. al., 2008).

Considering the importance of the isoflavones derived from soybean, and the microencapsulation process, this study had the following objectives: to extract the isoflavones from soybean molasses with different solvents, microencapsulate the extract with better characteristics by testing different encapsulating agents.

MATERIALS AND METHODS

Soy molasses was supplied by the Selecta industry (Goiânia, GO). Three solvent systems were tested: 80% methanol 1:10 (sample/solvent, v/v), 80% ethanol 1:10 (sample/solvent, v/v) and 80% and 50% grain alcohol 1:10 (sample/solvent, v/v). The extraction was performed according to CARRÃO-PANIZZI et al. (2003), with modification. Briefly, extraction was done under constant agitation for 2 hrs at room temperature (shaker at 250 rpm - Orbital Tecnal Mod. TE140), followed by centrifugation (Servilab MTD III Plus centrifuge) at 1083 g for 20 min to obtain two fractions: the supernatant and the precipitate. The supernatant was collected and the precipitate was subjected to exhaustive extraction for a further 2 hours, totalling a 4 hour extraction. After exhaustive extraction, the extracts were concentrated using a rotary evaporator and the identification of the isoflavones in extract was done using high performance liquid chromatography (HPLC). Afterwards, extracts were microencapsulated and other analyses such as antioxidant activity performed, the extracts were stored under refrigeration.

The determination of the phenolic content was performed using Folin-Ciocalteu method as described by SINGLETON et al. (1999), and total phenolic compound contents and expressed as milligrams of gallic acid/ 100 g of soybean molasses extract (mg GAE/100 g).

The antioxidant capacity was determined by the reduction of the stable radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) according to the methodology proposed by BRAND-WILLIANS et al. (1995). The ability to sequester free radical was calculated according to equation 1 and expressed as percentage of inhibition of oxidation of the radical.

$$\% \, Inhibition = ((Abs._{DPPH} - Abs._{Am/Trolox}) / Abs._{DPPH}) * 100$$
Equation (1)

Additionally the IC_{50} value was determined by the equation of the line plotted through the results containing the concentration values (mg/mL) used in the X axis and the percentages of protection reported in the Y axis. A standard Trolox curve in μmol versus % of inhibition was also constructed, where the result was expressed in μmol equivalents of Trolox/g sample (μmol TEAC/g)

The antioxidant capacity was determined by the ORAC method (Oxygen Radical Absorption Capacity) proposed by OU et al. (2001). In a 96-well microplate, 25 μL of the diluted extract (200 mg mL^{-1}) was added in phosphate buffer, pH 7.4 (75 mmol L^{-1}), followed by incubation in a microplate reader (HIDEX, Turku , Finland) for 10 min at 37 °C with 150 μl fluorescein working solution (81 nmol L^{-1}). After incubation, 25 μl of AAPH (152 mmol L^{-1}) were added to form peroxyl radicals. The results were compared with a standard Trolox curve (0-96 μmol L^{-1}) and expressed as μmol of Trolox equivalent to 1 g of sample. The AUC was calculated as follows:

$$AUC = 1 + f1 / f0 + f2 / (f0) + f3 / f0 + \cdots fn / f0 \quad (2)$$
Where
f 1...fn: fluorescence determined every minute.
f0: fluorescence at time zero.

The isoflavones were analysed using a high performance liquid chromatography (LC-20A Prominence, Shimadzu, Japan) equipped with a quaternary pump (LC-20AD), manual injector (CTA-20A) and diode arrangement detector (SPD-M20A). The data were processed in the LC solutions program (Version 3, Shimadzu, Columbia, U.S.A). Separation of the compounds was performed on reverse phase column (ODS C18 Microsorb-Mv, 25 mm x 4.6 mm - 5 μm) in gradient elution with flow rate of 0.8 mL / min. Acidified water (0.1% acetic acid) (A) and acidified methanol (0.1% acetic acid) (B) were the solvents used as the mobile phase. The gradient was 0 min, 30% B, in 7 min 40% B, in 15 min 50%, in 25 min 50% B, according to the methodology proposed by ROSTAGNO et al. (2005).

Prior to the injection, samples were treated for removal of interferences in polymer resin cartridges (500 mg, 6cc, Dionex™ SolEx™ HRPHS

Polymer-Based SPE Cartridge, Thermo Fischer Scientific) for solid phase extraction as described by KLEJDUS et al. (1999), with modifications quantification was performed by integrating peak areas at 254 nm. The limits of quantification (LoQ) and detection (LoD) of the method were: LoD = 0.78 and LoQ 2.36 for daidzein and LoD = 0.29 and LoQ = 0.89 for genistein determined by curve data analysis, which were used to quantify the different aglycone isoflavones present in soybean molasses. All compounds with UV-visible spectra similar to that expected for isoflavones and the standards used in this research were considered as compounds derived from this class and totalized for determination of the total isoflavone concentration in the sample. All compounds with UV-visible spectra similar to that expected for isoflavones and the standards used in this research were considered as compounds derived from this class and totalized for determination of the total isoflavone concentration in the sample.

Microencapsulation was performed on a laboratory scale using mini Spray drying (Buchi, B-290) with 1.5 mm feed nozzle with a flow rate of 0.45 L/h. A percentage of 50% grain alcohol 1:10 (sample/ solvent, v/v) was used as the standard solvent and the isoflavones extracts were mixed with maltodextrin MOR-REX®192, DE20 and Hi-maize® 260 (22000b00) (modified starch) at different concentrations. Treatment 1 corresponds to 18% maltodextrin (T1), treatment 2 to 18% modified starch (T2), treatment 3 to a mixture of equal portions of maltodextrin and 9% modified starch each (T3). All treatments were tested at different drying temperatures (inlet air) of 120, 130 and 140 °C.

The microcapsules were dissolved by the method proposed by ROBERT et al. (2010), with modifications. A total of 0.6 g of capsules were weighed and 3 mL of acetonitrile and 3 mL of a methanol: acetic acid: water (50:8:42 v/v/v) solution were added. The mixture was agitated for 1 min in vortex, and then placed in an ultrasonic bath for 60 min, centrifuged at 500 rpm/ 15 min.

The encapsulation efficiency was evaluated by the phenolic compounds content of the microcapsules. The determination of surface phenolic compounds followed the methods described by ROBERT et al., (2010), with modifications, which 0.4 g of microcapsules was weighed and 2 mL added of an ethanol: methanol (1:1 v/v) followed by the procedures established in item 2.6.2. Surface phenolic compounds (SFC) and encapsulation efficiency (EE) were calculated according to equations 1 and 2, respectively.

$$SFC\ (\%) = \frac{Surface\ phenolic\ compounds}{Theoretical\ content\ of\ phenolic\ compounds}\ x\ 100$$

$$EE\ (\%) = 100 - SFC\ (\%) \quad (3)$$

Statistical analysis

Extracts and analyses were conducted in triplicate. Data were submitted to analysis of variance (ANOVA), followed by the Tukey test for comparison of means each other. Results were considered significant when $p<0.05$. Statistical analyses were performed in the STATISTICA version 7.0 (StatSoft Inc, Tulsa - OK, USA).

RESULTS AND DISCUSSION

Results (Table 1) showed that 50% grain alcohol extraction (v/v) (244.89 ± 11.20 mg GAE/100 g) was better for the extraction of phenolic compounds from soybean molasses, differing statistically from the other solvents. These values were higher than the ones reported by MANTOVANI (2013), in which phenolics ranged from 26.8 ($\pm$ 3.6) to 45.7 ($\pm$ 1.9) mg GAE/100 g and 28.3 ($\pm$ 3.1) to 59.8 ($\pm$ 2.3) mg GAE/100 g of soy molasses, using as solvent 80% methanol and 90% ethanol, respectively.

Studies performed by MENEZES et al. (2013) showed that pure water was not efficient to extract phenolic substances and, in addition, that mixtures of alcoholic solvents with water are more efficient. Mixture of water with grain alcohol provided an increase in the polarity of the extracting solvent, may have contributed to a high extraction of phenolic compounds (HUSSAIN & CHAKRABORTY, 2012), which are results similar to those reported in this study.

The IC_{50} values of the extracts obtained with cereal alcohol show low values indicating high antioxidant activity, because according to ARBOS et al. (2010) high IC_{50} values (above 25 mg/g) have low antioxidant potential. Antioxidant activity determined by the ORAC method also showed that the cereal alcohol was the best solvent.

The levels of daidzein and genistein (15.38 ± 1.50 and 4.25 ± 0.53, respectively) were higher with 50% grain alcohol extraction (Table 2). This study presented high results when compared to those reported by MANTOVANI (2013), who used ultrafiltration of isoflavone aglycones from soybean molasses, resulting in values of daidzein 1.70 ($\pm$ 1.3) mg/100 g and genistein 0.014 ($\pm$ 1.2) mg/100 g of soybean molasses, with the use of 80% methanol as solvent.

The variation in the levels of isoflavones in soybean and its derivatives may change, since they depend on the grain variety, climate, soil and cultivation site. In addition, they may be altered by the temperature and type of processing that foods containing soy derivatives go through (LEE et al., 2003).

Table 1 – Total phenolics, IC_{50}, ORAC, daidzein, genistein, isoflavones aglycones and total isoflavones from the extract of soybean molasses obtained by different solvents.

---Antioxidant activity---			
Solvents	Total phenolics mg GAE/100 g soy molasses	IC_{50} mg/g soy molasses	ORAC µmol Trolox/g soy molasses
Ethanol 80%	153.09 [b] ± 1.36	7.89 [a] ± 0.26	211.30 [c] ±1.78
Methanol 80%	144.81 [c] ± 1.88	6.78 [a] ± 0.66	241.85 [b] ± 2.67
Grain alcohol 80%	159.42 [b] ± 0.0	5.13 [b] ± 0.75	266.97 [a] ± 2.56
Grain alcohol 50%	244.89 [a] ± 11.20	5.03 [b] ± 0.25	271.90 [a] ±1.80
---Isoflavones aglycones---			
Solvents	Daidzein mg/100 g soy molasses	Genistein mg/100 g soy molasses	Isoflavones aglycones mg/100 g soy molasses	Total isoflavones mg/100 g soy molasses
Ethanol 80%	11.60 [c] ± 2.4	3.47 [b] ± 0.1	15.07 [b]	56.84 [c] ± 2.39
Methanol 80%	13.52 [b] ± 0.6	2.36 [c] ± 0.0	15.88 [b]	63.31 [b] ± 4.19
Grain alcohol 80%	12.07 [c] ± 1.20	3.26 [b] ± 0.50	15.33 [b]	46.36 [d] ± 3.80
Grain alcohol 50%	15.38 [a] ± 1.50	4.25 [a] ± 0.53	19.63 [a]	72.03 [a] ± 1.54

Results expressed in Mean ± SD (n = 3). Equal lower case letters in the same column do not present significant difference by the Tukey test (p>0.05) for the different solvents with different concentrations. Gallic acid equivalent (GAE).

Another important factor to emphasize is the solubility of the isoflavones, the aglycone forms are less soluble in water than the glycosylated and conjugated forms. Studies by LUI, (2004) showed that increasing the polarity of the solvent decreased the solubility of the aglycone forms, thus increasing their concentration. This hypothesis was reinforced by the fact that the percentage of the aglycone forms in the supernatants increased with the alcohol concentration. However, this study showed opposite results, where the solvent with higher water concentration obtained higher aglycone results.

The extract obtained using 50% grain alcohol was selected owing to the aforementioned advantages, and subjected to the microencapsulation process.For all input temperatures tested, (Table 2) the outlet temperatures were 90 °C with the exception of T1 (18% maltodextrin DE20) which showed an oscillation at the outlet temperatures of 91, 110 and 111 °C. The input temperature is a variable that interferes with the encapsulation efficiency of the process allied with the characteristics of the encapsulating agent (KISSEL et al., 2006).

The encapsulation efficiency of T1 (18% Maltodextrin DE20) differs statistically from the other formulations, thus presenting itself higher in the different temperatures tested, followed by T3 (9% Maltodextrin DE20 and 9% Hi-maize).

Analysing the treatments with 18% Hi-maize encapsulating agent (T2) in Table 2, the values differed statistically from the other treatments, presenting a mean of 50% retention of the phenolic compounds analysed in this study, when compared to the other treatments that presented retention values of less than 24 and 26% for treatment T1 (18% Maltodextrin DE20) and T3 (18% Maltodextrin DE20 and 18% Hi-maize), respectively. The inlet temperature at 130 ºC for T2 presented total phenolic values (78.81 ± 0.17 mg GAE/100 g) and total isoflavones (20.44 ± 4.54 mg/100 mL) higher than the other treatments, as well as the treatment that presented higher values of aglycone isoflavones, with values of 2.40 (± 0.43) mg/100 g and 1.28 (± 0.28) mg/100 g of daidzein and genistein, respectively (Table 2). The results showed that 18% Hi-maize at 130 ºC is the best encapsulating agent in the retention of the phenolic compounds and isoflavones of soybean molasses, differing from other studies showing maltodextrin DE20 as the best encapsulating agent used in the retention of other bioactive compounds.

Maltodextrin is one of the most widely used encapsulating agents as a wall drying material for spray drying, due to its high solubility in water (ABADIO et al., 2004). However, the results reported in this study showed the 18% hi-maize as the encapsulating agent with higher retention of the studied bioactive compounds in all air temperatures tested. Analysing the total phenolic compounds and total isoflavones (Table 2), it is possible to observe that there was a degradation of 40 to 70% in all the treatments used. In addition, the hi-maize microcapsule submitted to 130 ºC better protected the

Table 2 – Total phenolics, isoflavones aglycones, total isoflavones, IC_{50} and ORAC from the extract with the best features, microencapsulated with different encapsulating agents and different inlet air temperatures.

			------Phenolics and Isoflavones aglycones------			
Microcapsules	Yield (%)	EE (%)	Phenolic mg GAE/100 g soy molasses	Daidzein mg/100 g soy molasses	Genistein mg/100 g soy molasses	Total isoflavones mg/100 g soy molasses
T1 120ºC	44.30 [c]	89.29 [a]	40.46 [e] ± 0.31	1.11 [g] ± 0.15	0.62 [f] ± 0.27	11.71 [h] ± 3.34
T1 130ºC	46.02 [b]	87.49 [ab]	39.17 [f] ± 0.02	0.98 [h] ± 0.22	0.60 [f] ± 0.20	8.14 [i] ± 1.94
T1 140ºC	39.72 [e]	89.65 [a]	36.64 [g] ± 0.01	1.70 [e] ± 1.20	0.69 [f] ± 0.28	15.41 [e] ± 4.69
T2 120ºC	37.48 [f]	76.36 [c]	76.46 [c] ± 0.19	1.55 [e] ± 0.24	0.88 [e] ± 0.27	16.62 [d] ± 4.68
T2 130ºC	40.23 [d]	76.69 [c]	78.81 [b] ± 0.17	2.40 [b] ± 0.43	1.28 [b] ± 0.28	20.44 [b] ± 4.54
T2 140ºC	37.83 [f]	76.34 [c]	78.27 [b] ± 0.09	2.41 [b] ± 0.35	0.61 [f] ± 0.14	18.77 [c] ± 3.04
T3 120ºC	41.71 [d]	88.92 [a]	41.24 [e] ± 0.13	1.34 [f] ± 0.13	0.83 [e] ± 0.31	10.83 [f] ± 1.37
T3 130ºC	34.95 [g]	88.78 [a]	41.16 [e] ± 0.03	1.61 [d] ± 0.27	1.02 [c] ± 0.41	13.50 [f] ± 3.83
T3 140ºC	53.44 [a]	87.51 [ab]	44.34 [d] ± 0.11	1.56 [e] ± 0.23	0.96 [d] ± 0.41	12.45 [g] ± 3.24
Free extract mg/100 g soy molasses	-	-	159.71 [a] ± 0.02	14.92 [a] ± 2.76	9.01 [a] ± 2.56	49.96 [a] ± 5.33
	------Antioxidant activity------					
Microcapsules			IC_{50} (mg/g)		ORAC (µmol Trolox/g)	
T1 120 ºC			41.05 [d] ± 6.86		25.59 [e] ± 3.40	
T1 130 ºC			39.79 [e] ± 3.43		30.83 [d] ± 2.53	
T1 140 ºC			36.38 [f] ± 3.56		22.74 [g] ± 2.22	
T2 120 ºC			31.05 [g] ± 0.34		90.54 [b] ± 0.97	
T2 130 ºC			29.7 [h] ± 0.24		90.35 [b] ± 0.17	
T2 140 ºC			28.06 [i] ± 1.63		76.62 [c] ± 1.63	
T3 120 ºC			62.99 [b] ± 3.11		23.63 [f] ± 1.40	
T3 130 ºC			55.46 [c] ± 4.03		10.40 [i] ± 0.26	
T3 140 ºC			61.28 [b] ± 2.15		14.39 [h] ± 0.96	
Free extract mg/g soy molasses			4.73 [a] ± 0.49		194.02 [a] ± 0.96	

Results expressed in Mean ± SD (n=3) [ab]Lowercase letters in the same column did not show significant difference by Tukey's test (P>0.05) (between 120, 130 and 140 °C). [**] T1= 18% Maltodextrin DE20. T2 = 18% Hi-maize. T3 = 9% Maltodextrin DE20 and 9% Hi-maize.

bioactive compounds when compared to the initial extract containing 159.71 mg GAE/100 g and 49.96 (± 5.33) mg/100 g total isoflavones.

According to MAHDAVI et al. (2016), phenolics and flavonols can form complexes with polysaccharides. OZDAL et al. (2013) described that some proteins are also capable of forming complexes with phenolic compounds, and that these bonds may be reversible and irreversible; however, the mechanism of the proteins influence on phenolic compounds is not yet known. Therefore, the interactions between the phenolic compounds and the encapsulating agents used may be responsible for the observed retention values.

Table 2 shows the results of the antioxidant activity of the microcapsules in relation to the free extract. The T2 treatment differs from the others statistically presenting higher antioxidant activity,

followed by microcapsules with 18% maltodextrin DE20 (T1). It is estimated that the decrease in antioxidant activity at all temperatures tested in relation to the free extract occurred due to the loss of retention of the encapsulated compounds. This is because microencapsulation process by spray drying occurs at high temperatures, which favours the loss of the antioxidant activity of the thermosensitive compounds possible.

CONCLUSION

The best solvent for the extraction of phenolics and total isoflavones was 50% cereal alcohol, this extract also presented higher antioxidant activity. Among the encapsulating agents tested, 18% Hi-maize with inlet air of 130 °C was shown to be the best to

encapsulate isoflavones. The ORAC method showed that microcapsules with the 18% Hi-maize encapsulating agent also had higher antioxidant activity.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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