Journal of Thermal Analysis and Calorimetry, Vol. 93 (2008) 2, 397–402

DSC STUDIES ON PROTEIN ISOLATE OF GUAVA SEEDS *Psidium guajava*

G. G. Fontanari[1*], *G. R. Souza*[2], *J. P. Batistuti*[1], *V. A. Neves*[1], *I. A. Pastre*[3] *and F. L. Fertonani*[3]

[1]Faculdade de Ciências Farmacêuticas, UNESP, 14801-902 Araraquara, SP, Brazil
[2]Instituto de Química, UNESP, 14800-900 Araraquara, SP, Brazil
[3]DQCA-IBILCE-UNESP, 15054-000 S. J. Rio Preto, SP, Brazil

Glutelin, the major protein fraction from guava seed, was obtained by fractioning as described by Osborne. The total proteins were extracted and the isolates obtained by isoelectric precipitation presented similar DSC curves, concordant with the results obtained by gel filtration chromatography and electrophoresis in polyacrylamide gel (PAGE-SDS). However, the DSC curves showed a higher enthalpy with regard to the denaturing protein isolate (PI) extracted at pH 10.0 when compared to a PI at pH 11.5. Such results are in accordance with those obtained for PI extracted at pH 10.0 using chromatography, this one being present in the form of molecular aggregates of greater molecular mass. The glutelin fraction, however, did not present a denaturation peak in the DSC curve, showing that the process for obtaining the same significantly altered its conformation.

Keywords: *chromatography, DSC, glutelins, protein isolate*

Introduction

One of the greatest problems caused by food industry processing is the by-products, which leads to economical and environmental problems. During processing, guava seeds are thrown away, as is done with other types of food. Several studies have been reported aiming the use of this rejected (waste) material as a source of oils, aromas, fibers and proteins [1–7]. The utilization of this rejected material for protein isolate preparation requires several information, e.g. the knowledge of the protein fractions as well as its physical-chemical properties [7, 8].

Protein fractioning studies are necessary to obtain a better understanding of both their functional and their thermal properties with regard their usage in industrial scale [8]. An important set of techniques used for quality control upon food processing is thermal analysis [9–14]. TG-DTG and DSC techniques have been applied to the study the isolated and purified proteins [14–16].

Both the emulsifying property and the thermal stability of the protein isolate extracted from guava seed flour (*Psidium guajava*) were recently evaluated. These showed promising results for their use in emulsification systems [2]. However, there is an observable lack of additional information such as the constitution and characterization of the major protein fraction, the best extraction conditions and also the denaturation temperature. All these are needed for a larger overall view of the use of PI as a food ingredient.

The specific objectives of this research were to obtain a protein isolate from guava seeds (*Psidium guajava*), identifying its main protein fraction using the protein fractional method and characterizing the thermal properties of its PIs and its major fraction.

Experimental

Material

Guava seeds were supplied by Predilecta Foods (São Lourenço do Turvo, SP, Brazil). They were washed with water to remove fibers, dried at room temperature, triturated in a knife grinder using a 1 mm sieve for 10 min and defatted under an ethylic ether flow in a soxhlet apparatus. The product was then dried at room temperature to remove the solvent in an exhausted gas cupboard; pulverized in a hammer grinder having a 0.4 mm sieve and classified using 60-mesh silk (0.250 mm particle size) to obtain the flour that was used for the extractions.

Methods

Centesimal composition

The determination of moisture, ash, fibers, crude fat and protein in guava seed flour was carried out according to procedures described by AOAC, 1995 [17].

* Author for correspondence: guganari@fcfar.unesp.br

1388–6150/$20.00

Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

Obtaining the protein isolate (PI)

The protein isolate was obtained according to the methodology described by Liadakis *et al.* [1], and freeze-dried. For some of the samples, the method was modified. The modification involved the addition of ascorbic acid at 1% AA or Na_2SO_3 at 0.25% during the protein extraction step.

Guava seed protein fractions

The extraction of the fractions was based on Osborne's procedure [18], with a small alteration according to Neves *et al.* [19]. Samples of defatted flour were dispersed in distilled water in 1:30 mass–volume proportion, agitated mechanically for one hour at 4°C, centrifuged at 8000 rpm for 40 min and the residue (R_1) was separated. The supernatant was dialyzed for 72 h at 4°C, and the precipitated protein (P_1) collected by centrifuging and then set apart. On the other hand, the supernatant was freeze-dried and represents the albumin fraction (water soluble protein). The residue (R_1) obtained was dispersed in NaCl (0.5 mol L^{-1}) and then agitated mechanically for one hour at 4°C and centrifuged. The residue obtained (R_2) was separated and the supernatant dialyzed for 48 h against distilled water at 4°C. The precipitate (P_2) was separated by centrifuging and the supernatant was discarded. Precipitates P_1 and P_2 were combined, forming the globulin fraction (salt soluble proteins). Residue R_2 was dispersed in 70% alcohol solution and submitted to mechanical agitation for one hour at 4°C, centrifuged separating the alcohol-soluble proteins, which are the prolamin. The insoluble residue (R_3) was dispersed in a solution of NaOH 0.1 N, agitated mechanically for 1 h at 4°C and centrifuged. The proteins from the supernatant form the glutelin fraction (protein soluble in extreme acids or bases). The final residue obtained resulted in the insoluble part.

Gel filtration chromatography

Aliquots with known protein-isolate concentration were dispersed in *tris*-HCl buffer, pH 8.6 and applied separately in a chromatographic column containing Sepharose CL-6B (100×2.5 cm), previously brought to equilibrium using the same buffer. During the elution, 5.6 mL fractions were collected and the elution profile was registered at 280 nm. From the fractions relative to the maximum-absorbance peaks in the chromatogram, quantities were removed to determine the protein using the method described by Lowry *et al.* [20].

Estimate of the molecular mass using gel filtration chromatography

An estimate of the molecular mass of the proteins in the PIs were undertaken using gel filtration chromatography in a Sepharose CL-6B column (100×2.5 cm), previously balanced with tris-HCl buffer, pH 8.6, containing 0.01% sodium azide. The standard proteins used were: cytochrome c (12.4 kDa), ovalbumin (45 kDa), catalase (120 kDa) and ferritine (480 kDa). The volume of the exclusion of the column (Vo) was determined using blue dextran 2000.

The protein content was measured at 280 and 550 nm wavelength was used for cytochrome c. Samples of the protein fraction taken from the protein isolates were applied to the column and eluted with the same buffer and each protein was observed and read at an absorbance of 280 nm.

Gel electrophoresis

The electrophoretic profile of the proteins was obtained by electrophoresis in a gel of polyacrylamide sodium dodecylsulphate, in a discontinued system using the procedure described by Laemmli [21] in the presence of 2-mercaptoethanol as reducing agent. The standard protein mixtures were: phosphorylase (97 kDa), bovine serum albumin-BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), tripsin inhibitor (20.5 kDa) and β-lactalbumin (14.4 kDa).

The electrophoresis was carried out in rectangular acrylic basins using tris/glicine 25 mM buffer at pH 8.3, containing 0.1% of SDS. The initial voltage was 100 V until the coloring reached the separation gel and then it was altered to 150 V. The gel remained under continuous current until the end of the run, which lasted about 90 min. The gel was colored by immersing into a solution of coomassie blue R-250 at 0.25% in acetic acid, methanol and distilled water for 30 min. The gel color was removed by immersing in a glacial acetic acid solution, methanol and distilled water in the ratio of 30:20:350 mL, respectively. The solution was exchanged several times.

Differential scanning calorimetry (DSC)

The DSC curves were obtained using a DSC25 Mettler (Greifensee, CH) calorimeter attached to a Mettler TC-15 processor in a dynamic environment of N_2 (100 mL min^{-1}), using a covered and perforated (Φ=1 mm) aluminium crucible. The sample mass was 4 mg of protein isolate or glutelin, both freeze-dried. The applied heating rate (β) was 10°C min^{-1}.

Results and discussion

Characterization of the flour

The average values of the guava-seed flour's chemical composition can be seen in Table 1. A lower protein content (9.2±0.1%) and higher fiber content (67±1%) is observed when compared to a previous study, where the protein and fiber contents are (22.7±0.8%) and (50.7±1.5%), respectively [2]. The decrease in the protein content is probably due to a more effective milling process using a hammer mill when compared to cutting with knives in the knife mill. Thus a higher fiber content was gained from seeds milled in the hammer mill and, consequently, a lower protein content, since the hammer mill grinds to a much finer powder allowing a greater usage of the seed's shell, such that the seed is 100% utilized in the flour. Whereas when the knife mill is used without the hammer mill, there is a greater proportion of the proteic content of the seed than fiber in the flour, since the knife mill only cuts the seed but does not in fact smash it totally as the hammer mill does.

Table 1 Chemical composition of defatted guava seed flour (*Psidium guajava*)

Method/analyses	Mass/mass% present work	Mass/mass% from [2]
Conventional*		
Moisture	8.3±0. 4	9.6±0.5
Ethereal extract (fat)	0.7±0.8	0.6±0.1
Protein	9.2±0.1	22.7±0.8
Fibers	67.0±1.0	50.7±1.5
Mineral residue (ash)	0.7±0.8	1.9±0.3
Thermogravimetry		
Moisture	6.70±0.02	9.74±0.05
TG mineral residue (ash)	0.82±0.03	2.06±0.04

*AOAC [17]

The moisture content and the mineral residue (ash) obtained by thermogravimetric methods presented better reproducibility when compared to the AOAC method [17]. The mineral residue obtained, heated to a temperature of 600°C, was identified as being the oxides of alkaline metals.

Recent studies, undertaken with guava seeds from different origins present protein contents similar to those obtained for Brazilian guavas (*Psidium guajava*): 1 – México (*Psidium guajava*), 7.60±0.20% [3]; 2 – Egypt 10.8% (species not stated) [4]; 3 – India (*Psidium guajava L.*) 9.0% [6].

The ash and fiber contents obtained for the guava flour (*Psidium guajava*) (Table 1) were also correlated to values encountered in literature. The ash content was close to the value presented in literature (0.9%) [6], (0.98%) [4] and (1.20±0.02%) [3]; whereas the fiber content was similar to that obtained for the species cultivated in México (67.7±0.12%) [3], and in India (64.0%) [6], and superior to the species cultivated in Egypt, (60.2%) [4].

Protein fractionation of the guava seed

The protein extraction and fractioning sequence of Brazilian guava seeds (*Psidium guajava*) is shown in Table 2 and compared to those obtained from literature [7].

Table 2 Proteins fractions from the guava seed flour (*Psidium guajava*)

Fraction	Protein * g (100 g)$^{-1}$	
	**Osborne's method	***Data from [7]
Albumin	2.25±0.09	1.50±0.05
Globulin	6.05±0.07	6.1±0.1
Prolamin	2.49±0.02	1.9±0.3
Insoluble residue (IR)	≅89	≅90
Glutelin from IR	44.01±0.02	59.1±0.5

*AOAC [17]; **method used [18]; ***Nicanor *et al.* (using Barba de la Rosa's method [22])

Table 2 shows that the fraction percentages, except the glutelin, presented similar results to those in literature for guava seeds from México [7]. Although the percentages of the insoluble residue were close, the glutelin content (44.01±0.02%) was less than that observed by Nicanor *et al.* [7].

The glutelin results obtained from guava flour (*Psidium guajava*), Table 2, are in agreement with those of Agboola *et al.* [23], obtained from Australian rice flour that have a major glutelin fraction protein of 50%.

Thermal characterization of the PIs and of the glutelin fraction

The PI obtained at pH 10.0 and 11.5, using different additives (Na_2SO_3 and ascorbic acid (AA)) in the extraction of proteins, gave yields which varied from 33.2±0.4 to 71.3±0.2% and protein contents varying between 91.1±0.8 and 98.0±0.4%. PIs were submitted to a DSC analysis using a sample of 4 mg, β=10°C min^{-1}, N_2 gas flow, 100 mL min^{-1} and an aluminium crucible.

The DSC curves obtained from PIs are shown in Fig. 1. The graph shows a considerable similarity between the characteristics of the curves obtained for both PIs, extracted at pH 10.0 and 11.5 in the presence of 0.25% Na_2SO_3 (mass/mass). This fact shows

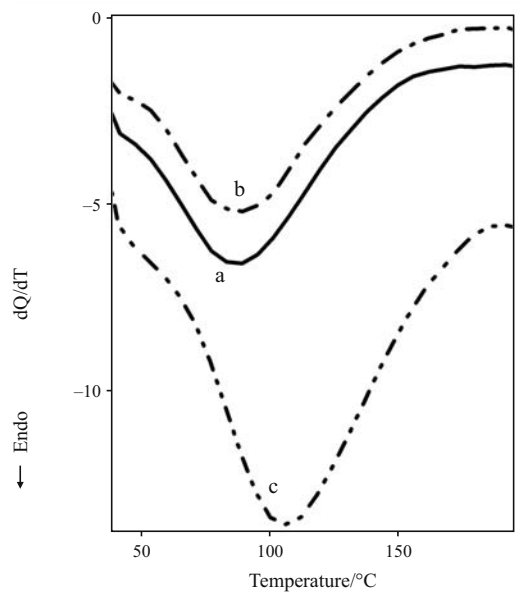

Fig. 1 DSC curves of the PI: a – pH 10.0 with 0.25% Na_2SO_3; b – pH 11.5 with 0.25% Na_2SO_3; c – pH 11.5 with1% AA

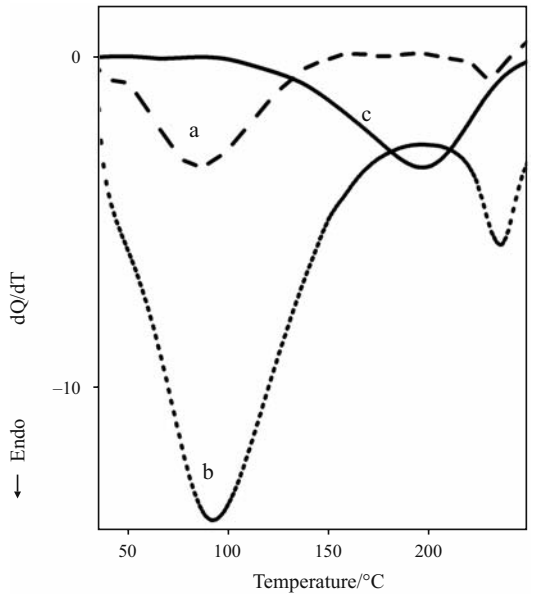

Fig. 2 DSC curves of a – PI at pH 11.5; b – PI at pH 10.0; c – glutelin

that the characteristics of the DSC curves do not depend on the pH used in the extraction (curves a and b).

The curves show similar peak temperatures (T=88.1°C and an approximate ratio of 1:1 between the values of ΔH (ΔH~1; $\Delta H_{\text{curve a}}/\Delta H_{\text{curve b}}$). However, the PI obtained at pH 10.0 yielded less (33.2±0.4%) in terms of mass but showed a higher protein (98.0±0.4%) and lower ash content (0.35±0.06%) when compared to the PI obtained at pH 11.5. However, for a pH of 11.5, the yield was higher (71.3±0.2%) in terms of mass but gave a lower protein content (91.1±0.8%) and a higher ash content (4%).

In Fig. 1, curve c was obtained from the PI extracted at pH 11.5 in the presence of 1 mass/mass% AA. A comparison of this curve with curves a and b show an accentuated temperature shift for the denaturation peak of the protein. The temperature peak shifted from T_p=88.1°C (curves a and b) to T_p=107.1°C (curve c), which coincides with a considerable increase in the ratio between the values of ΔH (ΔH~1.8; $\Delta H_{\text{curve c}}/\Delta H_{\text{curve a}}$). Such differences in relation to previous treatments employing Na_2SO_3, 0.25 mass/mass% and pH 10.0 and 11.5 suggest that the presence of AA induces a reduction in denatured protein during the extraction process and an increase in the thermal stability of the protein. However, the addition of AA gave a yield of (70.2±0.4%) and a protein content of 96.3±0.5% close to that observed for the PI extracted at pH 10.0 in the presence of 0.25 Na_2SO_3 mass/mass%. Although, it had an ash content of about 2% when compared to the treatments presented previously.

The PIs obtained at pH 10.0 and 11.5 in the absence of additives had extraction yields of 53.1±0.8 and 77.0±0.4% and a protein content of 96.4±0.5 and 93.5±0.4%, respectively. Figure 2 shows the DSC curves obtained for the PIs prepared in the absence of additives. Curve a (PI obtained at pH 11.5) appears similar to curve b (Fig. 1) with T_p=86.2°C and a ratio of ΔH values at 1:1 (ΔH~1; ΔH curve $b_{\text{Fig. 1}}/\Delta H$ curve $a_{\text{Fig. 2}}$). These results show that the treatment using a pH higher than 11.0 provoked a partial denaturation of the proteins in the PI.

The curve b (Fig. 2) obtained for the PI extracted at pH 10.0 showed a ratio of ΔH~2.8 (ΔH curve $b_{\text{Fig. 2}}/\Delta H$ curve $a_{\text{Fig. 2}}$) and a shift in the temperature peak (T_p=86.2°C, curve a, to T_p=91.8°C, curve b) when compared to curve a. This pattern shows a gain in the PI's thermal stability and the preservation of the original characteristics of the proteins present in the PI.

Figure 2 also shows a comparison between the patterns of the DSC curves obtained for a glutelin fraction (major fraction, curve c) in comparison to the patterns obtained for the PI prepared at pH 10.0 and 11.5 (curves a and b) in the absence of additive. The figure shows the following for the glutelin fraction: 1) the absence of the denaturing peak characteristic of the proteins present in the PI obtained at pH 10.0 (T_p=85.6°C) and pH 11.5 (T_p=92.3°C): 2) the presence of a peak with a maximum T_p=197.5°C above the denaturation region indicating the beginning of the glutelin degradation process, observed in TG curve [2].

The absence of a peak in the denaturation temperature range suggests that the condition of the extraction of the glutelin fraction (NaOH 0.1 N) promotes denaturation of the protein during this stage of the

fractioning process [8]. The glutelin thermal decomposition peak (T_p=197.5°C, curve c) is anticipated from a comparison with the PI decomposition peaks (pH 11.5, T_p=229.4°C, curve a and pH 10.0, T_p=235.1°C, curve b) suggesting a lower thermal stability for the glutelin fraction according to the TG curve [2].

Gel filtration chromatography

Chromatograms obtained on Sepharose CL-6B column for both PIs are shown in Fig. 3. The figure shows a similar profile for both isolates with the presence of two peaks for the eluted proteins. The calibration of the column with proteins of known molecular masses showed that the two isolates are constituted of protein aggregates of high molecular mass (over 2000 kDa) and in distinct proportions, although similar to each other. These chromatographic profiles suggest that isolation by precipitation at the isoelectric point encouraged a molecular aggregation between the albumin, globulin and glutelin protein fractions as constituents of the isolates. However, the glutelin fraction predominates and is responsible for the high molecular mass (Table 2).

These results are in accordance with the profiles of the DSC curves presented in Figs 1 and 2. The curves for PIs at pH 10.0 and 11.5 show significant profile differences, according to the ΔH values and the peak temperatures (T_p). Such differences suggest that the proteins present in the PIs prepared at pH 11.5 were shown to be considerably denatured when compared with the protein present in the PI at pH 10.0.

Gel electrophoresis from protein isolates of guava seeds (Psidium guajava)

SDS-PAGE analysis of the isolates and of the peaks eluted from the chromatography (Fig. 3) is shown in Fig. 4. The electrophoresis profile reveals, for both protein isolates, the same polypeptide constitution containing three fractions of molecular masses between 30 to 40 kDa, followed by 2 fractions between 20 to 30 kDa and two fractions less than 20 kDa.

A small protein band having a molecular mass around 66 kDa was observed and seems to consist of an aggregate of lesser fractions or even a component of the minority globulin fraction.

The differences in the molecular masses seem to be related to the content of each polypeptide chain between the corresponding fractions of the first and second peaks eluted in the chromatogram and is demonstrated by the reaction with coomassie blue on SDS-PAGE. The SDS-PAGE of the glutelin fraction coincides with the electrophoretic profile obtained for

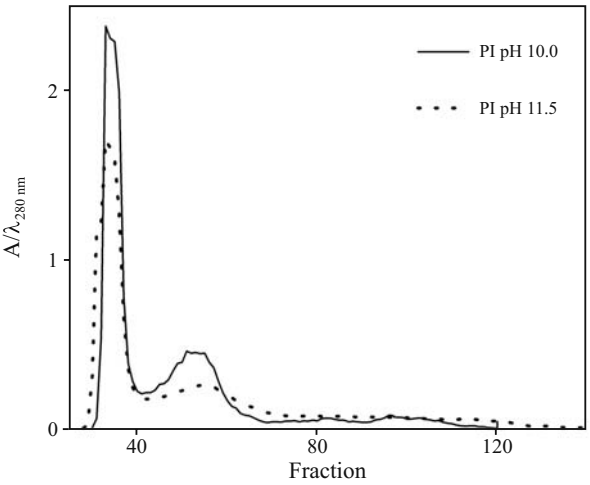

Fig. 3 Chromatogram of PIs. Suspensions of 9.5 mL of PI at pH 10.0 and 11.5, containing 5.85 mg protein/mL^{-1} and 5.55 mg protein/mL^{-1}, respectively, were applied separately. The protein was eluted in a buffer solution of Tris/HCl at pH 8.6 with sodium azide 0.01%

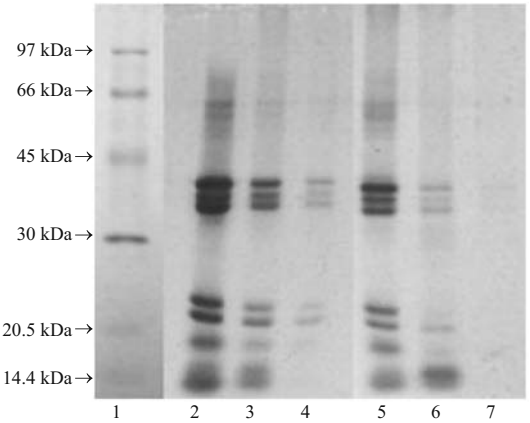

Fig. 4 Gel electrophoresis in polyacrylamide 10% gel with SDS for the PIs at pHs 10.0 and 11.5 and for the peaks obtained from the chromatography in gel: 1 – standard; 2 – PI 10.0; 3 – 1° peak PI 10.0; 4 – 2° peak PI 10.0; 5 – PI 11.5; 6 – 1° peak PI 11.5; 7 – 2° peak PI 11.5

the protein isolate and shows its predominance as a protein fraction [7].

However, these fractions are made up from aggregates of a high molecular mass that would probably be stabilized by non-covalent attachments, a fact also observed for different varieties of guava seeds (Nicanor *et al.* [7], El-Din and Yassen [4]). Thus, it is clear that, independent of the relative quantity of protein extracted from PI 10.0 and 11.5, the results indicate that the protein fractions present in both of the isolates are the same.

Conclusions

For the best extraction of the protein isolate from guava seeds, a pH of 11.5 was used and obtained a yield of 77.0±0.4% and a protein content of 93.5±0.4%. The protein fraction present in greatest quantity was that of glutelin (44.01±0.02%). The PIs presented distinct DSC profiles and depended on the pH and mode of the preparation. The glutelin fraction, the greatest protein content, was extracted, purified, and thermally characterized. The DSC profile showed that extracted glutelin was denatured, suggesting that the conditions of glutelin extraction promote its denaturation during the fractioning process. The denaturated protein presented a low thermal-stability temperature when compared to protein isolates obtained under similar experimental conditions.

The protein isolates presented similar molecular masses and polypeptide composition. However, the DSC curves suggest that the isolates have distinct conformations and the extraction pH is the differential element leading to larger water retention for the 'native folding' conformation (pH 10.0) in comparison with the partly open 'random coil' (pH 11.5). These differences are reflected in the physical-chemical properties of isolates influencing their functional properties when applied to food.

Acknowledgements

The authors wish to thank Predilecta Foods (São Lourenço do Turvo, SP, Brazil), CAPES Foundation (Brazil), FUNDUNESP Foundation (Unesp, Brazil), SIBAN Foundation for financial support, Ms Daniele Branta, Camila Mota Mariano and Ana Luiza Fachini for their support in obtaining the guava seed flour and Miss Maraiza Aparecida Silva for her technical assistance.

References

1 G. N. Liadakis, C. Tzia, V. Oreopoulou and C. D. Thomopoulos, J. Food Sci., 60 (1995) 477.
2 G. G. Fontanari, J. P. Batistuti, G. Bannach, I. A. Pastre, E. Y. Ionashiro and F. L. Fertonani, J. Therm. Anal. Cal., 83 (2006) 709.
3 A. B. Nicanor, A. O. Moreno, A. L. M. Ayala and G. D. Ortiz, J. Food Biochem., 25 (2001) 77.
4 M. H. A. Shams El-Din and A. A. E. Yassen, Food Nahrung, 41 (1997) 344.
5 G. G. Fontanari, M. C. Jacon, I. A. Pastre, F. L. Fertonani, V. A. Neves and J. P. Batistuti. Ciênc. Tecnol. Aliment., 27 (2007) 73.
6 N. B. L. Prasad and G. Aseemoddin, J. Am. Oil Chem. Soc., 71 (1994) 457.
7 A. B. Nicanor, A. A. Scilingo, M. C. Añón and G. D. Ortiz, Swiss Soc. Food Sci. Technol. (LWT), 39 (2006) 902.
8 E. N. Abugoch Martínez and M. C. Añón, J. Agric. Food Chem., 51 (2003) 4060.
9 J. A. Amorim, S. A. Elizário, D. S. Gouveia, A. S. M. Simões, J. C. O. Santos, M. M. Conceição, A. G. Souza and M. F. S. Trindade, J. Therm. Anal. Cal., 75 (2004) 393.
10 Y. H. Roos, J. Therm. Anal. Cal., 71 (2003) 197.
11 P. D. Meuter, H. Rahier and B. V. Mele, Int. J. Pharm., 192 (1999) 77.
12 A. Schiraldi and D. Fessas, J. Therm. Anal. Cal., 71 (2003) 225.
13 A. Raemy, J. Therm. Anal. Cal., 71 (2003) 273.
14 J. Magoshi, M. A. Becker, Z. Han and S. Nakamura, J. Therm. Anal. Cal., 70 (2002) 833.
15 A. A. Mohamed, Food Chem., 78 (2002) 291.
16 S. E. M. Ortiz and J. R. Wagner, Food Res. Int., 35 (2002) 511.
17 Association of Official Analytical Chemists, 16th Arlington, 1995.
18 T. B. Osborne, 2nd Ed., Longmans, Green, London 1924, p. 154.
19 V. A. Neves, E. J. Lourenço and M. A. Silva, Arch. Latinoameric. Nutr., 46 (1996) 238.
20 O. H. Lowry, N. J. Rosebrough, L. A. Farr and R. J. Randall, J. Biol. Chem., 193 (1951) 265.
21 U. K. Laemmli, Nature, 227 (1970) 680.
22 A. P. Barba de La Rosa, J. Gueguem, O. P. Lopez and G. Viroben, J. Agric. Food Chem., 40 (1992) 931.
23 S. Agboola, N. G. Darren and D. Mills, J. Cereal Sci., 20 (2005) 1.

Received: May 30, 2007
Accepted: January 10, 2008

DOI: 10.1007/s10973-007-8576-8