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ULTRASOUND-ASSISTED ZEIN EXTRACTION AND DETERMINATION IN SOME PATENTED MAIZE FLOURS

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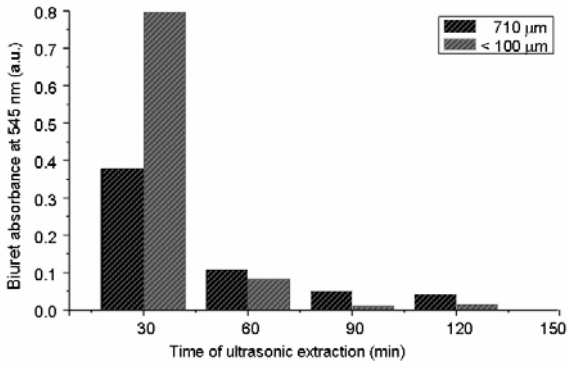
Zein, an alcohol soluble mixture of proteins with industrial applications, is the main storage protein found in the seeds of maize. Recently, a patent was awarded (RO128468-A2 dated June 28, 2013) for a process for producing corn meals each of them of different hardness, granulation and chemical composition. Since some of these maize flours are rich in starch and zein prolamin, whereas some other flours are rich in oil and non-zeinic proteins, both the extraction and determination of zeins should be investigated. Consequently, we proposed here the extraction of zein and determination in hard and normal corn meals. Besides, we characterized the extracted zeins by FTIR and UV-visible spectroscopy. Among the protein assays used to determine zein content of extracts, we found that biuret method is the most suitable procedure to follow the ultrasonic extraction of zein. Therefore, an adapted biuret micro-method was introduced herein and its feasibility and analytical parameters discussed. The alcoholic extracts proved to contain mostly α-zein.



INTRODUCTION

Corn is an abundant and renewable resource, which is processed into many food and industrial products, such as starch, sweeteners, corn oil, beverage and industrial alcohol, as well as ethanol fuel.[1] Zein is a unique and complex material, and it is one of the few cereal proteins extracted in a relatively pure form.[2] It is the most known cereal protein, being, in fact, a mixture of hydrophobic, water insoluble corn proteins, which has long been investigated for uses other than food and feed. Zein, is found prevalent in maize endosperm. It is also the major co-product of the bio-fuel industry with different applications in biomedicine (as drug-delivery compound, drug capsules that release the active substance in the body) as well as in industrial production of bioplastic, paper coating, and food products (chewing gum). Generally, zein molecule contains around 21.4% glutamine, 19.3% leucine, 9.0% proline, 8.3% alanine, 6.8% phenylalanine, 6.2% isoleucine, 5.7% serine, and 5.1% tyrosine. However, it is deficient in tryptophan, lysine and methionine.[3] In the dry-milling ethanol process, ethanol is used as a solvent to extract zein from dry-milled corn.[3]

Belonging to the family of proteins known as prolamins, the zeins are divided into four different classes, α-, β-, γ- and δ-zein, which differ one from another by their molecular weight and location in

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the protein body. Moreover, corn zein is also comprised α, β, γ, and δ zeins based on the zein solubility in 2-propanol. The α- and δ-zeins are found in the protein body core, while β- and γ-zeins are on the periphery of the protein, however, α- and δ-zeins are the only proteins that are considered as true prolamins. Based upon Osborne's solubility principles, β- and γ-zeins are considered glutelins, but they have been included in zein prolamins because of their inclusion within the zein protein bodies.[4,5]

It is also well-known that the proteins in cereals like maize are low in lysine, tryptophan, and methionine because of the high content in prolamins. Indeed, zein, the corn prolamin, is nearly devoid of such essential amino acids.[6] Since zein is a high valued co-product, which can be produced from corn seeds prior to the ethanol fermentation process, interest in zein production is growing again. Biopolymers, including corn zein, offer a noticeable potential of replacing conventional petroleum based polymers in food packaging materials. Due to its strong hydrophobicity and good resistance to the digestive processes of stomach, zein can be also used as a drug delivery system. For example, a zein-based colloidal delivery system to encapsulate poor water-soluble bioactive compounds was proposed for various food products.[7]

The microencapsulation of normal corn starch by zein and its slow digestion property have been investigated.[8-10] The hydrophobic physical barrier of zein matrix might limit the water accessibility and starch swelling leading to a dense packing of starch. In spite of the increased interest in zein in USA, there are little data on the nutritional quality of the maize grains in Romania, primarily dictated by the zein prolamins.[11,12] Other cereals such as wheat, barley, rye, and sorghum each contain prolamins with similar characteristics to zein.[13,14]

Zein can be extracted from three different corn materials: dry-milled corn (DMC), corn gluten meal (CGM), and corn fermentation coproduct, DDGS.[13-15] The basic source material is DMC, which contains from 6.8% to 8.0% protein, of which 52% is considered zein protein. However, many new improvements in zein extraction are still needed like the use of ultrasonic techniques, various solvents and their combination, reducing agents etc.[16-18]

Since a patent was awarded [19] for a process based on maize seed milling followed by sieving the resulted coarse flour to produce various corn meals each of them of different hardness, granulation and chemical composition, this work is aiming at presenting zein extraction and determination in hard and normal corn meals using ultrasonic conditions and ethanol as the main reagent. Zein extraction was followed using a variant of the biuret micro-method.

EXPERIMENTAL

Materials

Zein from maize (Z 3625 Product from Sigma-Aldrich, USA) was used for analytical purposes and comparison with the extracted zeins within our experiments. Zein(s) were extracted from commercial corn meal **(**S.C. Enache-Morarit SRL, Vaslui, Romania**)** and from that obtained by milling corn seeds **(**untreated hybrid corn KWS 3381, FAO group 450) from KWS Company (Germany). We started from an invention which relates to a process for separating corn floury fractions.[19] According to this patent, the maize seeds are milled and the resulted flour is sieved with differently meshed sieves for separating fractionations of flours with different granulation and chemical composition. The resulting flours have improved biological and industrial quality and can be used as food for mono-gastric animals or for producing starch and ethanol. Sieving was accomplished using a Retsch sieves (Germany) to obtain meal fractions with different granulation. Time of sieving was 7 min at 60 rpm. Experiments were done using a flour fraction with the particle diameter, $d > 710$ μm and another one, with $d > 250$ μm. The coarse fraction (710 μm) was grinded to a fine powder ($d < 100$ μm) using a laboratory electric cereal mill (SAMAP, Andolsheim, France), with adjustable millstones Mod F100. Solvents as ethanol, acetone, and petroleum ether were purchased from Merck (Darmstadt, Germany or Fluka). Aqueous solutions of 70% ethanol were prepared on a volume/ volume basis. The maize flours as well as those of zein were kept in a desiccator with anhydrous $CaCl_2$ before spectral measurement. All reagents were of analytic purity and the solutions were prepared using MilliQ grade water (18.2 MΩ·cm).

Sample preparation

Samples of corn meal were degreased with petroleum ether using a Soxhlet equipment for 5 hours. Duplicates of 100 mg of degreased corn meal were weighted in eppendorf vials and 2 mL of 70 % ethanol was added. Separately, 150 mg of hybrid corn meal was weighted and 1.5 mL of ethanol was added. Eppendorf vials were sonicated for 30 min using an Ultrasons JP Selecta.

The other eppendorf plastic vials were agitated using a Thermomixer Compact Eppendorf AG 22331 Hamburg to compare the results with the ones obtained by the ultrasonic method. All samples were centrifuged for 10 min at 18,000 rpm and 20 °C using a Hettich centrifuge (Germany). Extracts were analysed spectrophotometrically and by the biuret reaction. In the last case, the alcoholic extracts were treated with insoluble copper phosphate and KOH solution and sonicated. After centrifuging the mixture, the violet-colored supernatants were read at 545 nm.

KWS 3871 maize hybrid seeds were milled and defatted. In each of two test tubes, 100 mg of 710 μm meal was introduced. Separately, the defatted 710 μm meal was further

milled to pass the 100 μm mesh sieve. Then, samples of 100 mg of such flour were weighed in other two test tubes. In all four tubes, 2 mL of 70% ethyl alcohol were added. The mixtures were sonicated in a sonication bath for 30 min, followed by centrifugation at 18,000 rpm. The supernatants were used in the spectrophotometric measurements. The process of ultrasonic extraction and alcoholic solution removing was carried out many times.

Instruments and methods

UV-vis measurements were recorded in the range from 190 to 590 nm using a LIBRA S35 spectrophotometer (Biochrom, Cambridge, England) in 1 cm quartz cuvette against a blank containing the solvent. As for the biuret reaction, the spectrophotometric reading range was 400-700 nm. UV and visible measurements for all extracted fractions were also performed at 280 nm, where some essential aromatic amino acids such as tryptophan absorb, and 545 nm to evaluate the content of zeins by their reaction with copper ions. In the last case, to 1 mL of alcoholic sample, 0.2 mL of 6% KOH was pipetted and 10 mg of $Cu_2(PO_4)_3$ powder was added with stirring. The extraction of zein(s) was studied by kinetic measurement at 545 nm over different periods of time (30 min, 60 min, 90 min, and 120 min, respectively) and at 2 different temperatures: 25 °C and 60 °C. When necessary, the samples were diluted 1:1, for the absorbance maximum to fall within the range from 0 to 1.

The infrared spectra of the alcoholic extracts, after evaporation to dryness, were recorded in solid KBr using a JASCO 660+ FT-IR spectrophotometer. Thus, the crude zein samples (1-2 mg) were mixed with KBr powder and formed into a disk-shaped pellet. The FT-IR spectra were recorded in the frequency region 4000–400 cm^{-1}, under a resolution of 2 cm^{-1}, with a scanning speed of 2 mm sec^{-1} and 20 scans per sample.

RESULTS AND DISSCUSION

The effect of particle size on zein extraction

When used seeds of KWS 3871 maize hybrid, the defatted d < 710 μm corn meal was selected and tested before and after milling to obtain the fine flour (d < 100 μm). At room temperature, we observed that the biggest amount of zein was extracted in the first 30 min of sonication from the 100 μm granulated flour. The extracted zein from the residue left from the 30-min extraction was only 12.5% of the first amount of zein, extracted at 30 min (Fig.1). Even though the 710-μm corn meal was rich in zeins, the amount of such protein extracted in the first 30 min was 2 times lower than that extracted from the 100 μm flour, as also demonstrated in a previous paper.[18]

On increasing the temperature up to 60 °C, the ultrasonic-assisted extraction of zeins from both granulations (> 710 μm and < 100 μm) was more effective during the first 30 min of sonication than that carried out at room temperature. Thus the absorbance of zein was almost constant at the three times of extraction, namely at 10, 20, and 30 min (Fig. 2).

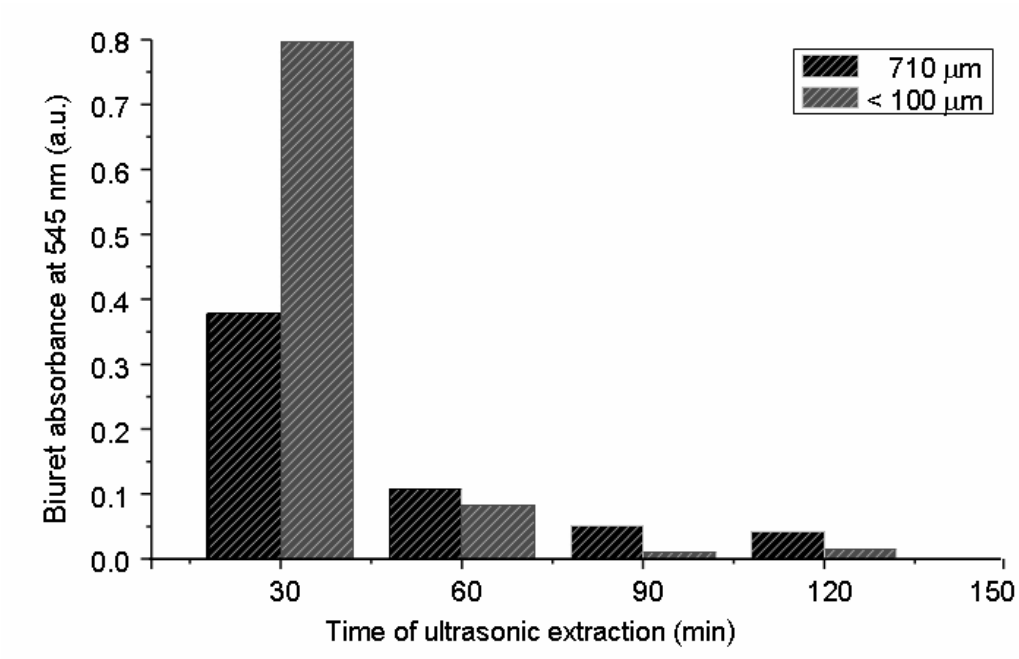

Fig. 1 – The biuret absorbance of alcoholic extracts sampled at different extraction times under ultrasonication at 25 °C.

On studying electrophoretically the alcoholic extracts, they proved to contain mostly α-zein, having a molecular weight of about 23-24 kDa (not shown). However, some electrophoretic bands were assigned to dimers and oligomers of α-zein with higher molecular weights.[18] Beside α-zein, some other weak bands were found and assigned to β and γ-zeins, which are also present in the alcoholic extracts.

The mass spectrometric experiments indicated the presence of at least two major α-zein isoforms, having the following molecular weights: 23,360 and 24,018 Da, respectively.[18]

Extraction of zein from 250 µm and 500 µm granulated flours

In the next experiment, we used commercial corn meal which was milled to obtain different fractions. Here, we investigated only the 250 µm and 500 µm fractions using a thermomixer and a ultrasonic bath. The extraction of soluble components in 70% alcohol was performed using either the ultrasonic bath or the thermomixer under automated mixing conditions. The amount of zein extracted over time respected polynomial equations. The UV-vis data confirmed both the presence of free essential amino acids and the zein(s). The effectiveness of zein(s) extraction was studied by kinetic measurement at 545 nm over different periods of time.

The extraction from the 250 µm flour on thermomixer reached a maximum at 45 min, when the absorbance was about 0.4 arbitrary units (Fig. 3). The ultrasound-assisted extraction reached a maximum at 60 min, the point at which the relative absorbance had the value 0.8. From the coarse granulation, with 500 µm particle diameter, only half of the amount of zein was extracted with the thermomixer as compared with the ultrasonic extraction. Regarding the 250 µm particle diameter flour, the differences between two extraction techniques proved to be even higher than in the case of the 500 µm flour (Fig. 4).

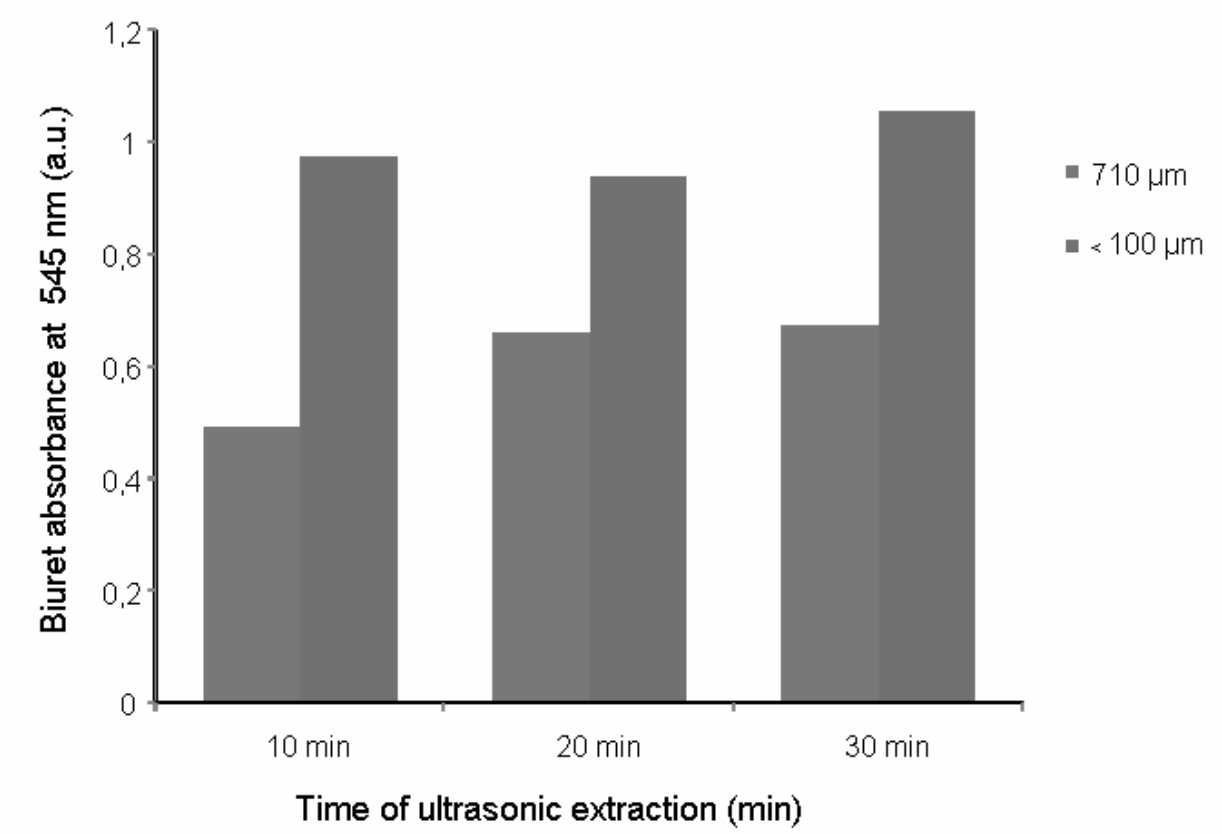

Fig. 2 – The biuret absorbance of the extracts sampled at different extraction times under ultrasonication at 60 °C.

On searching the Figs. 3 and 4, many differences are to be noticed. First of all, the thermomixer conditions induced a weaker extraction of zeins from the coarser flour (500 µm diameter), as expected.[18] Contrary, the ultrasonic extraction provided almost the same amount of zein regardless of grit. Moreover, the sonication was more effective when applied to coarser flours, even if the extraction was modest in the first 5 min of experiment (Fig. 4).

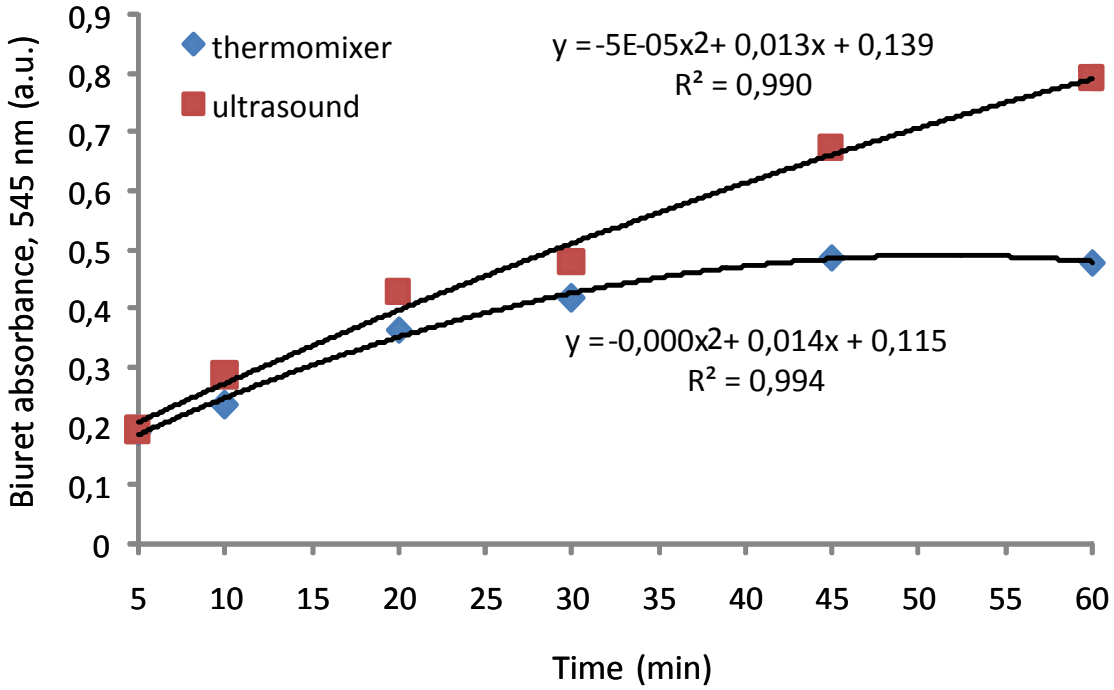

Fig. 3 – The evolution of biuret absorbance during the zein extraction from 250 µm maize flour.
The 545 nm spectrophotometric reading was made at 5 min, 10 min, 20 min, 30 min, 45 min, and 60 min, respectively.

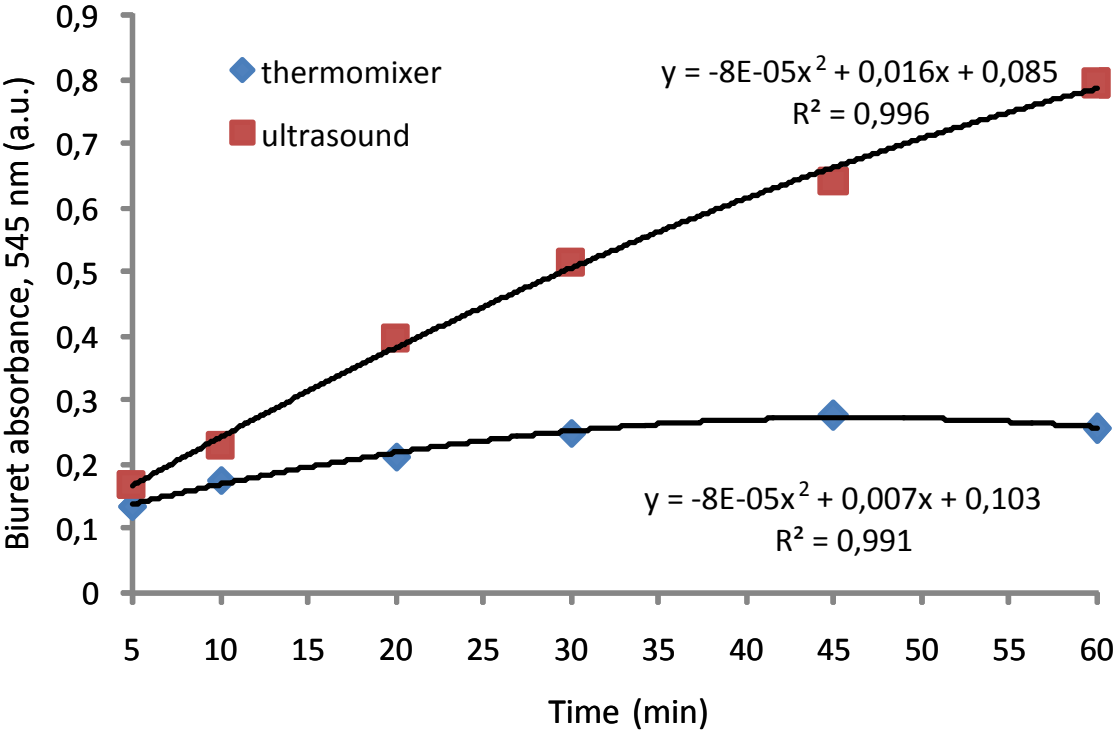

Fig. 4 – The kinetic of zein extraction from the 500 µm maize flour followed by the biuret absorbance measurement. The biuret absorbance was read at 545 nm and at 5 min, 10 min, 20 min, 30 min, 45 min, and 60 min, respectively.

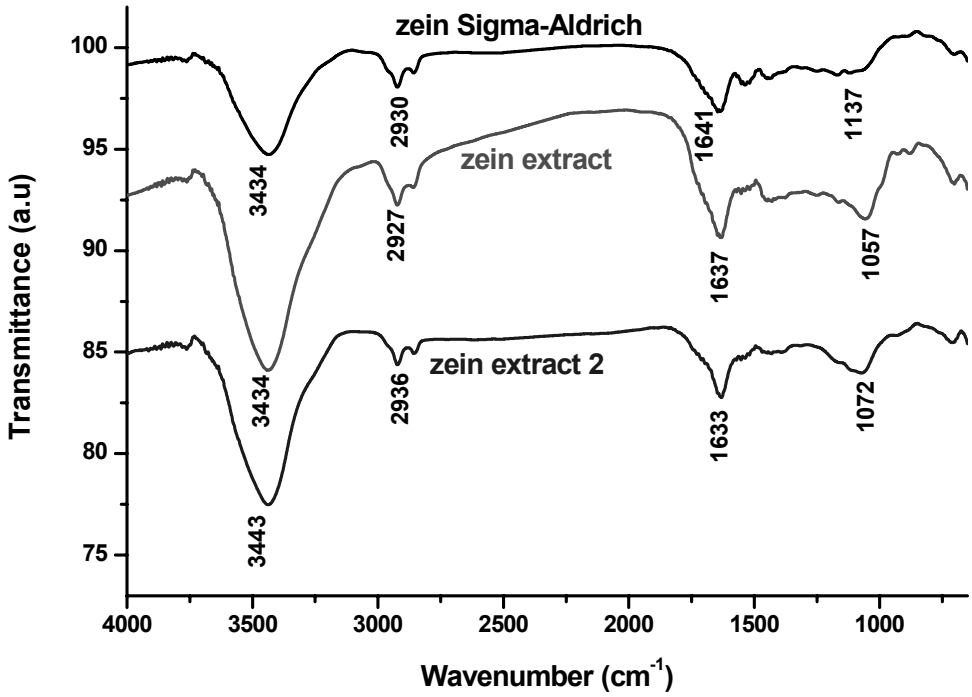

Fig. 5 – FT-IR spectra of zein extracts: zein from Sigma-Aldrich, used as control sample, the crude zein from the alcoholic extract (zein extract) and zein from zein extract, purified by two time washing with water (zein extract 2).

FT-IR measurements

Amide I and II bands are two major bands of the proteins that are conformationally sensitive. FT-IR spectra of proteins are complex and difficult to interpret, but can be used to structural characterization and in the investigation of conformational changes of peptides and proteins.[20] At first view, the spectra of zeins extracted in 70% alcohol are similar to that of zein from Sigma Aldrich (Fig. 5). The carboxyl groups are present in the spectrum at 3443 cm^{-1} to 3434 cm^{-1} and about 2936 to 2927 cm^{-1}, although such bands may interfere with those of hydrogen bond in water or the N-H groups. The amide I band from 1633-1641 cm^{-1} is an important and relatively intense band, being mainly associated with the C=O stretching vibration and directly related to the backbone conformation. The amide I band was found composed of many absorption bands, suggesting a high proportion of α helical conformers of extracted zeins. Since the above mentioned band is related to protein conformation, while the other compounds extracted together with zeins are relatively less concentrated in the alcoholic extracts, it was found almost similar in all samples being analyzed (Fig. 5).

Usually, amide II band results from the N—H bending vibration and from the C—N stretching vibration.[21] Important bands associated with various peptide conformations were also found below 1400 cm^{-1}. The signals from 1057 to 1137 cm^{-1} were due to the polysaccharides present in the gluten which slightly interfered with the analysis. Similar results were previously reported.[18]

Extracted zeins can be used o generate value-added, acid-insoluble reaction products with enhanced metal-binding properties.[22-24] The corn zeins extracted in this study are biodegradable and could be produced at a considerably lower cost compared with petroleum-based resins. Moreover, zein can overcome the limitations of inorganic nanoparticles that tend to accumulate in the organs and tissues and is therefore preferable for drug delivery systems.[25]

CONCLUSIONS

The ultrasound extraction of zeins from commercial corn meal and hybrid maize seeds was studied. Our results show that the ultrasonication of alcohol-flour mixtures improve the extraction of zein from differently granulated corn meals. The influence of temperature on the extraction was also investigated. As expected, at higher temperature, the extraction was favored. Besides, the biuret assay is suggested to be the best choice in estimating the total proteins (zeins) in the alcoholic extracts of maize flours.

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