QUANTIFICATION AND PHYSICOCHEMICAL CHARACTERIZATION OF STARCH IN MALTED BARLEY

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ABSTRACT

Sixteen samples of malted barley -grown in Buenos Aires Province (Argentina)- were selected for this study. The starch was isolated and purified using a modified version of Morrison Method. The starch concentration in the samples ranged from 53.2 to 63.3 %. The starch samples were characterized physicochemically, obtaining the following results: a) the concentration of lipids, phosphorus and nitrogen was too low; b) the values of apparent amylose ranged from 19.0 to 23.9 % whereas the values of total amylose ranged from 23.6 to 31.2; c) starch presents a bimodal granule size distribution: type A (big and lenticular) and type B (small and irregular or spherical); d) the gelatinization temperature ranged from 62.18º C to 64.68ºC; e) the amylopectin is the responsible for the crystallinity of starch granules; f) granules are birefringent. This property disappears when starch is heated above its gelatinization temperature.

Key words: malted barley, starch, quantization, physicochemical characteristics.

INTRODUCTION

Malted barley is one of the most important crops in the world, occupying the fourth position after wheat, corn and rice. MERCOSUR produces approximately 1% of the world's total production, Argentina being the country that makes the greatest contribution, followed by Uruguay, Brazil and Bolivia. The province of Buenos Aires concentrates more than 90% of the national production as its climate and soils are favorable for the harvest of this crop. Besides, the presence of a thriving malting industry contributes to the production of malted barley in this area (*1*).

As many other cereals, malted barley is extremely important in the primary production of foods. In Argentina, the most important application of this cereal is the production of malt.

Malted barley belongs in the family of the gramineaes, which produce one-seeded cereal grains, called caryopsides. In their structure, caryopsides present a pericarp, a testa, an aleurone layer, an endosperm and an embryo or germ (Figure 1). The endosperm makes up 70% of the total weight of the caryopsis. Having the greatest capacity to store the grain, this organ becomes the main host for the accumulation of carbohydrates, presenting 65-70% of its weight in the form of starch (*2*).

Starch constitutes a potential source of energy for the plant. It occurs in the form of granules, which are synthesized in the amyloplasts. There are two kinds of granules: Type A (big and lenticular) and Type B (small and spherical). When the grain is fully ripe, granules B considerably outnumber granules A, at a rate of 10:1. Being much smaller (<10 µm) than granules A (20-45 µm),

granules B represent less than 30% of the total weight of accumulated starch (*3*).

Starch granules are anisotropic due to the presence of crystalline regions in their mass, which is preeminently amorphous. As a result of this, the phenomenon of birefringence takes place. Birefringence allows for the possibility of looking at the starch granule through the polarizing microscope. Under the microscope, the starch granule appears as two different gray zones which resemble a Maltese cross. When starch is heated in water, its structure absorbs the water, becoming “swollen”. At a certain temperature, this swelling becomes irreversible and birefringence is lost because of the destruction of the crystalline state. This temperature is called gelatinization temperature.

Starch is fundamentally made up of two polymers: a polymer of amylose glucose (lineal) and a polymer of amylopectin glucose (strongly ramified). It also presents a series of minor constituents such as polar lipids, nitrogen and phosphorus in the form of phospholipids (*4*). As it has been mentioned before, the starch granule is found partially crystallized as a result of the presence of the amylopectin molecule. There is a relatively constant relation between amylose and amylopectin (23:77).

The aim of this study is twofold: to establish an accurate quantitative methodology which enables the isolation and quantification of starch in malted barley and to provide a physical and chemical characterization of the isolated starch.

MATERIALS AND METHODS

Grain samples

Sixteen samples of malted barley were processed from Quilmes Palomar cultivar (liberated in Argentina and registered in 1994). The samples were collected in six cities of Buenos Aires Province: Alberti (S35°08'-W60°13'), Bordenave (S37°51'-W63°01'), Cascallares (S38°30'-W60°28'), Coronel Suárez (S37°28'-W61°56'), Puán (S37°33'-W62°46') and San Mayol (S38°23'-W60°17'). These samples were quartered to obtain representative samples of each site.

Starch isolation and purification

Applying the technique proposed by McDonald and Stark in 1988 (*5*), barley grains were triturated using a small mill so that grains would only be reduced to parts avoiding powder formation. Then smaller parts were steeped for 17 hours in 0.02 M HCl at 4º C (10 mL / 1 g of grain).

The steeped grains were neutralized using 0.2 M NaOH and then filtered to recover the solids. A solution of 0.1 M TRIS HCl; pH 7.6; with 0.5 % of Na_2SO_3 and 0.01 % of thiomersal (10 mL / 1 g of grain) was added to the recovered solids, which were then agitated for 1 hour at room temperature. After that, Proteasa Sigma type XIV (5 mg / 1 g of grain) was added to the grains, which were constantly agitated at 4º C for a period of 12 hours. Once the time for the enzymatic action had passed by, the remaining solids were rubbed with a pestle to release the starch as a suspension, which was next filtered, recovering the filtered liquid. The solids which remained in the funnel were put back in the mortar with distilled water to ensure the full release of

starch. A new filtering process was carried out. This process was repeated until the total release of starch was achieved.

The solution obtained was centrifuged for 5 minutes at 5000 rpm, producing two layers: a lower layer of starch (white) and an upper very thin layer of protein (brown). The brown proteid layer was softly removed and reserved for later treatment. The white layer was washed in distilled water and centrifuged again. Once more, two layers were formed: the upper layer was removed and set apart. This procedure was repeated until the brown layer no longer formed at the moment of centrifuging.

The layers of starch and protein were washed separately, three times in distilled water and once in acetone. Then they were put to dry at room temperature. The brown layer was purified to separate the starch granules from the protein matrix.

The brown layer was weighed before starting the purification process and a solution 0.1 M TRIS HCl; pH 7.6, containing 0.5 % of Na_2SO_3 and 0.01 % of thiomersal, and proteinase K were added, knowing that for 1 g of brown layer, 30 mL of the solution and 1.25 mg of proteinase K are required. The solution was agitated for 24 hours at room temperature. The resulting solution was centrifuged for 5 minutes (5000 rpm).The supernatant was discarded and the precipitate obtained was washed six times in distilled water and once in acetone. Then it was dried at room temperature.

The brown layer, dry and treated with the enzyme, was weighed and 0.2 M NaCl and toluene were added to the solution, knowing that for every 5 g of brown layer, 500 mL of solution NaCl and 71.5 mL toluene are required. This suspension was agitated for 12 hours at room temperature and centrifuged,

forming three layers: a layer of toluene, an aqueous layer and a precipitate of starch. The toluene layer and the aqueous layer were removed and set apart.

The toluene layer was washed in distilled water and centrifuged to recover starch granules. The aqueous layer was centrifuged directly to obtain the same result.

The layers of starch obtained were dried at room temperature and weighed.

All procedures were conducted in duplicate in all the samples.

Physicochemical characterization of starch

Lipids concentration

The concentration of lipids was determined using the technique devised by King in 1980 (*6*). Starch was agitated for 4 hours at room temperature, with a mixture of chloroform- methanol (2:1), at a rate of 27 mL/500 mg of starch. The solution was centrifuged for 5 minutes at 5000 rpm, then it was filtered and transferred into a tared distillation balloon flask. The mixture of solvents evaporated in a rotary evaporator and the distillation balloon flask was placed in a vacuum furnace for 2 hours at 100º C and 6.66 x 10^2 Pa (5 Torr). The balloon flask was next weighed again and the percentage of lipids in the sample was estimated by the difference of weight registered.

Nitrogen concentration

The concentration of nitrogen was determined by means of the method Kjeldahl (*7*). Starch was weighed and introduced into the digestion tube. Concentrated H_2SO_4 (8 mL / 0.05 g of starch) and a catalytic mixture of salts (10 g K_2SO_4: 1 g $CuSO_4$: 0.1 g Se) were added and the digestion process was carried out. 40 mL of distilled water and some drops of Tashiro Indicator (a

mixture of methyl red and methylene blue) were added to the solution. Then, a given volume of 0.1000 M NaOH was added to neutralize the solution. The mixture was distilled for 5 minutes in a distillation unit including an automatic steam generator. The distillate was received in a 50 mL Erlenmeyer which contained 10 mL of 0.1000 M HCl and some drops of Tashiro Indicator. Finally, the distillate was titrated using 0.1000 M HCl until the indicator turned to bright green.

The following formulae were used to make the calculation:

$$(10\ \text{mL HCl} - \text{mL HCl used})\ \text{x}\ [\text{HCl}]\ \text{x}\ [\text{NaOH}]\ \text{x}\ 14 = \text{mg N}$$

$$\%\ \text{total N} = \frac{\text{mg de N(sample)} - \text{mg de N(control)}}{\text{mg sample}}$$

Phosphorous concentration

Following the method devised by Johnson and Ulrich in 1959 (*8*), starch was introduced into a 50 mL tube, and a mixture of HNO_3 and $HClO_4$ (2:1), at a rate of 1 mL / 0.1 g of starch, was added. Then the mixture was digested. Distilled water was added until the solution reached 25 mL. The content (ppm) of phosphorous (P) was determined using a spectrometer of atomic absorption with inductive coupling plasma (ICP).

The following formulae were used to make the calculation:

$$(\text{ppm P in plasm}) \times \left(\frac{25\ \text{mL}}{\text{starch weight}} \right) = \text{ppm P sample}$$

$$\text{ppm P in sample} \times 10^4 = \%\ \text{P sample}$$

Amylose concentration

Following the method devised by Morrison and Laignelet in 1983 (*9*), 70-80 mg of starch were weighed in a 20 mL tube placed on a magnetic shaker. 10

mL of a urea-dimethyl sulfoxide solution (9 volumes of dimethyl sulfoxide : 1 volume of 6M urea) were added and the tube containing the suspension was placed in a calefactor plate with agitation until homogenization was achieved. Then the suspension was introduced into a furnace at 100º C for 1 hour, after which it was cooled at room temperature. (*).

Concentration of apparent amylose

0.5 mL of the solution (*) were taken and transferred into a 50 mL flask. This step was made in triplicate. The 0.5 mL added to each flask were weighed. After that, 25 mL of distilled water and 1 mL of solution I_2/IK (2 mg of I_2, 20 mg of IK/mL) were added to the solution. Distilled water provided the required volume and the solutions were mixed until they became homogeneous. Simultaneously, the control sample was prepared with distilled water. The samples were settled for 15 minutes for stabilization. In order to read the absorbency, the spectrophotometer was adjusted to zero with the control sample. The absorbency was read at 635 nm and the “blue value” and the percentage of apparent amylose were calculated.

Total amylose concentration

0.5 mL of the solution (*) were transferred into a 10 mL tube and weighed. This step was made in triplicate. 5 mL of ethanol 99.5 % (v/v) were added to remove the lipids. Then the content of the tube was mixed in a vortex mixer, centrifuged for 30 minutes at 5000 rpm and the supernatant was discarded. 1 mL of the urea- dimethyl sulfoxide was added and mixed in a vortex mixer and a clear solution was obtained 10 seconds later. The content was transferred into a 50mL flask and 25 mL of distilled water and 1 mL of I_2/IK were added. Distilled water provided the required volume and the solution was

mixed. After 15 minutes the absorbency was read at 635 nm and the “blue value” and the percentage of total amylose were calculated.

The following formulae were used to make the calculation:

$$\text{Blue value} = \frac{\text{Absorbency} \times 100}{2 \times \text{g solution x mg starch}}$$

$$\text{Percentage of amylose} = 28{,}414 \times \text{blue value}$$

Scanning Electron Microscopy (SEM)

The microscope used in this study was a scanning electron microscope (JEOL 35 CF) equipped with a detector of secondary electrons, with a nominal resolution of 60 Å. An acceleration voltage of 15 KV was used with a magnification of 1000 X. The images were obtained with IDX, a software program designed to acquire images digitally (IDX), with a resolution of 1024 x 800 pixels. The statistic treatment was conducted applying AnalySis Pro 3.0, a software program used for the processing and analysis of images.

Determination of gelatinization temperature by differential scanning calorimetry (DSC)

The tests were made in a DSC Polymer Laboratories (Rheometric Scientific Surrey, UK) with a PL-V5.41 software program, using a heating program of 10ºC/minute from 10 to 120ºC. Suspensions of starch were prepared. The samples were weighed in aluminum capsules and then they were closed hermetically. An empty capsule was used as reference. The enthalpy of gelatinization was determined in mJoule/mg of the dry sample, and the initial and peak temperatures (ºC) were measured using the software mentioned before. The dry weights of the samples were obtained using a 105º C furnace until a constant weight was achieved.

X ray diffraction spectroscopy

The tests were made in a diffractometer equipped with an X Ray tube with a copper anode (PW1710 BASED), which produces a radiation of 1.54 Å, and is operated at 45 KV and 30 mA.

Polarizing microscopy

A suspension of starch was prepared, presenting a 5% concentration. The suspension was then centrifuged and a drop from the bottom of the tube was removed and observed under the polarizing microscope. The suspension was next heated above its gelatinization temperature and a drop of the solution was removed and observed under the polarizing microscope.

RESULTS AND DISCUSSION

1. Starch concentration

Previous research (*10*) proved that a considerable number of small starch granules is lost if the brown protein layer is removed from the surface of suspensions of raw starch during the isolation process. Following the method selected for this study, the brown protein layer was purified in order to recover the starch granules which are associated with the protein matrix, obtaining representative samples.

The percentage of starch found in the dry samples ranged from 53.2% to 63.3%, with an average value of 59.7%. This variation is similar to that reported in the bibliography (*2*), and is probably close to the real values.

Starch chemical composition

Phosphorous, lipids and nitrogen concentration

The concentration of phosphorous (P), lipids and nitrogen (N) was determined with the sole aim of showing the purity of the starch samples under study.

The method selected for the determination of P is a modified version of the method used for determining micronutrients. The percentages of P ranged from 0.060 to 0.075, with an average value of 0.067.

The method employed in the determination of lipids is based on the premiss that lipids associated with starch are essentially polar and require polar solvents so that they can later be extracted and quantified. The percentages of lipids ranged from 0.73 to 0.97, with an average value of 0.86. This range is correspondent with the values found in the bibliography (0.5 – 1 %).

In order to determine the concentration of N, all N in the sample was converted to ammonium through a digestion process made with concentrated sulfuric acid. Also, K_2SO_4 was used to increase the digestion temperature and catalysts such as Se were employed to increase the rate of oxidation. The ammonium was determined by the quantity of ammonia released by the distillation of the digest with an alkali. The advantage of this method is that it requires only a few samples and reagents and that it offers results which are highly reproducible. In all cases, the samples presented a very low level of N (< 0.05 %), part of which belongs to the lipids. The rest might be proteid, probably the remains of enzymes involved in the synthesis of starch.

In order to corroborate that the enzymatic treatment with proteinase K - developed in the purification of starch- can effectively separate proteins from

granules, the percentage of N in one of the samples was determined before and after its purification. It was confirmed that the concentration of N in the sample which was not purified was greater than that found in the sample purified with the enzyme, the results being 0.513 and 0.042 % respectively.

The percentages of P, lipids and N found allow us to conclude that the method used for isolating the starch led to starch samples which were highly purified.

Amylose concentration

The method selected to determine the concentration of amylose is based on the premiss that amylose fraction has affinity with iodine to form complexes, which can be quantified colorimetrically.

Amylose forms complexes with the lipids present in starch granules. These complexes hinder the bond between amylose and iodine, impeding the determination of total amylose at this stage of the process (*2*). The value of apparent amylose was determined, which corresponds with the amylose found in starch without the separation of lipids. The study also determined the value of total amylose, which corresponds with the real value of amylose in starch given that lipids had previously been separated from starch. The values of apparent amylose ranged from 19.0 and 23.9 %, with an average value of 21.3 % and the values of total amylose ranged from 23,6 to 31,2 %, with an average value of 26.4 %.

Table 1 shows the chemical composition of starch in malted barley.

Morphology and size distribution of starch granules

The study conducted with SEM showed two types of granules: type A, corresponding to big granules, and type B, to small ones (Figure 2). Some of

the small granules presented an irregular shape while others were practically spherical. In contrast, big granules presented a lenticular shape with better defined rims (Figure 3).

In order to study the size distribution of starch granules, a histogram was made (Figure 4). Each rectangle represents the amount of particles counted for each class and each class corresponds with a certain range of average diameters of starch granules (class 1: 0–2 μm; class 2: 2–4 μm, etc.). A bimodal distribution is observed. Granules A present average diameters greater than 20 μm, whereas granules B have average diameters smaller than 10 μm and represent 83% of the total number of granules.

Gelatinization temperature

Diverse changes are observed when starch is hydrated and heated. The process of gelatinization implies the loss of molecular order which is revealed in the starch granule. This transformation is accompanied by certain irreversible changes such as water absorption, grain swelling, crystalline part fusion, loss of birefringence, increment of viscosity and solubility of the granule. The differential scanning calorimetry (DSC) is a thermo-analytical technique which monitors how the physical or chemical properties of materials are affected when temperature is modified and detects changes in heat. The principle underlying the procedure is based on the comparison between the influence of the heat flux on the sample and on some inert material of reference, both of which are heated and cooled at the same rate (*11*).

Figure 5 shows a thermogram of the starch samples under study. Thermograms show how the heat flux varies with temperature. The beginning of

the peak is correspondent with the beginning of the loss of birefringence and the area below the curve represents the energy (enthalpy) necessary for the loss of molecular order. Concerning these parameters, Table 2 shows the values of starch in the samples under study.

X Ray diffraction spectroscopy

As starches are crystalline, they have the capacity of diffracting X rays, which allows for the possibility of obtaining information about their structure (*11*). In fact, starch is a semi-crystalline material which produces different responses when subject to the action of X Ray. In general, four different patterns can be observed (Figure 6).

Pattern A: starch in cereals.

Pattern B: starch in tubercles.

Pattern C: an intermediate form between A and B.

Pattern V: complexes of helicoidal inclusion of crystalline amylose.

The X Ray diffraction specters of amylose and of commercial amylopectin were made in order to show their crystalline quality (Figure 7). The specter of the amylose presents some peaks, probably due to the small ramifications of its structure. The amylopectin specter is similar to the pattern of X ray diffraction registered in type A cereals. Therefore, crystallinity in the starch granule should be the product of amylopectin.

The specter of X Ray diffraction of one of the starch samples under study is shown in Figure 8.

A study using Correlation Coefficients was carried out in order to establish statistic correlations between the X Ray diffraction specters of all starch samples and the patterns of amylose and amylopectin. It was found that

all samples present a high correlation of crystallinity with amylopectin, as all coefficients were greater than 0.9. On the other hand, the correlation with amylose was very low, smaller than 0.25 in all cases. This clearly shows that the crystallinity of the starch granule is a consequence of the amylopectin polymer.

Polarizing microscopy

Given their high degree of structuring, starch granules showed birefringence under the polarizing microscope. The birefringent material is seen as bright areas when the granules are observed through the polarizing microscope. Figure 9 shows a microscopy of one of the starch samples under study. Birefringence was destroyed when the samples were heated above their gelatinization temperatures and the bright areas were no longer observed. Figure 10 shows a microscopy of one of the starch samples after it was heated above its gelatinization temperature.

CONCLUSIONS

The method selected for the isolation and purification led to the isolation of starch samples from malted barley grains, which are both representative and quantitative.

The variation of starch found (53.2 and 63.3 %) was similar to that reported in the bibliography, showing that the technique used for isolating the starch was appropriate to produce quantitative samples. Besides, the values of concentration of lipids, phosphorous and nitrogen allow us to conclude that the method of isolation used led to highly purified samples.

The starch studied presented a bimodal granule size distribution, evidencing small granules (type B) and big granules (type A). Type B granules present an irregular or spherical shape, while most granules in Type A have a lenticular shape.

The gelatinization temperature of the starch studied coincided with the data reported in the bibliography. X–Ray diffraction spectroscopy determined that amylopectin is the responsible for the crystallinity of the starch granule.

Besides, the use of polarizing microscopy allowed us to prove that starch granules are birefringent. This characteristic disappears when the samples are heated above their gelatinization temperature.

403 **LITERATURE CITE**

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CAPTIONS FIGURES

Figure 1. Structure of malted barley grain.

Figure 2. Scanning Electron Micrography of starch granules in malted barley, Quilmes Palomar cultivar.

Figure 3. Scanning Electron Micrography of starch granules in malted barley, .Quilmes Palomar cultivar.

Figure 4. Size Distribution of Starch granules in malted barley, Quilmes Palomar cultivar.

Figure 5. Thermogram of starch in malted barley, Quilmes Palomar cultivar.

Figure 6. X Ray Specters of different starches: **A**: cereals, **B**: tubercles, **C**: intermediate form between A and B and **V**: complexes of helicoidal inclusion of crystalline amylose.

Figure 7. X Ray Specters of commercial amylose and amylopectin.

Figure 8. X ray Diffraction Specter of starch in malted barley, Quilmes Palomar cultivar.

Figure 9. Polarizing Micrography of starch granules in malted barley, Quilmes Palomar cultivar, before the sample reached its gelatinization temperature.

Figure 10. Polarizing Micrography of starch granules in malted barley, Quilmes Palomar cultivar, heated above its gelatinization temperature.

TABLES

Table 1. Chemical composition of starch in malted barley, Quilmes Palomar cultivar.

Phosphorous [a] (%)	0.067 ± 0.004
Lipids[a] (%)	0.86 ± 0.10
Nitrogen[a] (%)	0.042 ± 0.000
Apparent amylose[b] (%)	21.3 ± 1.46
Total amylosel[b] (%)	26.4 ± 2.19

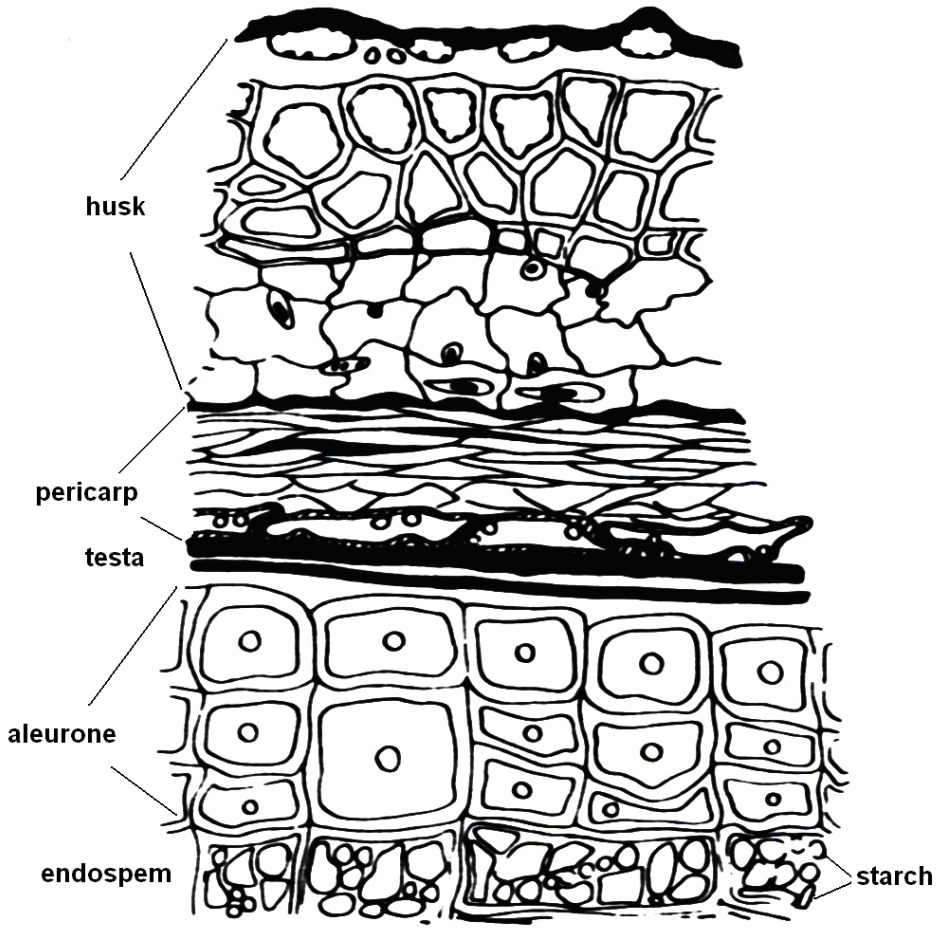
[a] Average of two determinations

[b] Average of three determinations

Table 2. Enthalpies, initial temperature of peaks and gelatinization temperatures, determined by Differential Scanning Calorimetry (DSC) of starch in malted barley, Quilmes Palomar cultivar.

ΔH[a] (mJ/mg)	Initial temperature of peak[a] (ºC)	Gelatinization temperature[a] (ºC)
9.24 ± 0.38	55.99 ± 0.76	63.43 ± 0.64

[a] Average of two determinations



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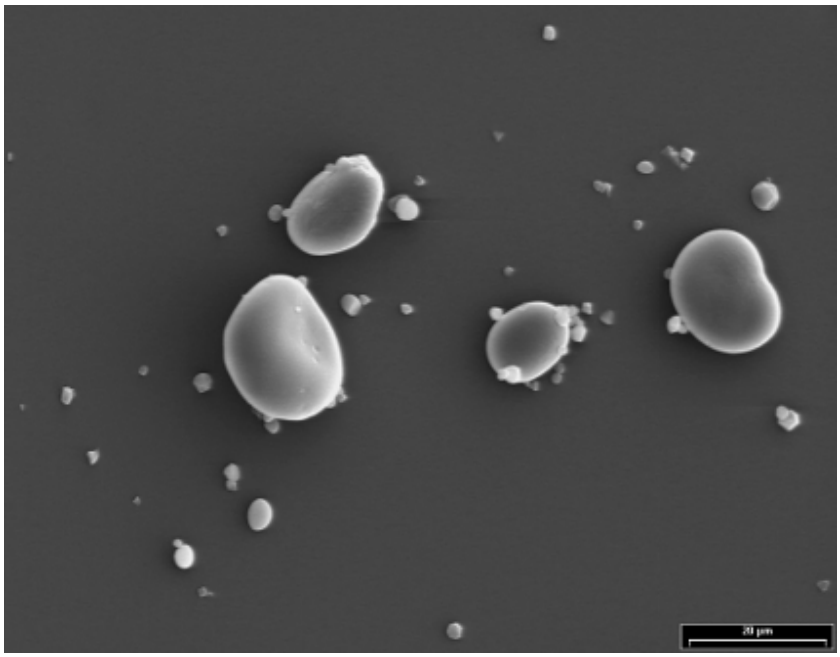

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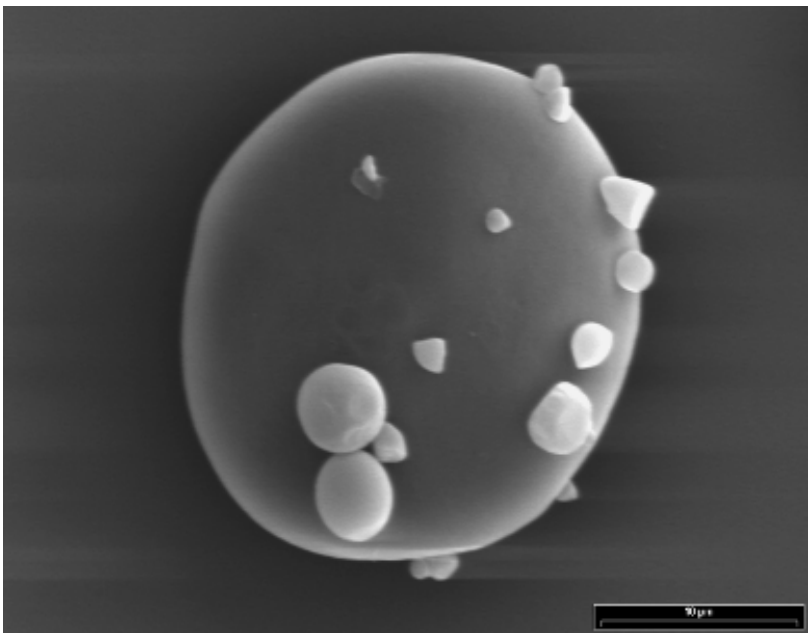

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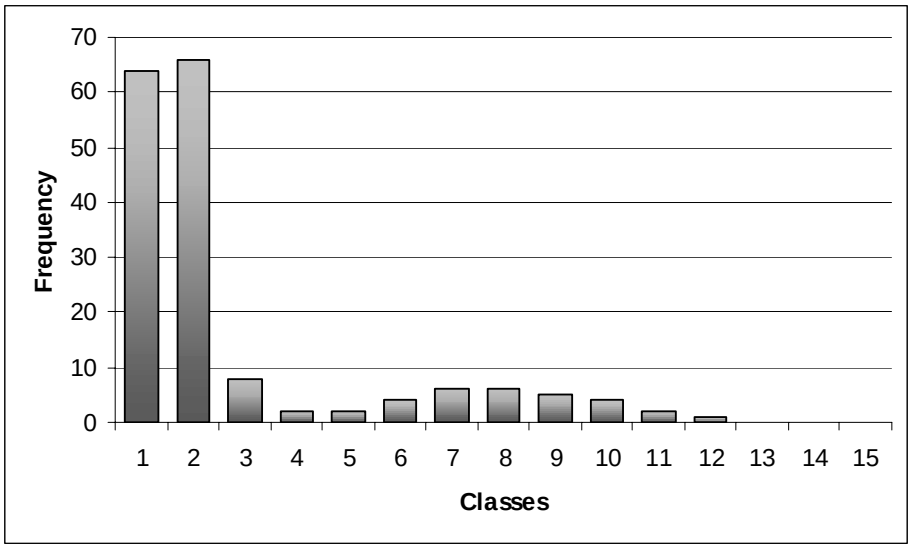
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Figure 4

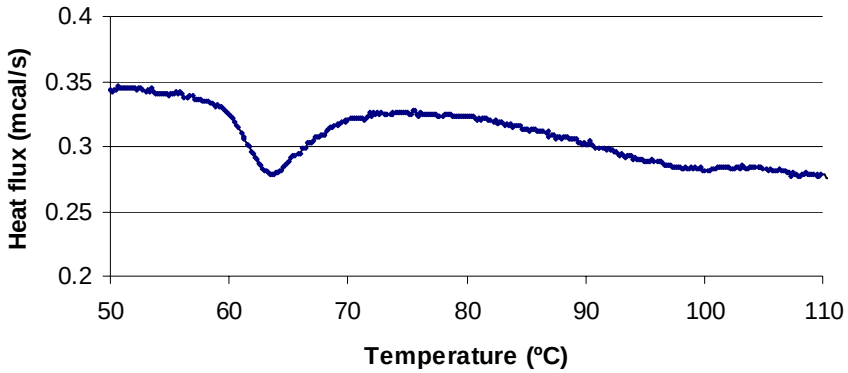
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Figure 5

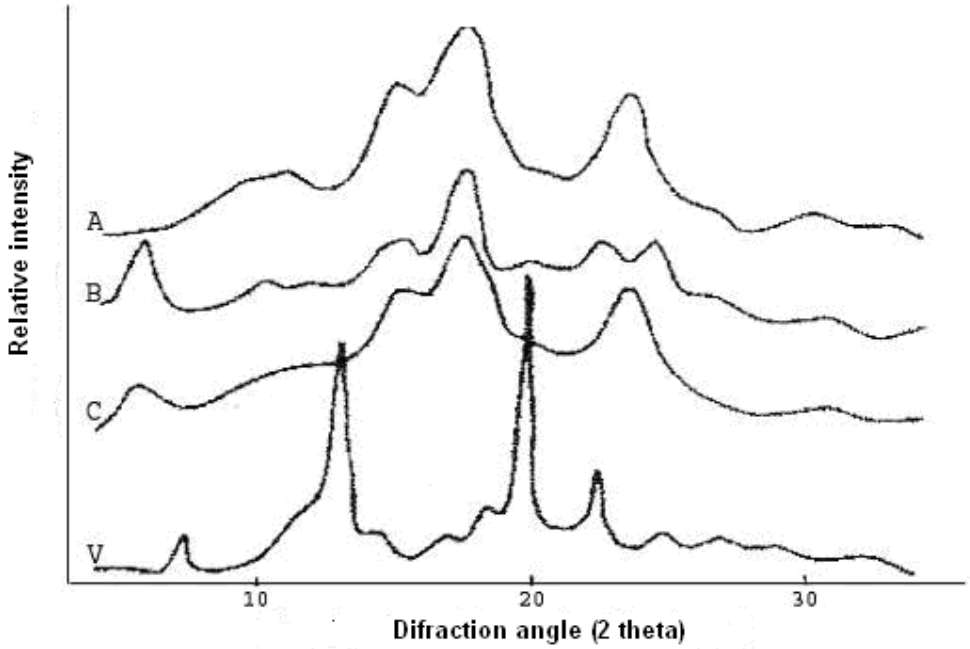

Figure 6

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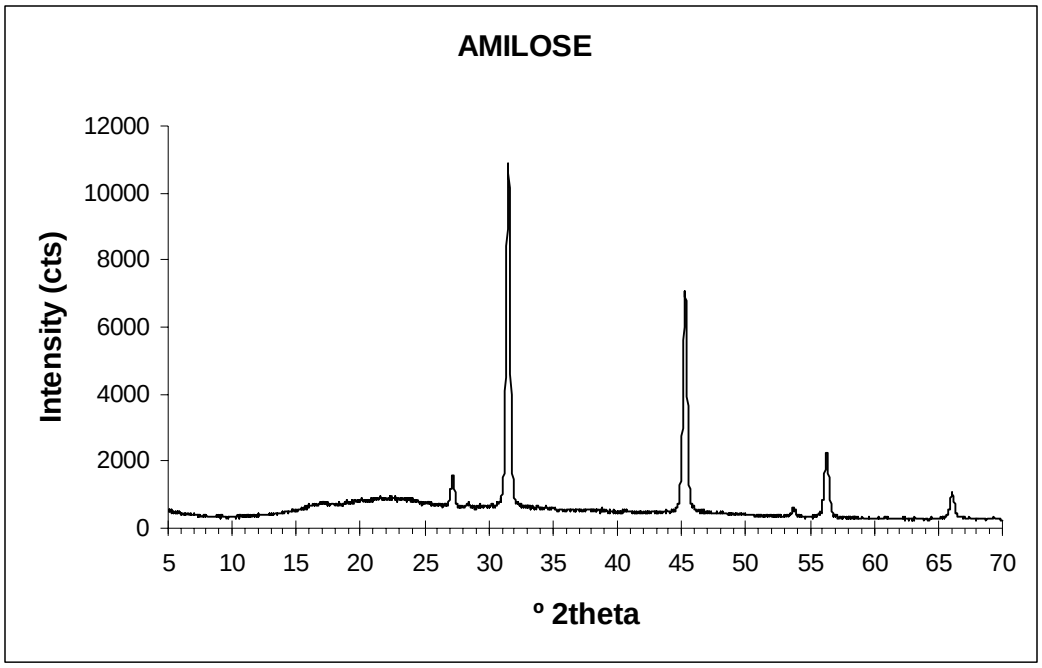

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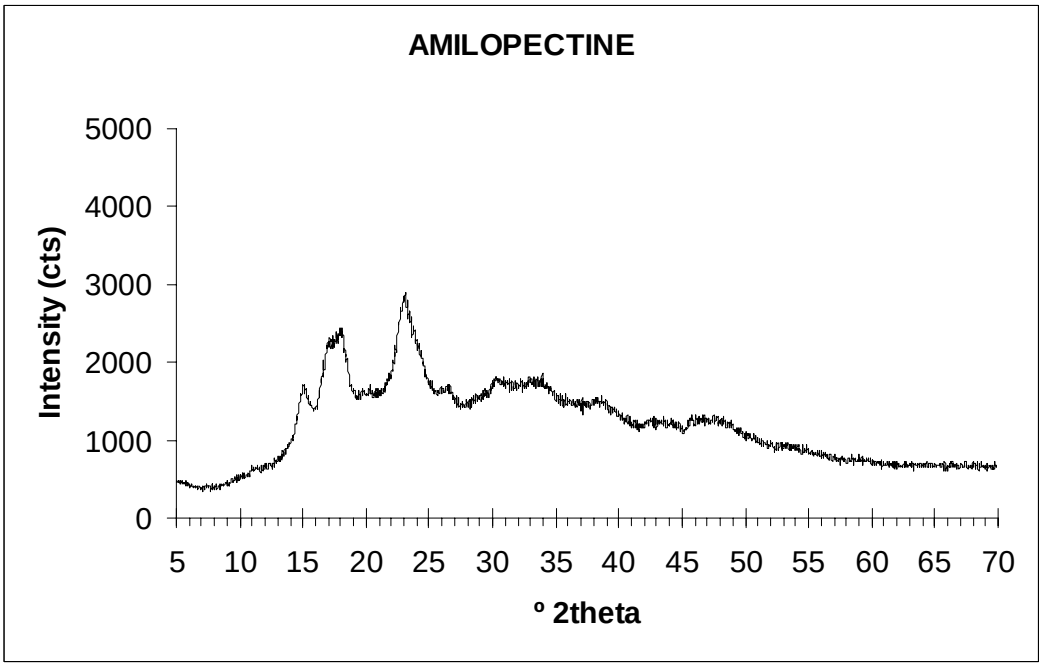

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611 **Figure 7**

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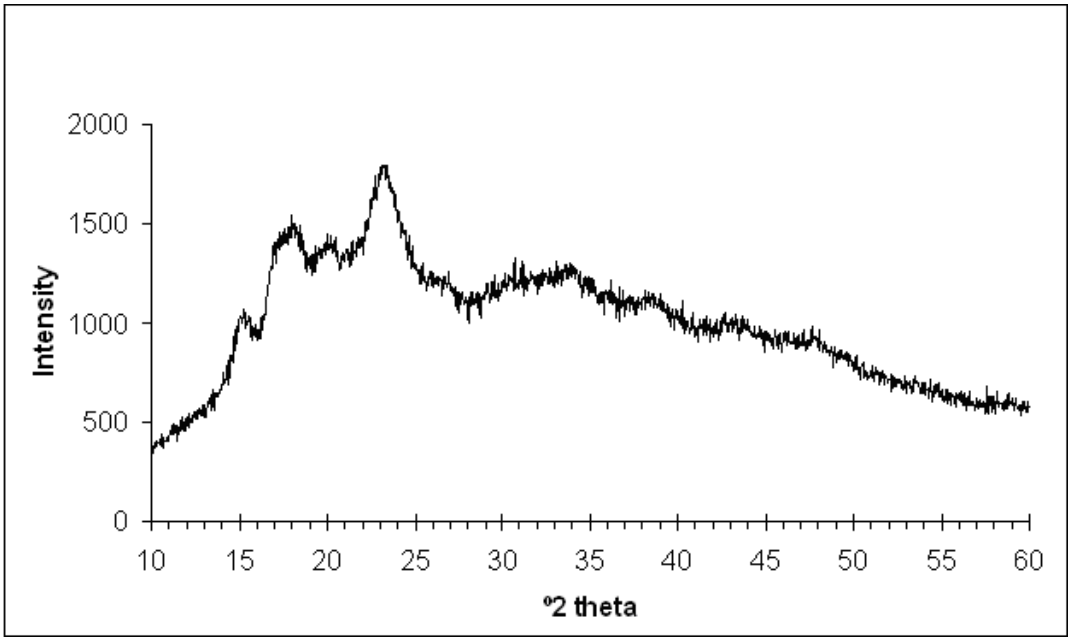
2000
1500
1000
500
0
Intensity
10
15
20
25
30
35
40
45
50
55
60
º2 theta

Figure 8

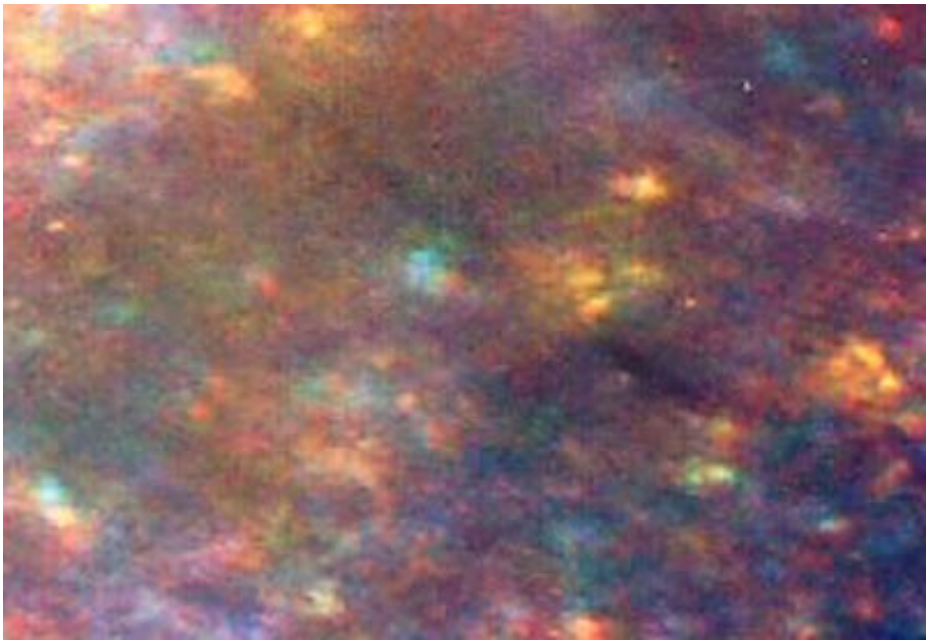

Figure 9

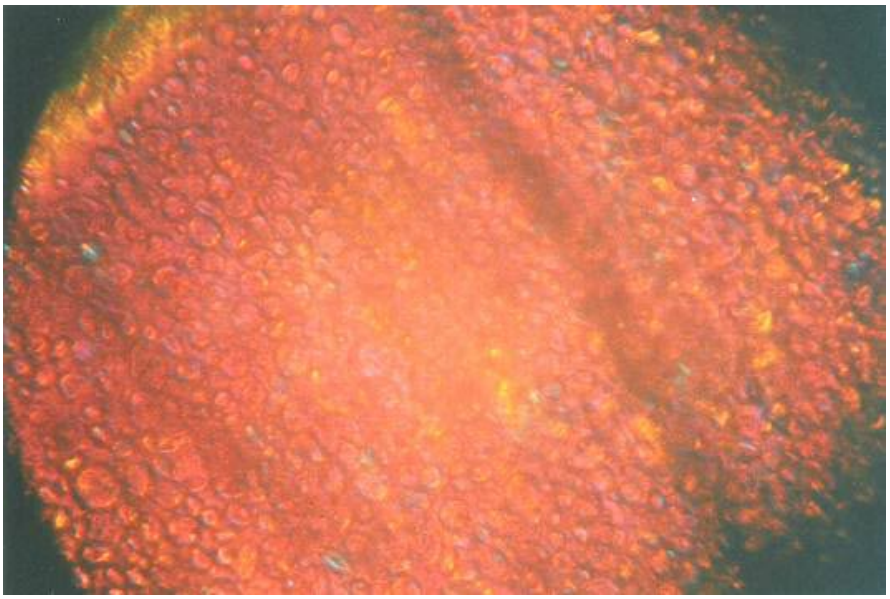

Figure 10

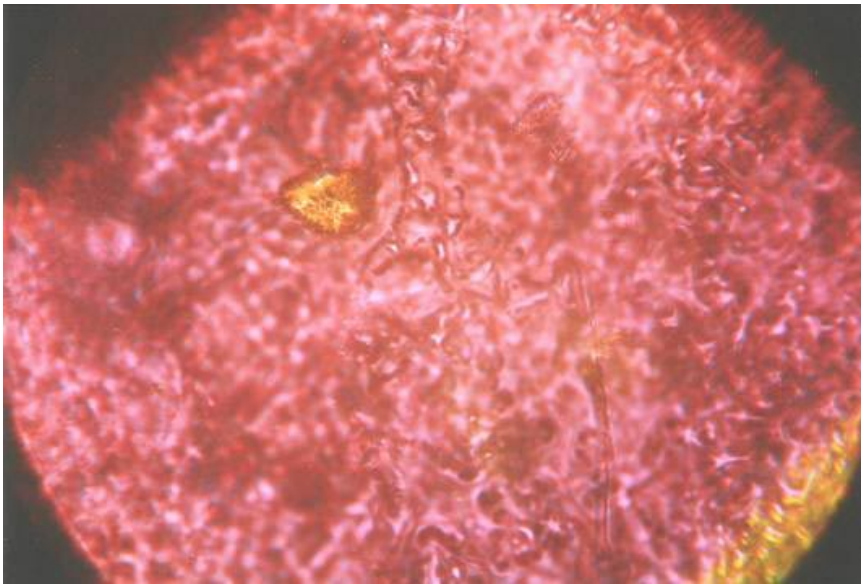

Figure 11