



American Journal of
Food Technology

ISSN 1557-4571



Academic
Journals Inc.

www.academicjournals.com

Effect of Adsorption Medium, Hydrolytic Parameters and Ultrafiltration on the Phenylalanine Removal from Pancreatic Whey Hydrolysates

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Abstract: With the aim of producing dietary supplements for phenylketonurics, using whey hydrolysates as protein sources, the effect of some procedures over phenylalanine (Phe) removal was evaluated. Twelve whey hydrolysates were prepared by the action of a pancreatin, in three enzyme:substrate (E:S) ratios and two temperatures. Half of the samples were submitted to the ultrafiltration (UF) through 10,000 Da cut-off membranes. The activated carbon and the polymeric adsorbent XAD-4 were used for removing Phe from the hydrolysates. The results showed that the activated carbon was more advantageous than the resin, since it led to the lowest final Phe content (2.3 to 38.2 mg Phe/100 g hydrolysate). The effect of E:S ratio, temperature and ultrafiltration was also evaluated. The least final amount of Phe was obtained in absence of UF for three situations: E:S = 0.01% and 25°C, E:S = 0.1% and 50°C, E:S = 1.0% and 50°C.

Key words: Whey, activated carbon, amberlite XAD-4, pancreatin, protein hydrolysis, ultrafiltration

Introduction

The introduction in a diet of enzymatic hydrolysates rich in oligopeptides, especially di- and tripeptides, represents a way of improving protein utilization (González-Tello *et al.*, 1994). These preparations have been used in some countries in the manufacture of special food for several groups, such as those with protein intolerance or allergy, phenylketonuria, cystic fibrosis, chron disease, etc. (Boza *et al.*, 1995; Synowiecki *et al.*, 1996; Clemente, 2000). Moreover, these hydrolysates may be useful in the dietetic supplementation for old people, athletes as well as for weight control diets (Frøkjær, 1994).

The necessity of removing phenylalanine (Phe) from protein hydrolysates is associated to their use in the preparation of dietetic supplements for phenylketonuria (PKU). In fact, the nutritional therapy for PKU is based on limitation of protein ingestion, reducing Phe supply to the minimum and promoting the normal growth of patients with other nutrients (Lopez-Bajonero *et al.*, 1991; Acosta and Yannicelli, 1997; Shimamura *et al.*, 1999).

Free amino acid mixtures (Trahms, 1994; Lajolo and Tirapegui, 1998) or Phe-poor protein hydrolysates (Tesmer *et al.*, 1998) may be used in the nutritional therapy of phenylketonurics. Several hydrolytic conditions have been reported in literature to cleave Phe bonds and help the subsequent removal of this aminoacid (Lopez-Bajonero *et al.*, 1991; Moszczynski and Idziac, 1993; Outinen *et al.*, 1996).

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Among several protein sources that may be used for preparing dietary supplements for phenylketonurics, isolated casein, the main milk protein, is the choice in most cases (Lopez-Bajonero *et al.*, 1991; Outinen *et al.*, 1996; Shimamura *et al.*, 1999). However, in underdeveloped countries, this protein needs to be imported which represents an important increase in production costs. Thus, the use of less expensive alternative sources must be investigated.

Among them, the whey may be tested since its proteins are ready assimilated by the organism, showing a high protein efficiency ratio (Nicolau *et al.*, 2005). Moreover, the use of whey may contribute to reduce the environment pollution.

Considering that in our country the formulations normally used as dietetic supplements must be imported and, consequently, are high-price products, our interest turned to the preparation of these formulations, having protein hydrolysates as the main source of amino acids in a high available form, that is, in oligopeptide form, especially di- and tripeptides. This is the reason we have been preparing several protein hydrolysates and testing different hydrolytic conditions for obtaining peptide profiles appropriate for nutritional purposes (Silvestre *et al.*, 1994a,b; Morato *et al.*, 2000; Barbosa *et al.*, 2004; Carreira *et al.*, 2004; Morais *et al.*, 2005; Lopes *et al.*, 2005a).

Most of the methods used for Phe removal from protein hydrolysates are based on the principle that a sufficient amount of Phe is liberated by enzymatic hydrolysis and the free Phe is, then, removed by gel filtration, adsorption by activated carbon or resins (Lopez-Bajonero *et al.*, 1991; Outinen *et al.*, 1996)

In two studies developed by our group (Lopes *et al.*, 2005b; Soares *et al.*, 2004) using skim milk as protein source to prepare low-Phe products, we used a protease from *Aspergillus oryzae*, isolated or in several associations with papain and pepsin, for preparing the hydrolysates and we tested some parameters for removing Phe from these hydrolysates using activated carbon.

In order to evaluate the efficiency of Phe removal, its amount must be determined either in the protein source or in their hydrolysates, after having used an appropriate adsorption method. Several techniques to quantify Phe among other amino acids are available in the literature, including the ion-exchange chromatography using the amino acid analyzer (Kan and Shipe, 1981), reverse high performance liquid chromatography (Badoud and Pratz, 1984; Bidlingmeyer *et al.*, 1984; Carisano, 1985; Vendrell and Avéles, 1986; Alaiz *et al.*, 1992), hydrophylic interaction chromatography (Alpert, 1990) and spectrophotometric methods of second order (SDS) (Brandts and Kaplan, 1973; Matsushima *et al.*, 1975; O'Haver, 1979; Silvestre *et al.*, 1993; Rojas *et al.*, 1998).

Some authors reported the great reliability of using SDS, between 250 nm and 270 nm, for quantifying Phe in proteins, since parameters such as pH and the addition of other elements are controlled (Brandts and Kaplan, 1973; Ichikawa and Terada, 1979; O'Haver, 1979; Cahill and Padera, 1980; Grant and Bhattacharyya, 1985; Rojas *et al.*, 1998).

Our group has been testing the SDS for several purposes. Thus, we used successfully this technique for determining the hydrolysis degree of protein hydrolysates (Silvestre *et al.*, 1993), for evaluating the encapsulation rate of protein hydrolysates (Morais *et al.*, 2005), as well as for estimating the Phe removal of skim milk hydrolysates (Soares *et al.*, 2004; Lopes *et al.*, 2005a).

The aim of the present study was to evaluate the use of the activated carbon and an adsorption resin, the effect of the ultrafiltration as well as of the E:S ratio and the temperature for removing Phe from pancreatic whey hydrolysates, in order to prepare dietary supplements for phenylketonurics.

Materials and Methods

Materials

L-phenylalanine, L-tyrosine, L-tryptophan, adsorption resin XAD-4, pancreatin (p-1500) and activated carbon (20-60 mesh) were purchased from Sigma (St. Louis, MO, USA). The whey (powder)

was kindly furnished by a food industry (Minas Gerais, Brazil). Whey was kindly furnished by a food industry (Prolácteos, Contagem, MG, Brazil).

The HPLC system consisted of one pump (HP 1100 Series) and an UV-VIS detector, coupled to a computer (HPChemstation HP1100, Germany). A poly (2-hydroxyethylaspartamide)-silica (PHEA) column, 250×9.4 mm, 5 µm, 200 Å pore size (PolyLC, Columbia, MD), was used for HPLC. The freeze-dryer was from Labconco (7750 model, Kansas City, MI, EUA).

For HPLC, water was purified by passage through a Milli-Q water purification system (Aries-Vaponics, EUA). All solvents used for the HPLC were carefully degassed by sonication for 10 min before use.

Methods

Determination of the Chemical Composition of Whey

The contents of moisture, protein, lipid, minerals, calcium and lactose of whey were determined according to the Association of Official Agricultural Chemists methods (AOAC, 1995).

Preparation of Whey Hydrolysates

Twelve hydrolysates were prepared from solutions of whey (1.06 g/100 mL) in 0.01 mol L⁻¹ phosphate buffer (pH 7.5). Initially, they were pre-heated in a water-bath, at 80°C for 10 min. Then, the temperature was adjusted to 25°C or 50°C and the enzyme was added in such a concentration to attain the desired enzyme:substrate ratios. The total reaction time was 5 h for all samples. The hydrolytic reactions were stopped by the reduction of pH to 3.0 by adding formic acid and the samples were, then, freeze-dried. Six hydrolysates were submitted to ultrafiltration by transmembrane pressure (Amicon 8400, Millipore Corporation, MA, USA), using 10,000 Da cut-off membranes (Cellulose, Millipore Corporation, MA, USA) and the permeates were freeze-dried. The other parameters of hydrolysis are listed in Table 1.

Removal of Phenylalanine from Whey Hydrolysates

Use of Activated Carbon

The activated carbon, previously hydrated for 10 min, was placed inside a disposable syringe of 20 mL containing a filter of nylon and wool glass, manufactured in our laboratory. Then, a hydrolysate solution (80 mg/100 mL) was added to the column and the eluate was collected and filtered through qualitative paper (Whatman, number 1, Maidstone, England).

Use of Adsorption Resin

An amount of 5.0 g of XAD-4 was added to the hydrolysate solutions (80 mg/100 mL). After stirring in a beaker for 10 min at 30°C, the mixture was filtered.

Table 1: Hydrolytic conditions employed for preparing whey hydrolysates

| Hydrolysate | E:S (%) | Temperature (°C) | Use of Ultrafiltration |
|-------------|---------|------------------|------------------------|
| H1 | 0.01 | 25 | No |
| H2 | 0.01 | 25 | Yes |
| H3 | 0.01 | 50 | No |
| H4 | 0.01 | 50 | Yes |
| H5 | 0.1 | 25 | No |
| H6 | 0.1 | 25 | Yes |
| H7 | 0.1 | 50 | No |
| H8 | 0.1 | 50 | Yes |
| H9 | 1 | 25 | No |
| H10 | 1 | 25 | Yes |
| H11 | 1 | 50 | No |
| H12 | 1 | 50 | Yes |

E:S = enzyme:substrate ratio

Evaluating the Efficiency of Phe Removal

For evaluating the efficiency of Phe removal, its content in whey and in its hydrolysates was estimated by second derivative spectrophotometry, (Lopes *et al.*, 2005b). Briefly, the samples were hydrolysed (5.7 mol L⁻¹ HCl, 110°C, 24 h) and their absorbance measured from 250 to 280 nm. Second derivative spectra were drawn (CECIL spectrophotometer, CE2041 model, Buck Scientific, England) and the areas of negative peaks were used to calculate the amount of Phe in the samples, employing a standard curve. In case of protein hydrolysates, this same procedure was employed after the treatment with activated carbon and the resin. A software GRAMS-UV (Galactic Industries Corporation, Salem, NH, EUA) was used to draw the second derivative spectra.

Then, the efficiency of Phe removal was calculated according to Eq. 1:

$$\text{Phe removal(\%)} = \frac{\text{Initial of Phe} - \text{Final amount of Phe}}{\text{Initial amount of Phe}} \times 100 \quad (1)$$

Where: Initial amount of Phe = amount of Phe in whey (mol L⁻¹) and Final amount of Phe = amount of Phe in hydrolysates (mol L⁻¹) treated by activated carbon or by the resin.

Statistical Analysis

All experiments were carried out in triplicate. Differences between means of areas of negative peaks were evaluated by analysis of variance (ANOVA) and Duncan test (Pimentel-Gomes, 2000). Differences were considered to be significant at p<0.05 throughout this study.

Results and Discussion

Chemical Composition of Whey

Table 2 shows the amount of the components evaluated in this work are close to the values of the United States Department of Agriculture (USDA, 2001) as well as to those cited by other authors (Berlot *et al.*, 1996), although several factors may influence the composition of whey, such as the original milk, the type of cheese and the fabrication process.

Removal of Phenylalanine

The data in Table 3 show that the activated carbon was efficient to remove Phe from whey hydrolysates. The reduction changed from 75 to 99% and the final content of Phe from 2.3 to 38.2 mg/100 g of hydrolysate. The amount of Phe in the whey was 456 mg/100 g.

It is worth stating that the level of Phe found for all samples was much less than the maximum value established by the Brazilian Legislation for products intended for phenylketonurics, that is 0.1 g/100 g (Brasil, 2002).

In previous studies developed by our group with enzymatic hydrolysates from skim milk, similar removal levels of Phe were found (94 to 99%), using activated carbon as the adsorption medium (Soares *et al.*, 2004; Lopes *et al.*, 2005a).

Table 2: Chemical composition of whey

| Nutrients | Values found* | USDA (2001)** | Berlot <i>et al.</i> (1996) |
|-------------------|---------------|---------------|-----------------------------|
| Moisture (g%) | 3.51±0.07 | 3.51 | - |
| Protein (g%) | 11.82±0.38 | 11.73 | 11.41-15.71 |
| Lipids (g%) | 0.85±0.03 | 0.54 | 0.4-1.0 |
| Total ash (g%) | 8.72±0.02 | 10.11 | 8.30-11.33 |
| Total sugars (g%) | 67.47±0.64 | 73.45 | 63.20-72.12 |
| Calcium (mg%) | 981.5±0.06 | 2054.0 | 790.0-2400 |

*Values found in the present work. **USDA Nutrient database for standard reference

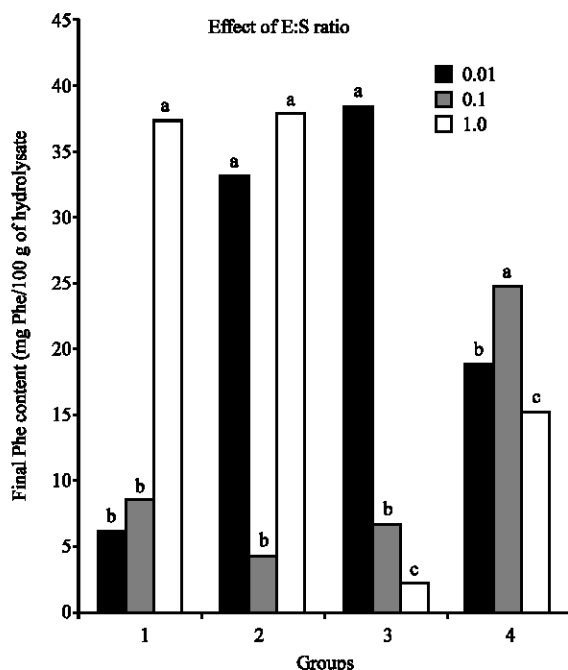


Fig. 1: Effect of E:S ratio over Phe removal. Groups of samples, according to the conditions employed for preparing the hydrolysates: group 1 = 25°C and no UF; group 2 = 25°C and UF; group 3 = 50°C and no UF and group 4 = 50°C and UF. UF = ultrafiltration. Different letters are significantly different ($p < 0.05$) within each group

Table 3: Efficiency of Phe removal from whey hydrolysates by activated carbon and adsorbent resin

| Hydrolysates | Removal (%) | | Final Phe content* (mg Phe 100 g of hydrolysate) | |
|--------------|-------------|-------|--|--------------------|
| | AC | XAD-4 | AC | XAD-4 |
| H1 | 98 | 89 | 6.2 e,f/2 | 37.3 ^{af} |
| H2 | 75 | - | 33.0 a,b | - |
| H3 | 89 | - | 38.2 a | - |
| H4 | 84 | - | 18.7 d | - |
| H5 | 97 | 90 | 8.6 e/2 | 24.5 ^{bf} |
| H6 | 97 | 83 | 4.3 f/2 | 25.7 b/1 |
| H7 | 98 | 94 | 6.7 e,f/2 | 19.5 b/1 |
| H8 | 86 | - | 24.9 b,c | - |
| H9 | 84 | - | 37.2 a,b | - |
| H10 | 78 | - | 37.8 a | - |
| H11 | 99 | 95 | 2.3 g/2 | 16.9 b/1 |
| H12 | 94 | - | 15.4 d,e | - |

AC = activated carbon; XAD-4 = adsorption resin. *Final Phe content = Phe content after treatment with activated carbon or XAD- resin. Different letters are significantly different ($p < 0.05$) for different hydrolysates and the same removal medium. Different numbers are significantly different ($p < 0.05$) for different removal media and the same hydrolysate

Other authors also used activated carbon to remove Phe from protein hydrolysates and reported results near to those of the present work and to the other works of our group. Thus, Kitagawa *et al.* (1987), after hydrolysing whey proteins with actinase, in pH 6.5 at 37°C, treated these preparations with activated carbon and removed 97% of Phe. However, the conditions for the treatment with activated carbon were not mentioned. Lopez-Bajonero *et al.* (1991) reduced 92% the level of Phe from hydrolysates of skim milk or sodium caseinate obtained by the action of papain and a protease from

Aspergillus oryzae. Using a mixture of three enzymes (chymotrypsin, carboxypeptidase A and leucine aminopeptidase), Moszczynski and Idziak (1993) removed 95% of Phe from casein hydrolysates. However, these authors employed more severe conditions than those used here, i.e., a very long time for hydrolysis (72 h) and for the treatment with activated carbon (5.5 h).

The hydrolysates containing the lowest Phe amount (H1, H5, H6, H7, H11), after the treatment with activated carbon, were submitted to Phe removal process using XAD-4 resin.

Table 3 shows that this resin was efficient having removed from 83 to 95% of Phe from the hydrolysates, corresponding to final Phe contents of 37.3 to 16.9 mg/100 g, respectively. No significant difference was observed among hydrolysates H5, H6, H7 and H11. H1 showed the highest Phe amount, possibly due to the use of the smallest E:S ratio in its preparation leading to a lesser exposition of Phe which reduces Phe removal.

Outnen *et al.* (1996) reported similar results for casein hydrolysates obtained by the action of pancreatin (E:S = 3%, 6h, pH 7.0). Thus, the treatment with XAD-4 removed 92 to 100% of Phe from the hydrolysates. However, in this study the amount of enzyme used was at least 3 times higher than those of the present work, which increases the costs of the process.

Other authors described Phe removal lower than those found here, using pepsin (De Holanda *et al.*, 2003) and pronase E (De Holanda *et al.*, 1989) for hydrolysing casein. However, in both cases, only around 62% of Phe were removed after using XAD-4 resin.

Finally, Table 3 shows that the activated carbon was much more efficient than the resin for removing Phe from the five hydrolysates (H1, H5, H6, H7, H11). The final content of Phe in the hydrolysates using activated carbon was 3 (H5 and H7), 6 (H1 and H6) and 7 times (H11) lower than using the resin.

Effect of Different Treatments on the Phenylalanine Removal

This study was performed with all whey hydrolysates submitted to the treatment with activated carbon.

Effect of E:S ratio

For evaluating the effect of E:S ratio, the hydrolysates were divided in 4 groups in order to keep constant the other parameters (temperature and treatment by ultrafiltration -UF): group 1 = 25°C and no UF; group 2 = 25°C and UF; group 3 = 50°C and no UF and group 4 = 50°C and UF.

The data in Fig. 1 show that the E:S ratio affected the Phe removal of whey hydrolysates. The desirable effect associating the decrease of E:S with the least final amount of Phe (in mg/100 mg of hydrolysate), related to the reduction of production costs of dietary supplements for phenylketonurics, occurred in two groups (1 and 2) when passing from 1 to 0.1%. Thus, in group 1 the Phe content passed from 37.2 to 8.6 and in group 2 from 37.8 to 4.3. In the other two groups, this procedure was disadvantageous leading to a larger amount of Phe in the final product: groups 3 (from 2.3 to 6.7) and 4 (from 15.3 to 24.9). The further reduction of E:S ratio from 0.1 to 0.01% was beneficial to group 4 (from 24.9 to 18.7), disadvantageous to groups 2 (from 4.3 to 33.0) and 4 (from 6.7 to 38.2) and unaffected to group 1 (from 8.6 to 6.2).

In an earlier study with skim milk, the 10-fold decrease of E:S ratio from 10 to 1% and from 20 to 2% for a protease from *Aspergillus oryzae* and for papain, respectively, used in association, was advantageous leading to a decrease in the final content of Phe from 0.82 to 0.21 mg/100 g of hydrolysate (Lopes *et al.*, 2005b).

Effect of the Temperature

In this case, the hydrolysates were divided in 6 groups in order to keep constant the other parameters (E:S ratio and treatment by ultrafiltration-UF): group 1 = 0.01% and no UF; group 2 = 0.01% and UF; group 3 = 0.1% and no UF; group 4 = 0.1% and UF; group 5 = 1.0% and no UF; group 6 = 1.0% and UF.

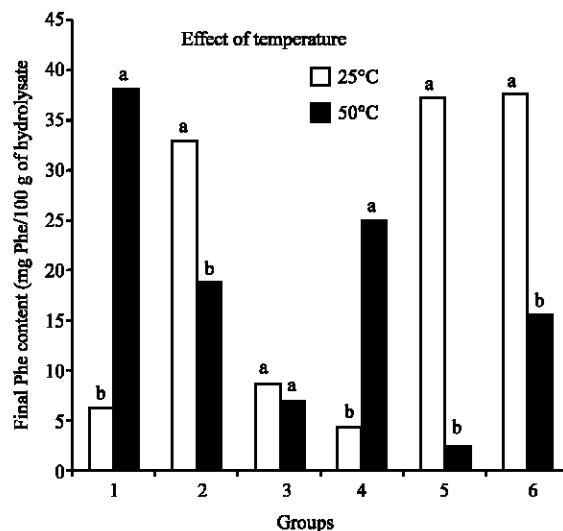


Fig. 2: Effect of the temperature over Phe removal. Groups of samples, according to the conditions employed for preparing the hydrolysates: group 1 = 0.01% and no UF; group 2 = 0.01% and UF; group 3 = 0.1% and no UF; group 4 = 0.1% and UF; group 5 = 1.0% and no UF; group 6 = 1.0% and UF. UF = ultrafiltration. Different letters are significantly different ($p < 0.05$) within each group

Figure 2 shows that, the Phe removal was also influenced by the temperature. The desirable effect associating the decrease of the temperature with the least final amount of Phe (in mg/100 mg of hydrolysate), related to the reduction of production costs of dietary supplements for phenylketonurics, occurred in groups 1 (from 38.2 to 6.2) and 4 (from 24.9 to 4.3). In group 3 this procedure had no effect on Phe removal (from 6.7 to 8.6). The decrease of the temperature was disadvantageous in groups 2 (from 18.7 to 33.0), 5 (from 2.3 to 37.3) and 6 (from 15.4 to 37.8).

No data concerning the effect of the temperature on the Phe removal was found in the literature.

Effect of the Ultrafiltration

The estimation of this treatment required that the hydrolysates were divided in 6 groups in order to keep constant the other parameters (E:S ratio and the temperature): group 1 = 0.01% and 25°C; group 2 = 0.01% and 50°C; group 3 = 0.1% and 25°C; group 4 = 0.1% and 50°C; group 5 = 1.0% and 25°C; group 6 = 1.0% and 50°C.

As described earlier for E:S ratio and temperature, the UF also influenced the Phe removal (Fig. 3). The desirable effect associating the absence of UF with the least final amount of Phe (in mg/100 mg of hydrolysate), related to the reduction of production costs of dietary supplements for phenylketonurics, occurred in three groups: 1 (from 33.0 to 3.2), 4 (from 24.9 to 6.7) and 6 (from 15.4 to 2.3). No effect of the UF was observed in group 5 (from 37.8 to 37.3). The lack of UF was disadvantageous in groups 2 (from 18.7 to 38.2) and 3 (from 4.3 to 8.6).

On the other hand, not only the economical aspect of UF but also other approaches may be taken into consideration. Although the UF is an expensive process, it shows some immunological and nutritional advantages such as reduction of the allergenicity of proteins and their enzymatic hydrolysates as well as the improvement of dietary formulas by increasing their oligopeptide content (Van Beresteijn *et al.*, 1994; Exl, 2001; Chandra, 2002). Thus, a precise evaluation of the costs and benefits must be done before choosing the best procedure to follow.

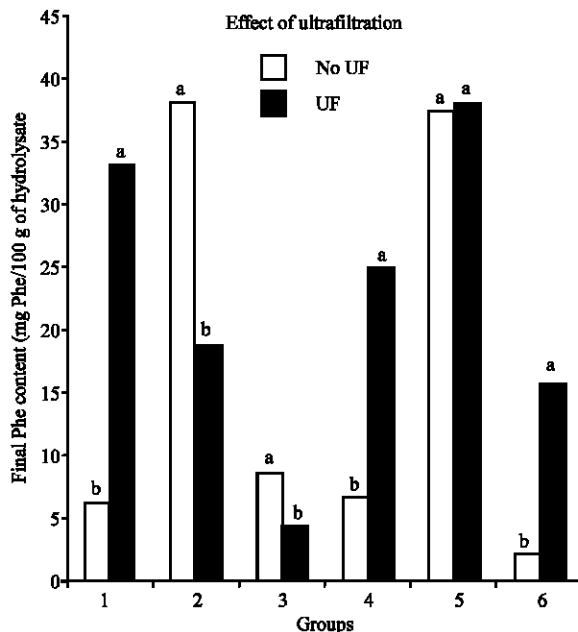


Fig. 3: Effect of ultrafiltration over Phe removal. Groups of samples, according to the conditions employed for preparing the hydrolysates: group 1 = 0.01% and 25°C; group 2 = 0.01% and 50°C; group 3 = 0.1% and 25°C; group 4 = 0.1% and 50°C; group 5 = 1.0% and 25°C; group 6 = 1.0% and 50°C. UF = ultrafiltration. Different letters are significantly different ($p < 0.05$) within each group

Conclusions

The activated carbon and the adsorption resin XAD-4 were efficient for removing Phe from pancreatic whey hydrolysates. The final content of Phe changed from 2.3 to 38.2 mg/100 g of hydrolysate and from 16.9 to 37.2 mg/100 g of hydrolysate, respectively, for the samples treated by both methods. These values show the superiority of activated carbon over the resin. The E:S ratio, the temperature of hydrolysis and the ultrafiltration affected diversely the Phe removal, depending on the conditions employed for preparing the hydrolysates.

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