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Production of phenylalanine-reduced soymilk for phenylketonuria patients

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RESEARCH ARTICLE

Abstract

Phenylketonuria (PKU) is a hereditary disease caused by the deficiency of phenylalanine (Phe) hydroxylase enzyme or its cofactor tetrahydrobiopterin. Treatment involves a Phe-restricted diet, although food options are limited. Soymilk, derived from soybeans, is a functional food with nutritional benefits. In this study, soymilk was produced and hydrolyzed with protease of *Aspergillus oryzae* and papain, and then activated carbon was used to remove Phe for PKU patients. The second-derivative spectrophotometry method was used to determine Phe content in soymilk. The results showed no significant difference in dry matter, fat, and crude fiber between soymilk and Phe-extracted soymilk with respect to the control group ($P < 0.05$). Soymilk's protein content was calculated as 2.74% (w/w) and that of Phe-reduced soymilk as 1.94% (w/w). Similarly, consecutive Phe content was 40.53 mg/L and 5.09 mg/L. After hydrolization, the calculated Phe removal rate was 87.44% (w/w), and the protein content was reduced by 29.19% (w/w). The study demonstrates that Phe-reduced soymilk is suitable for PKU patients, thus reducing the need for imported products and offering new market opportunities.

Keywords: phenylketonuria; soymilk; phenylalanine; activated carbon

Introduction

Phenylketonuria (PKU) is a metabolic disease caused by the absence of phenylalanine hydroxylase (PAH) enzyme, which converts phenylalanine (Phe) to tyrosine, or its cofactor tetrahydrobiopterin (BH_4) because of autosomal recessive inheritance (Blau *et al*., 2010; Neto *et al*., 2018). The disease was first described by Ivar Asbjørn Følling in 1934. Untreated PKU leads to progressive mental impairment characterized by eczematous rashes, autism, seizures, and other clinical manifestations (Blau *et al*., 2010). PKU patients account for approximately 32% of global cases, with the highest prevalence observed in Turkey. Owing to the high rate of consanguineous marriages, approximately one in every 3,500–6,500 individuals in Turkey suffer from this disease, while the rate is lower in the United States, with one in every 10,000–15,000 individuals having PKU (Dobrowolski *et al*., 2011). It is reported that there is no known drug treatment for the disease. PKU can be treated if diagnosed early and a Phe-restricted diet is initiated from infancy. The main goal of dietary treatment is to maintain a Phe level of below 6 mg/dL in the serum while ensuring that patients must receive the minimum amount of Phe necessary for vital functions (Kısa *et al*., 2017; Özboy, 2002). Therefore, the products used in diet therapy should be special medical foods with reduced or no Phe content. Since the special diet required for this disease should also be adequate and balanced in terms of protein, vitamins, energy, minerals, and Phe, it is important to select foods from free or limited food groups. One of the main differences of PKU from other diseases requiring a special diet is the very limited variety of foods that patients can consume and the limited production or availability of foods specifically meant for these patients in our country, resulting in the majority of products being imported from abroad. Another important aspect of PKU is that in an optimal dietary regimen, foods must be tasty, easily accessible, and consumable (MacDonald, 2011). The Phe-restricted diet generally comprises fruit- and vegetable-based diet,

while dairy and meat products, grains, and legumes are restricted due to their high protein contents. Even if successful, maintaining this diet throughout life is challenging for patients because of the limited variety of foods available. Therefore, the search for alternative foods is essential (Pimentel *et al*., 2014).

Soy, which is considered to be very close to animal-based foods and provides an alternative for patients who have difficulty consuming such foods, stands out. Soybean plant belongs to the *Papilionoideae* family of the *Leguminosae* order, and its cultivated variety *Glycine max. (*L*.) Merrill* is grown as soybean (De *et al*., 2022). Soybean and its products have gained popularity as a quality protein source and are used in the production of functional foods. In addition to its high-quality protein content, soy is rich in dietary fiber and isoflavones. It is also notable for its cholesterol-free and low saturated fat content. Soy has three main functions as food: oil products (glycerol, refined soybean oil, and soy lecithin), whole soy products (soybean, soy sprouts, soymilk, soy flour, and tofu), and soy protein products (soy protein concentrates and isolates) (De *et al.*, 2022).

The most practical and nutritious among these varieties is soymilk. Soymilk, consumed in China for many years, is obtained by the water extraction of soybeans. According to the US Food and Drug Administration (FDA, 1999), a daily threshold consumption of 25 g of soy protein is necessary to reduce cholesterol. According to a US epidemiologic research, the average daily soy protein consumption for vegans is 13.1 g and that for nonvegetarians 4.9 g. These intakes are much higher than the average soy protein intake by the US population (Zamora-Ros *et al.*, 2012). In Japan, people following a traditional diet typically consume 7–10 g of soy protein per day, or around 10% of their total protein consumption from food (Messina *et al.*, 2006). Chinese women consume 15.1–24.9 g of soy protein per day (Yang *et al.*, 2005). Soymilk is considered as an alternative to human milk because of its amino acid composition, and has the best ratio among plant proteins. Soymilk is produced in flavored, condensed, and reconstituted variants. Soymilk is used as a substitute for cow's milk, as it contains all essential amino acids required for human consumption according to Food and Agriculture Organization (FAO) and World Health Organization (WHO; Chen *et al.*, 2012). Since the early 1990s, soy has been recognized as a functional food because of its bioactive components, including protease inhibitors, phytosterols, saponins, phenolic acids, phytic acid, tannins, and isoflavones, which are discovered to reduce the risks of cancer, human immunodeficiency virus (HIV), cardiovascular diseases, and osteoporosis (Özcan *et al.*, 2015). The digestibility of soymilk by humans is 91%, which makes it a valuable food product. However, undesirable flavors and odors caused by daidzein and genistein isoflavones, produced by soaking soybeans, are eliminated by the use of activated carbon, as demonstrated in a study conducted by Shahidi and Naczk (1995), which also showed effectiveness of soybeans in development of color and aroma.

Phenylalanine from protein solutions is either removed through the enzymatic hydrolysis of proteins, or through ultrafiltration, active carbon adsorption, or resin adsorption. In order to remove maximum Phe, proteins are usually hydrolysed thoroughly, generating various amino acids and oligopeptides. Because ultrafiltration results in the loss of a significant quantity of low molecular weight nutrients, that is, amino acids and oligopeptides, its application is restricted for post-processing. Therefore, adsorption appear as a more promising post-processing technique for Phe removal (Su *et al.*, 2021). The methods that are frequently used involve the selective adsorption of Phe on solid matrices, such as activated carbon or ion exchange resins, which is followed by its desorption and crystallization. In the process of creating Phe-free dietary formulae for PKU patients, adsorption is also used to extract Phe from protein hydrolysates (Clark *et al.*, 2012). Numerous investigations on the adsorption of Phe on various materials, including polymeric resins, activated carbons, zeolites, and ion exchangers, have been documented in scientific literature (Díez *et al.*, 1998; Fei-Peng *et al*., 2012; Ghosh *et al.*, 2011; Titus *et al.*, 2003). However, the generation and regeneration of these adsorbents is expensive; however, using low-cost adsorbents could lower these expenses (Clark *et al.*, 2012).

Different techniques are used for detecting amino acids in alternative foods for patients with PKU, or in different areas. The first of these is ion exchange chromatography. High-performance liquid chromatography (HPLC) and gas chromatography–mass spectroscopy (GC-MS) are also used. Derivatization method is generally required when HPLC is used for separation of amino acids (Paramás *et al*., 2006; Piecyk *et al.*, 2007; Su *et al*., 2021). The last method used is spectrophotometry. Tyrosine, tryptophan, and Phe are aromatic amino acids, and they have characteristic absorption bands in the ultraviolet region of 240–310 nm. Using the absorbance of Phe at 280 nm in spectrophotometer, determination is carried out in the sensitivity range of 0.05–2.0-mg/mL protein. However, the intensity of peaks in the second-derivative spectrum of proteins and peptides is related to the exposure of aromatic amino acids, and how close they are to the C- or N-terminal position of the aromatic group. In addition, since interference from various substances that absorb at 280 nm is observed, in some studies it is preferable to take measurements spectrophotometrically at 280 nm and 260 nm and make the calculation using the correction factor given as follows:

$$\text{Protein (mg/mL)} = 1.55A_{280} - 0.76A_{260},$$

where A_{280} = absorbance value at 280 nm, and A_{260} = absorbance value at 260 nm (Barbosa *et al.*, 2002).

Derivative spectrophotometry is an analytical technique used for extracting and analyzing both qualitative and quantitative information from spectra consisting of unresolved bands. Derivative spectra are produced by processing spectrophotometer output. The use of derivative spectra increases the sensitivity of detecting small spectral features and reduces the error caused by overlapping of analyte spectral bands by interfering with bands of other species in the sample (Eskandari *et al.*, 2006). The differentiation process in ultraviolet (UV)-visible field spectroscopy is applied as follows:

Zero order

$$A = \varepsilon bc$$

First order

$$\frac{dA}{d\lambda} = \frac{d\varepsilon}{d\lambda} = bc$$

n^{th} order

$$\frac{d^n A}{d\lambda^n} - \frac{d^n \varepsilon}{d\lambda^n} bc$$

where:

λ: wavelength (nm)

ε: molar extinction coefficient (L/mol cm)

b: sample path length (cm)

c: sample concentration (mol/L)

The derivative spectrum is obtained by plotting absorbance values against the wavelengths of the rays sent on a substance having absorption in a visible region. Derivative spectroscopy is expressed with the equation, $A = f(x)$, where A is the absorbance and x is the wavelength. The derivative at each point of the function is calculated as $dA/d\lambda$, and if these derivative values are plotted against wavelength, a derivative spectrum is formed. Derivative studies show the slope of the spectrum and clearly reveal shoulder and elbow formations (Figure 1), as well as peaks and inflection points in the spectrum, to achieve accurate results. At the same time, a deformation in the spectrum line shows an impurity in the sample. The application of derivatives dilutes bands in the spectrum and allows their complete separation. Thus, it is possible to separate substances or their bands that make up a mixture. With the application of derivative spectroscopy, mixture samples are prevented from affecting each other by interfering in the spectrum, and it is possible to perform spectroscopic analysis of such substances. Derivative spectra enable the determination of two compounds together by facilitating the separation of intertwined bands in the mixture of two

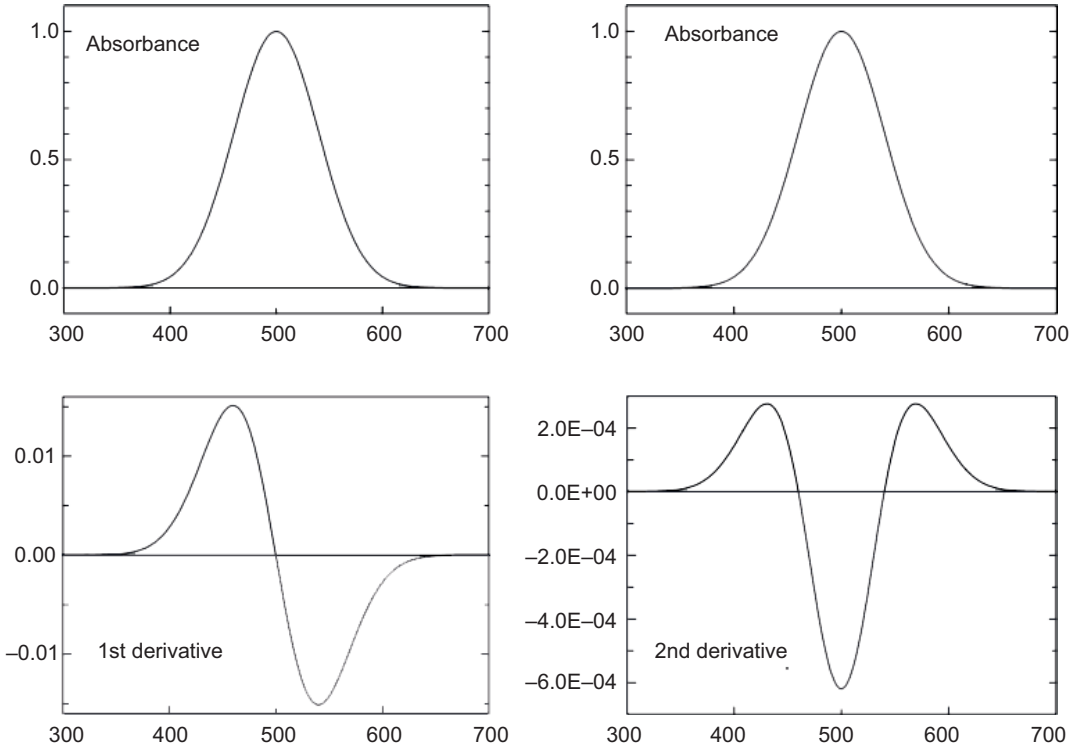

Figure 1. Order of derivative spectra (Owen, 1995).

compounds in quantitative analyses. Moreover, derivative spectra are used in the identification of mixtures in qualitative analyses, in impurity controls, and in many pharmaceutical analyses, such as toxicology and industrial applications, to analyze easily without requiring any separation while working in turbid environments, such as suspensions. Derivative spectra are also used widely in field analyses and in the analysis of proteins, amino acids, and food dyes in foods (Owen, 1995).

Driven by the limited selection of foods for PKU patients and the lack of sufficient production of proper foods for these patients, the aim of this paper was to add a new alternative to the restricted diets of PKU patients, considering the high nutritional value of soymilk. In this study, Phe was removed for PKU patients by protease hydrolysis of *Aspergillus oryzae* and papain, consequtively removed with activated carbon via adsorption from soymilk, obtained by the water extraction of soybeans. The removal rate of Phe from soymilk was calculated by second-derivative spectrometry, and dry matter, fat, protein, and dietary fiber contents of soymilk and the newly obtained product were examined.

Materials and Methods

Material

Soybeans were purchased from Ingro-Asaf Bilişim ve Pazarlama (İstanbul, Turkey) and stored at +4°C in a refrigerator.

Preparation of soymilk: Soybeans were washed and soaked in drinking water at +4°C for 16 h. After soaking, bean hulls were purged from soybeans. The soaked soybeans were then blended with water at a ratio of 5:1 (H_2O:soybeans) at 90°C for 5 min using a blender (AR1052 Technoart, Arzum, İstanbul, Turkey). The resulting slurry was filtered with a generic plastic household sieve, and insoluble residue was discarded from soymilk. The soymilk was heated at 90–95°C for 15 min. Approximately 500 g of soymilk was obtained from 100 g of soybeans (Kwok *et al.*, 1999; Metussin *et al.*, 1992).

Chemical analysis of soymilk and phenylalanine-reduced soymilk

The gravimetric method 934.01 of Association of Official Analytical Chemists (AOAC, 2006) was used to determine the dry matter content of soymilk. The crude protein content of soymilk was calculated using the Kjeldahl method (AOAC gravimetric method 991.20). The factor of 6.25 was used for milk products. The Gerber method was used to determine fat content in soymilk and the final product after removal of Phe (Pearson, 1970). For determining crude fiber, 1 g of dried soymilk sample was boiled for 30 min in a flask with 0.255-N sulfuric acid (Merck, Germany). The solution was cooled and filtered through a Büchner funnel and then washed with distilled water. The resulting solution was transferred into a flask containing boiling 0.313-N NaOH (Merck) and kept for 30 min. The solution was filtered through a pre-dried and weighed filter paper, and sequentially washed with HCl (1% v/v) (Sigma-Aldrich, USA), water, ethanol (80% v/v) (Sigma-Aldrich), and finally with diethyl ether (Sigma-Aldrich). The filter paper was collected carefully and placed in a pre-weighed crucible. It was dried in an oven (ED 53; Binder, Germany) at 102±2°C until a constant weight was obtained. The crucible with filter paper was ashed in a muffle furnace (PAF 120; Protherm, Turkey) at 500–550°C (Food and Agriculture Organization [FAO], 1986):

Crude fiber (%) = (A – B ÷ sample weight) × 100,

where:

A: weight (g) after ashed in a muffle furnace,
B: weight (g) after oven-drying.

Preparation of protein hydrolysates

Soymilk solution (0.40 g/mL) was prepared in a 0.01-M phosphate buffer (Sigma-Aldrich) at pH 6. Hydrolization was performed with 1 g of protease of *Aspergillus oryzae* (Sigma-Aldrich) at 50°C for 1 h, followed by treatment with 2 g of protease of papain (Merck) at a constant temperature of 50°C for 4 h. The hydrolysis was stopped by keeping it in an ice bath at 10°C (Amiri-Rigi *et al.*, 2012; Lopes *et al.*, 2005; Silvestre *et al.*, 2009).

Removal of phenylalanine from hydrolysate

Prepared protein hydrolysate, 100 mL, was heated and stirred with a magnetic stirrer (MSH 20 A; Wisestir, South Korea) at 25°C for 30 min. Then, 8 g of activated carbon (Thermo Fisher Scientific, UK) were added to the solution and again stirred for a while. After the addition of activated carbon, the hydrolysate was centrifuged at 5,000 rpm for 10 min at 25°C (Model 2-16 PK; Sigma-Aldrich, Germany) and filtered through filter paper (Whatman No. 1; England) (Lopes *et al.*, 2005; Soares *et al.*, 2006).

Determination of phenylalanine in soymilk and phenylalanine-reduced final product

Absorbance of prepared samples was measured at 250–280 nm at 1-nm intervals, and the second-derivative

spectra were plotted. For standard curve, stock solutions of Phe (6.05×10^{-4} mol/L), tyrosine (5.52×10^{-4} mol/L), and tryptophan (4.90×10^{-4} mol/L) were prepared in a 0.01-M phosphate buffer solution (Sigma-Aldrich) at pH 6. Then, successive dilutions of Phe standard solution were prepared to have Phe concentrations in a range of 0.30×10^{-4}–1.82×10^{-4} mol/L. Spectra of these diluted solutions were recorded between 250 and 280 nm using spectrophotometer (SP-3000 Plus; Optima, Japan), and third negative peak heights from second-derivative spectra were used to plot standard graph. In all, 10-mL samples were taken from soymilk and Phe-reduced products, and 0.1 mL of 5.7-M HCl (Sigma-Aldrich) was added to each sample. The samples were hydrolyzed in an étuve (ED 53; Binder, Germany) at 110°C for 24 h. After removing from étuve, the residue was dissolved in 30 mL of distilled water and adjusted to pH 6 with 1-M sodium phosphate buffer (Sigma-Aldrich). The absorbance values were measured and their second derivatives were calculated. The height of third negative peak was compared with the standard graph to determine Phe content. The removed Phe was calculated using the following equation (Ichikawa and Terada, 1977, 1979; Lopes *et al.*, 2005; Silvestre *et al.*, 2009):

$$\text{Removed Phe (\%)} = \frac{\text{Phe content in soymilk} - \text{Phe content in Phe} - \text{reduced soymilk}}{\text{Phe content in soymilk}}$$

Statistical analysis

All experiments were replicated in triplicate. Significant difference between the produced soymilk and the soymilk after Phe extraction was determined by a one-sample *t*-test using the IBM SPSS Statistics 22 software. A significance level of 0.05 was used for statistical analysis.

Results

Chemical analyses of soymilk and phenylalanine-reduced soymilk

Total dry matter content

The total dry matter content of soymilk samples was calculated as 8.61±0.20% (w/w), and as 8.49±0.03% (w/w) in Phe-reduced soymilk. No significant difference between soymilk and Phe-reduced soymilk was observed based on the *t*-test ($P < 0.05$) (Table 1). Cruz *et al.* (2007) reported a dry matter content of 8.27% (w/w) in soymilk, Pathomrungsiyounggul *et al.* (2007) discovered a moisture content of 5.69% (w/w), and Artık (1989) reported a dry matter content of 9.2% (w/w).

Crude protein content

The average protein content in soymilk was determined as 2.74±0.46% (w/w) and the same in Phe-reduced soymilk was 1.94±0.14% (w/w). The reduction in protein content was 29.19%, which was statistically significant ($P < 0.05$) (Table 1). Bricarello *et al.* (2004) reported the protein content in soymilk as 2.5% (w/v). Liu and Chang (2013) analyzed the nutritional and physicochemical properties of commercial soymilk products and performed crude protein determination using Kjeldahl method. Among the 39 products analyzed, only three had a protein content exceeding 3% (w/w), with an average value of 2.6% (w/w). The standard protein content for soymilk set by the Soyfoods Association of America (SAA) (1996) is 3% (w/w). Hajirostamloo (2009) reported protein content of soymilk as 2.74% (w/w) whereas Artık (1989) reported protein content in soymilk as 3.6% (w/w). The highest crude protein values for soymilk appeared as 6.74–6.84% (w/w) (De et al., 2022). Variations in the protein content of soymilk are attributed to the type of raw material used and production process and conditions.

Fat content

The fat content of soymilk was found as 2.20±0.26% (w/w) and as 2.73±0.87% (w/w) in Phe-reduced soymilk. No statistically significant difference was observed between Phe-reduced soymilk and the control ($P < 0.05$). De *et al.* (2022) reported a fat content of 3.5–3.9% (w/w), Cruz *et al.* (2007) discovered a fat content of 1.86% (w/w), and Hajirostamloo (2009) noted a fat content of 0.77% (w/w) in soymilk. Our results conformed to the range values of fat content reported in the literature.

Crude fiber determination

Crude fiber content determined in soymilk samples was 0.41±0.51% (w/w) and in Phe-reduced soymilk, it was 0.45±0.47% (w/w). The results showed nonsignificant difference ($P < 0.05$) (Table 1). This could be considered a positive outcome of the study. Previous studies have also found low levels of crude fiber content in soymilk.

Table 1. Chemical properties of soymilk and phenylalanine-reduced soymilk.

	Soymilk (control)	Phenylalanine-reduced soymilk
Dry matter content (% w/w)	8.61±0.20[a]	8.49±0.03[a]
Protein content (% w/w)	2.74±0.46[a]	1.94±0.14[b]
Phenylalanine content (mg/L)	40.53±0.65[a]	5.09±0.13[b]
Fat content (% w/w)	2.20±0.26[a]	2.73±0.87[a]
Crude fiber content (% w/w)	0.41±0.51[a]	0.45±0.47[a]

Means on the same row with different superscript letters differ significantly ($P < 0.05$)

De *et al.* (2022) discovered 0.14–0.16% (w/w) crude fiber content in soymilk. Gandhi (2009) reported a wide range of 0–4% (w/w) crude fiber content in soymilk. The production method of soymilk and the type of soybeans used in production are the factors that contribute to variation in crude fiber content. Adebayo-Tayo *et al.* (2009) investigated the physicochemical quality of powdered soymilk and revealed the lowest crude fiber content as 2.05% (w/w) and the highest as 2.34% (w/w) among branded and non-branded soymilk powders, while Liu and Chang (2013) examined commercial soymilk products and reported that 67% of the products contained less than 0.6% (w/w) crude fiber. Bricarello *et al.* (2004) found that soymilk contained 0.13% (w/w) of crude fiber. Some studies have reported that soymilk does not contain any crude fiber (Al and Oladimeji, 2008; DeMan *et al.*, 1987; Granata and Morr, 1996). In spite of the fact that raw soybean is known as a good source of crude fiber (5.4–7.5% [w/w]), compared to other legumes, it is obvious that soymilk cannot be considered as a healthy source of fiber (Siulapwa and Mwambungu, 2014).

Determination of phenylalanine content in soymilk and phenylalanine-reduced final product

Standard dilutions were prepared with Phe concentration ranging from 0.30 × 10^{-4} to 1.82 × 10^{-4} mol/L in 0.01-M phosphate buffer at pH 6.0 and measurements were taken at 250–280 nm with 1-nm intervals (Figure 2). The collected absorbance data were used to plot an absorbance versus wavelength graph (Figure 3). Then, in order to calculate the second derivative using spectrophotometry, the following mathematical equation provided by Owen (1995) was used:

$$D_\lambda = (A_{\lambda-\Delta\lambda} - 2A_\lambda + A_{\lambda+\Delta\lambda}) \div \Delta\lambda^2,$$

where D_λ is the derivative amplitude, λ is a particular wavelength (nm), $\Delta\lambda$ is a very close wavelength to a particular wavelength (nm), and A_λ is absorbance at a particular wavelength.

In the second-derivative spectra, each graph exhibited seven negative peaks (Figure 2). In addition, a standard graph was drawn based on the wavelength (262 nm) at which third negative peak took place (Figure 4) (Ichikawa and Terada, 1977; Lopes *et al.*, 2005; Soares *et al.*, 2006). After drawing the standard graph, measurements were taken at 250–280 nm with 1-nm interval to determine how much Phe content was reduced in soymilk and Phe-reduced soymilk samples (Figures 5 and 6).

Ichikawa and Terada (1977) demonstrated that at pH 7, the absorption bands of Phe were masked in the absence of derivative spectra of amino acid mixtures (tryptophan, tyrosine, and Phe). They found that these effects were reduced in the first derivative, but in the second derivative, tyrosine and tryptophan showed completely flat spectra of 245–270 nm, while Phe exhibited characteristic spectral bands. Thus, the authors strongly suggested that the presence of Phe could be determined by the second-derivative spectrum, even in the presence of other amino acids. Miclo *et al.* (1995) used the first- and

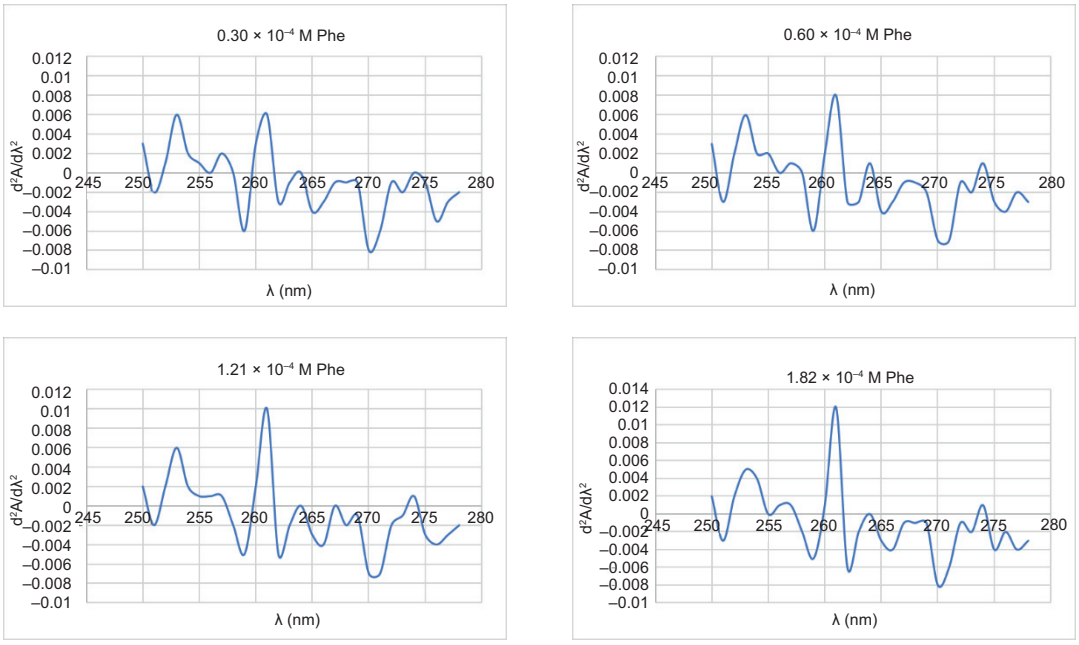

Figure 2. Second-derivative spectrometry graphs of standard phenylalanine solutions.

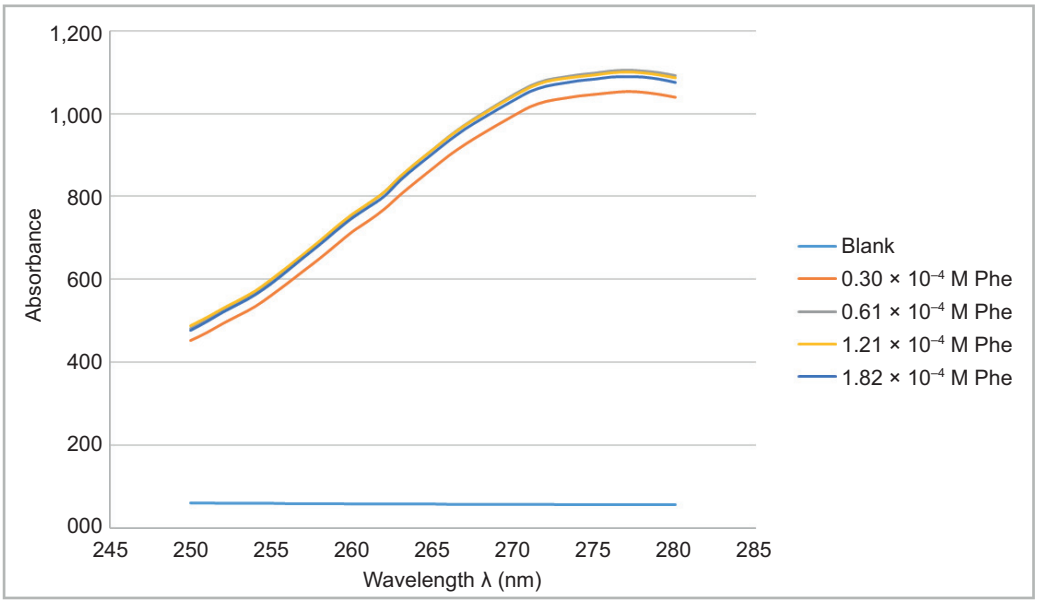

Figure 3. Absorbance versus wavelength graph.

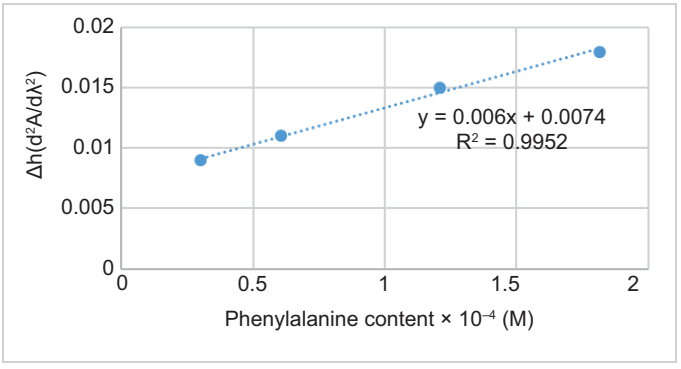

Figure 4. Standard graph of second-derivative spectrometry.

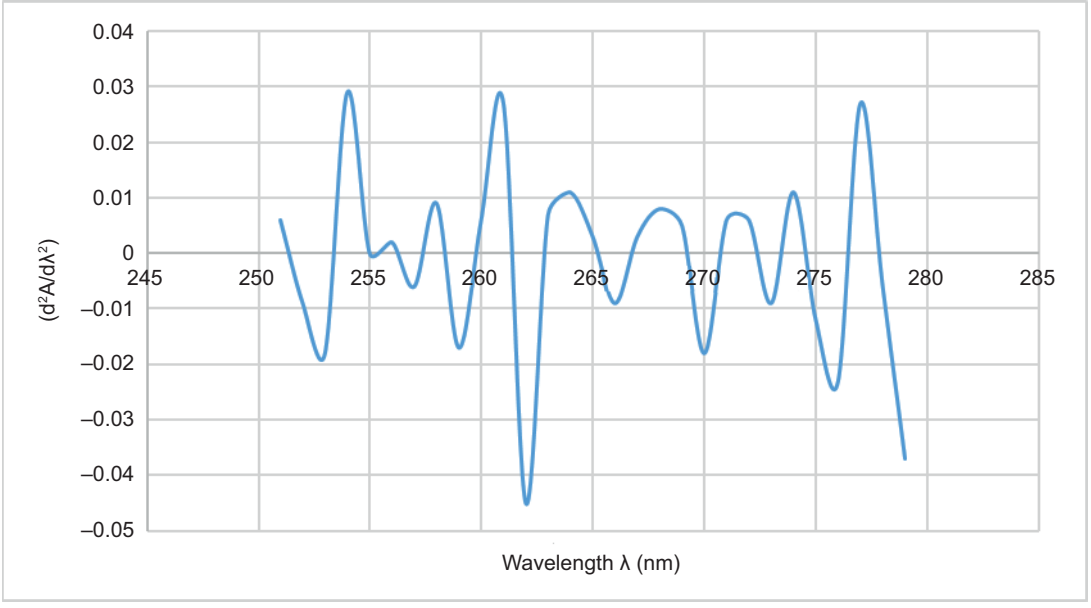

Figure 5. Second-derivative spectrometry wavelength–absorbance graph of soymilk.

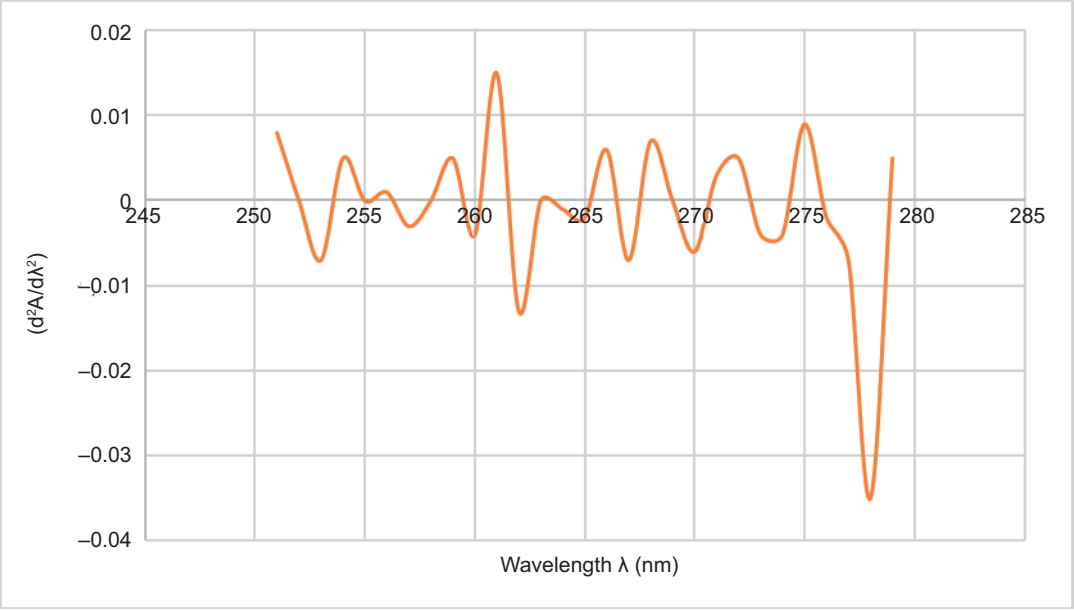

Figure 6. Second-derivative spectrometry wavelength–absorbance graph of phenylalanine-reduced soymilk.

second-derivative spectrometry for the characterization of aromatic amino acid residues. Phe exhibited six peaks and six negative peaks at pH 1.9 from 240- to 275-nm wavelength. In a mixture of Phe and tryptophan, Phe had a specific difference between the peak at 254.7 nm and the negative peak at 257.6 nm, and this range of wavelength was used to characterize Phe. Nozaki (1990) reported that determining the amino acid content using spectrophotometry is quite challenging due to factors, such as natural composition, impurities, or turbidity. Therefore, the second derivative method was applied for accurate detection of Phe. Phe was determined by measuring the heights of the peaks at 264 nm in specific dilutions of aromatic amino acid mixtures. The number of peaks and range of wavelength in Phe spectra depend on the instrument used, spectrophotometer, software, solvent type, pH value of the process, or the amino acid used for standard solutions (free or N-acetyl ester) (Barbosa *et al.*, 2002).

The number of peaks in these spectra is higher than that of the prepared samples for the standard graph, which is normal considering the possibility of slight interference from other amino acids. The Phe content was computed by calculating the height (Δh) of the peaks at 262 nm in soymilk and soymilk hydrolysates and substituting them into the equation of standard graph (Figure 4). The computed Phe content is presented in Table 1. From these results, the Phe reduction rate in soymilk was calculated as 87.44%.

Lopes *et al.* (2005) treated skimmed milk powder with the protease of *Aspergillus oryzae* and papain, followed by Phe removal using activated carbon, to prepare dietary supplements for PKU patients. They achieved a Phe removal rate of 96%–99%, and Phe concentration in the final product was reported as 0.060×10^{-4} mg/100 mg protein. Carreira *et al.* (2008) aimed to produce wheat flour with low Phe content that could be consumed in PKU diet. They extracted proteins enzymatically, followed by hydrolysis using enzymes, and evaluated the effectiveness of Phe removal using activated carbon with second-derivative spectrometry. They achieved a Phe removal of 66.28% and reported Phe content in the final product as 5224.4 mg/kg. Capobiango *et al.* (2007) utilized *Bacillus licheniformis* protease for extraction, pankreatin for hydrolysis, and activated carbon as an adsorbent for Phe removal from corn using the second-derivative spectrometry method. They accomplished a Phe removal rate of 97.55%, with final Phe content as 2,408 mg/kg.

Conclusions

In this study, soymilk was produced from soybeans, and to facilitate its use for PKU patients, protein hydrolysis was performed using protease of *Aspergillus oryzae* and papain, followed by Phe removal with activated carbon. Phe concentration in soymilk was determined using the second-derivative spectrometry method, used for the first time for determining Phe in soymilk. These results would serve as a guide for the future laboratory studies.

As stated in the Introduction section, it is not mandatory to produce a complete Phe-free food for PKU patients, because the main treatment strategy is to regulate Phe concentration in the serum at certain levels. In

Phe-reduced soymilk, the Phe content was reduced by 87.44%, compared to the control, and the crude protein content was reduced by 29%. The data obtained from this study demonstrate that soymilk is suitable for Phe extraction and can be used as an alternative food for PKU patients. Imported products used for PKU patients in Turkey impose a significant financial burden on consumers. Soymilk can be produced domestically by conducting continuous research and adding new flavors, thus providing a new option for PKU patients, and creating a new market for the industry. In order to improve the quality of the final product, further optimization studies are conducted on soymilk by utilizing different proteases and with different processing conditions for production.

Conflict of Interest

The authors declared no conflict of interest.

Author Contributions

EG was responsible from conceptualization, project administration, visualization, draft review and editing. YT did the formal analysis, methodology, validation, visualization and prepared the original draft. All authors have read and agreed to the published version of the manuscript.

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