PRINCIPAL COMPONENT ANALYSIS (PCA)-COMBINED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FOR DIFFERENTIATION OF BOVINE AND PORCINE GELATIN IN VITAMIN C GUMMIES

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PRINCIPAL COMPONENT ANALYSIS (PCA)-COMBINED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FOR DIFFERENTIATION OF BOVINE AND PORCINE GELATIN IN VITAMIN C GUMMIES

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ABSTRACT

Gelatin is a polymer derived from skin and bone of vertebrate, bovine and porcine. Gelatin is used as a gelling agent in the pharmaceutical and food products including in the preparation of vitamin C gummies. Porcine gelatin is forbidden to consume by Muslim and Jewish. The aim of this study was to differentiate and classify of bovine and porcine gelatin in vitamin C gummies using a combination method of High-Performance Liquid Chromatography (HPLC) and Principal Component Analysis (PCA). The analysis procedure involved complete hydrolysis of samples by 6N hydrochloride acid in order to release their amino acid residues. To get an amino acid composition of gelatin, all of the samples were performed by reversed-phase (RP) HPLC. The peak height of HPLC chromatogram was analyzed using PCA to classify both bovine and porcine gelatin. The Results of PCA showed a clear distinction between bovine and porcine gelatin in vitamin C gummies. Hence, this method could be successfully used for differentiation of bovine and porcine gelatin in vitamin C gummies.

Keywords: bovine gelatin, porcine gelatin, PCA-combined HPLC, Vitamin C gummies

INTRODUCTION

Gelatin is a soluble protein compound from land mammalian collagen obtained through the partial hydrolysis reaction. It is a popular biopolymer applied in the food, pharmaceutical, cosmetics, medic and photography industry. Due to the wide applications, the global demand for gelatin has been increasing. Recent reports relieved that the world gelatin production reac 326,000 tonnes per year and the highest source of gelatin is pig skin (44%), followed by bovine skin (28%), bovine bone 27% and other sources 1%. [1]

Bovine and porcine gelatin is more desirable because the produced gelatin provides better quality, although both these sources have disadvantages. Muslim (25% of the world population) and Judaism community ban to use porcine gelatin for consumption, cosmetics, and pharmaceutical product. Bovine gelatin is permitted by Muslims, but it is more expensive than porcine gelatin. Additionally, bovine gelatin is prohibited for the followers of the Hindus. [2,3]

Gelatin has unique properties so that the use of gelatin is preferred in the pharmaceutical, food and cosmetic industries. The pharmaceutical industries use gelatin in tablets (as a binder), in hard or soft gelatin capsules, in implantable delivery systems (as a biodegradable matrix material), and in the microencapsulation of drugs. The food industries have been using gelatin in innumerable products like jellies, desserts, aspics, milk products, namely, yogurt, ice cream, desserts and sweets such as marshmallows. [4]

In recent years, there is a new innovation of pharmaceutical product that uses gelatin called vitamin C gummies. Vitamin C gummies are soft candies containing vitamin C used gelling agent and flavor (strawberry, pineapple, and lemon) to make them elastic and yummy in the mouth. [5,6] Commonly, gelatin is used as a gelling agent. Therefore, differentiation of bovine and porcine gelatin in vitamin C gummies is needed.

Several methods for differentiation of gelatin in pure material or in foodstuff have developed, namely fourier transform infrared spectroscopy,[7] chemical precipitation, [8] fluorescence-combined HPLC, [9] mass-spectrometer, [10] enzyme-linked immuno-sorbent assay, [11] and DNA-based technique using polymerase chain reaction. [12] Based on the literature review, there is no available report regarding the differentiation between porcine and bovine gelatin in vitamin C gummies.

In this study, we developed high-performance liquid chromatography using a PDA detector combined with PCA analysis for differentiating bovine and porcine gelatin in vitamin C gummies. PCA is a statistical procedure that is one of the menus in Chemometrics software. The main idea of PCA is to simplify the complex data. [13] In our hypothesis, PCA-combined HPLC could be used to distinguish between bovine and porcine gelatin in vitamin C gummies.

MATERIALS AND METHODS

Materials

The materials used in this research were standard porcine and bovine gelatin (pro analysis grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tartrazine (as coloring), citric acid, sucrose, glucose syrup, vitamin C, gelatin (pharmaceutical grade) and distilled water were purchased from a local pharmacy distributor. Commercial vitamin C gummies were purchased from Ciputat Market, South Tangerang. The chemical for hydrolysis and HPLC analysis were hydrochloride acid (Merck), sodium hydroxide (Merck), alpha-aminobutyric acid (waters), AccQ Fluor borate buffer (Waters), AccQ Fluor reagent contain 6-aminoquinolyl-N-hydroxysuccinimide carbamate (Waters), standard solutions of amino acids (waters), AccQTag Eluant A (waters) and acetonitrile (Merck).

Instrumentation

The amino acid composition was measured by HPLC from Waters.

Methods

Preparation of vitamin C gummies (experimental vitamin C gummies)

3.5 g Bovine or porcine gelatin (pharmaceutical grade) was dissolved in 70 ml of distilled water and the solution was heated at 70 oC. The gelatin solution was added with 0.5 g citric acid and 0.5 g vitamin C. In the other erlenmeyer, 0.05 g tartrazine, 17.5 g sucrose and 17.5 glucose syrup were dissolved in distilled water and the mixture was also heated to 70 °C. The mixture was poured into gelatin solution and heated at 80 °C. The mixture was poured into a case and dried until gummies were formed. The gummies then were placed in the refrigerator and ready to use. [14]

Hydrolysis for sample preparation

100 mg of sample (standard porcine and bovine gelatin from Sigma, experimental vitamin C gummies, and commercial vitamin C gummies) was carefully weighed and added with 5 mL HCl 6 N. The solution was placed into an autoclave at 110 °C for 22 hours. After being autoclaved, the sample was neutralized with NaOH or HCl solution. The Sample was transferred to the 50-mL volumetric flask, then diluted with bidistilled water until 50 mL and shaken smoothly. Hydrolyzed gelatin was moved to a volumetric flask and adjusted to 50 ml with distilled water. The solution was filtered through a 0.45 µm membrane filter. The filtrate (500 µL) was added with 40 µL alpha-aminobutyric acid as an internal standard in 0.1M HCl and 460 µL distilled water.

Derivatization procedure for amino acid analysis

The filtrate (10 µL) was added with 70 µL AccQ Fluor borate buffer and mixed using a vortex for 5 minutes. The solution was added with 20 µL of AccQ Fluor reagent (Waters) and then mixed by vortexing for several seconds. It was then heated on a heating block at 55 °C for 10 min, then cooled to room temperature. Derivatives were stable at room temperature for up to 1 week.

Chromatographic conditions

The sample was injected into High-Performance Liquid Chromatography (Waters), flow rate 1 mL/min, eluent consisted of AccQTag Eluant A (waters) and 60% acetonitrile (HPLC grade) with gradient system temperature 37 °C, column Waters AccQ Tag 3.9x150 mm. Fluorescence detection was carried out by λ irradiation at 250 nm excitation and 395 nm emission wavelength. The measurement was performed in duplo for all of the samples.

Statistical analysis

Furthermore, the Principal Component Analysis (PCA) for differentiation and classification of samples was carried out using the Minitab software version 15 (Pennsylvania, USA).

Table 1: Amino acid composition of porcine and bovine gelatin from Sigma, experimental gummy vitamin C and commercial gummy vitamin C

Amino acid	Amino acid residues in % b/b (ppm)					
	BG	PG	EG Vit C contain		CG Vit C	
			BG	PG	1	2
L-Aspartic acid	5.116 ± 0.065	5.173 ± 0.086	0.493 ± 0.015	0.402 ± 0.053	0.253 ± 0.027	0.372 ± 0.011
L-Serine	3.539 ± 0.074	3.309 ± 0.009	0.374 ± 0.004	0.299 ± 0.004	0.203 ± 0.002	0.264 ± 0.000
L-Glutamic acid	10.510 ± 0.086	10.550 ± 0.010	0.996 ± 0.001	0.809 ± 0.014	0.573 ± 0.003	0.755 ± 0.005
Glycine	26.600 ± 0.077	25.860 ± 0.106	2.478 ± 0.003	1.990 ± 0.034	1.388 ± 0.014	1.940 ± 0.005
L-Histidine	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
L-Arginine	8.228 ± 0.085	8.608 ± 0.309	0.715 ± 0.001	0.599 ± 0.013	0.358 ± 0.000	0.410 ± 0.012
L-Threonine	2.035 ± 0.098	2.233 ± 0.080	0.232 ± 0.008	0.171 ± 0.001	0.109 ± 0.002	0.150 ± 0.002
L-Alanine	8.106 ± 0.057	8.260 ± 0.092	0.789 ± 0.016	0.687 ± 0.012	0.429 ± 0.005	0.654 ± 0.014
L-Proline	12.610 ± 0.054	12.930 ± 0.650	1.202 ± 0.027	1.032 ± 0.016	0.644 ± 0.005	0.937 ± 0.011
L-Cystine	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
L-Tyrosine	0.324 ± 0.002	0.489 ± 0.074	0.032 ± 0.001	0.024 ± 0.000	0.013 ± 0.001	0.006 ± 0.000
L-Valine	2.170 ± 0.011	2.447 ± 0.027	0.236 ± 0.007	0.199 ± 0.002	0.119 ± 0.002	0.180 ± 0.002
L-Metheonine	1.248 ± 0.106	1.117 ± 0.024	0.084 ± 0.005	0.043 ± 0.005	0.049 ± 0.001	0.061 ± 0.002
L-Lysin HCl	4.093 ± 0.047	4.607 ± 0.009	0.456 ± 0.000	0.386 ± 0.000	0.188 ± 0.001	0.243 ± 0.003
L-Isoleucine	1.434 ± 0.026	1.183 ± 0.002	0.182 ± 0.005	0.105 ± 0.000	0.089 ± 0.000	0.108 ± 0.002
L-Leucine	2.654 ± 0.061	2.695 ± 0.028	0.306 ± 0.004	0.219 ± 0.003	0.144 ± 0.001	0.208 ± 0.001
L-Phenylalanine	1.894 ± 0.044	2.055 ± 0.035	0.179 ± 0.002	0.152 ± 0.002	0.106 ± 0.002	0.130 ± 0.001
Triptophan	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

BG: bovine gelatin, PG: porcine gelatin, EG: experiment gummy, CG: commercial gummy

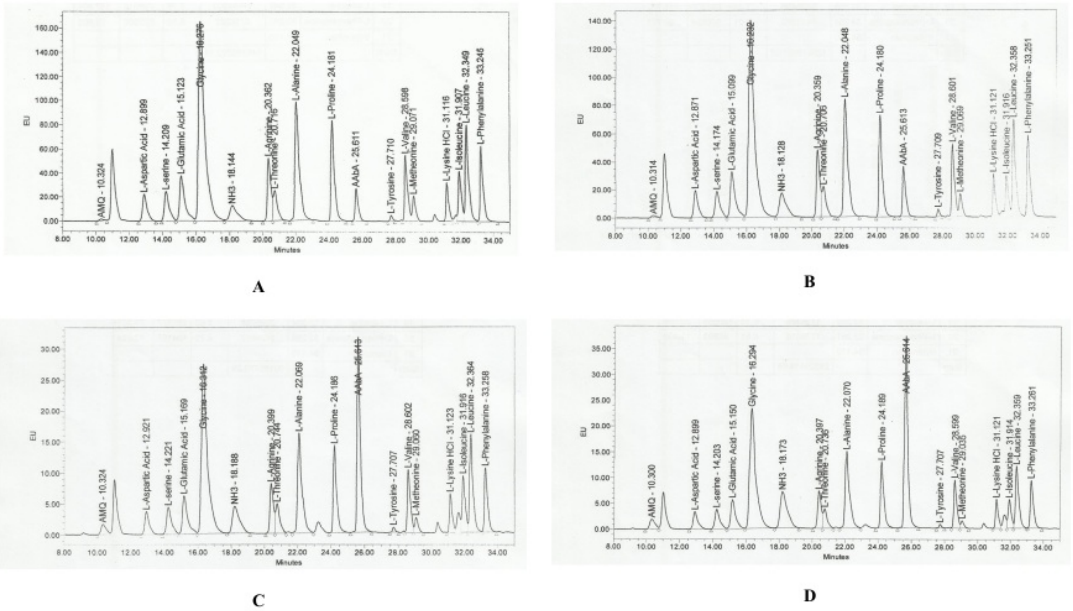

Figure 1: Chromatogram of amino acid extracted from gelatin; A: bovine gelatin, B: porcine gelatin, C: bovine gelatin from experimental vitamin C gummies, and D: porcine gelatin from experimental vitamin C gummies

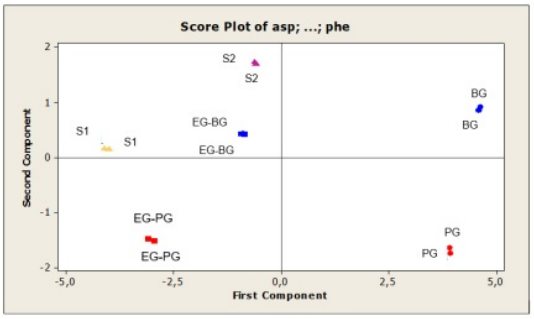

Figure 2: The score plot for the first two principal components (PC) for bovine gelatin (BG), porcine gelatin (PG), experiment gummy contain bovine gelatin (EG-BG), experiment gummy contain porcine gelatin (EG-PG) and commercial gummy as sample (S1 and S1)

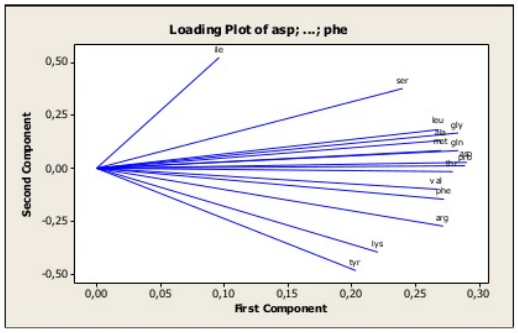

Figure 3: Principal component analysis (PCA) loading plot of PC1 versus PC2

RESULTS AND DISCUSSION

Amino acid composition of gelatin in vitamin C gummies

Bovine and porcine gelatin could be distinguished by amino acid composition. To get amino acid composition, all samples (standard bovine and porcine gelatin, experimental vitamin C gummies and commercial vitamin C gummies) should be hydrolyzed by using hydrochloric acid. This hydrolysis method is commonly used for a limited sample. The amino acid composition of the samples was quantified with pre-column derivatization HPLC. Amino acid could be reacted with 6-aminoquinolyl-N-hydroxysuccinimide carbamate to produce derivate of an amino acid having the fluorescence properties, with excitation and emission wavelengths of 250 and 395 nm, respectively. The amino acid derivatization using 6-aminoquinolyl-N-hydroxysuccinimide carbamate is highly sensitive and selective for HPLC analysis. [15] Figure 1 showed chromatograms of the amino acid obtained from gelatin. Chromatogram revealed all of the amino acid could be separated clearly. However, gelatin from different sources could be very similar in their amino acid sequences.

Base on the chromatogram, the amino acid composition of gelatin (% b/b) and peak height could be counted accurately, as shown in Table 1. Standard bovine and porcine gelatin have glycine as their major amino acid (26.600% and 25.864%, respectively) and relatively high of glutamic acid (10.515% and 10.547%, respectively). However, the next major compounds of bovine gelatin are arginine (8.226%), alanine (8.217%) and proline (7.319%), furthermore, the next major compound of porcine gelatin are proline (12.934%), arginine (8.608%) and alanine (8.260%). The different composition of amino acid was caused by different species. Karim and Bhat (2008) stated that glycine, alanine, and proline are the most three abundant amino acids in gelatin. In addition, there is no cysteine and tryptophan in bovine

and porcine gelatin. Cysteine (an amino acid in hair) has not been detected in this research, indicating unhairing process in raw material (skin) was running well. [16] Tryptophan has not been detected too. The preparation of amino acid monomers from gelatin through the acid hydrolysis process led to the destruction of the indole ring of tryptophan. Cysteine and tryptophan are not commonly in collagen and gelatin. [17]

The amino acid composition of gelatin in experimental vitamin C gummies and commercial samples were lower than that of the standard gelatin. The lower amino acid in samples was caused by the formulation process. However, the pattern of the amino acid composition of gelatin in experimental and commercial gummies was as same as the pattern of the amino acid composition of standard gelatin. The main amino acid composition of experimental gummies was glycine, proline, glutamate acid dan alanine. The experimental gummies made from bovine gelatin have higher aspartate, serine, glutamate, glycine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, lysine, isoleucine, leucine, phenylalanine than experimental gummy made from porcine gelatin. The amino acid composition of experimental vitamin C gummies made from bovine and porcine gelatin was different too. The amino acid composition of commercial vitamin C gummies has glycine, proline, and glutamic acid as a major compound, followed by alanine, aspartic acid, and serine.

Although amino acid could be separated well, and the amino acid composition could be counted, the source of gelatin in all samples could not be distinguished. Therefore, the results obtained will be further analyzed using chemometrics, as especially PCA to differentiate the sources of gelatin.

In this study, the variables used in PCA were peak height of amino acids in the chromatogram. According to Nemati et al (2004), the peak height of amino acids could distinguish both bovine and porcine gelatin. The peak height was chosen because it is proportional to the amino acid concentration. [9] The peak height used in this study was 15 peak height (excluding histidine, cystein, and tryptophan). PCA will recognize the peak height and clasify the samples based on their similarities and interpret the relationship between the variables.

PCA was able to detect the patterns, grouping, differences, and similarities of the input data. The result of PCA analysis was presented in the score plot (Figure 2) and loading plot (Figure 3). Score plot shows the location of bovine and porcine gelatin, experimental vitamin C gummies, and commercial vitamin C gummies. Score plot manipulates the multivariate data that could be displayed on an x,y coordinate system. X,y coordinate system is divided into 4 quadrant. There is principal component 1 (PC1) in the x-axis explaining the most variation among samples, and principal component 2 (PC2) in y-ordinate explaining the second largest amount of variation. [8,13]

Figure 2 shows bovine gelatin from Sigma found in I quadrant, while porcine gelatin from Sigma is in the IV quadrant. Bovine and porcine gelatin have a different quadrant. Bovine gelatin from experimental vitamin C gummies is in II quadrant and porcine gelatin from experimental vitamin C gummies is in III quadrant. Based on the score plot, gelatin from porcine and bovine are in different quadrants and they could be classified as their sources. The commercial gummy 1 and 2 are in the same quadrant with the bovine gelatin from experimental gummy vitamin C. This score plot shows that they have similar chemical and physical properties to the experimental vitamin C gummies from bovine gelatin. Therefore, the source of gelatin in commercial vitamin C gummies could be predicted from bovine gelatin.

Figure 3 shows the loading plot interpreting the relationship of the variables. It is used to determine which amino acid variables contribute most to the formation of principal component values. From loading plot in figure 3, it is known that aspartate, proline, glutamate, and glycine contribute greatly to the formation of PC1 value because it has a horizontal distance far from the line x = 0, with the coefficient value of 0.290; 0.289; 0.283 and 0.283 respectively. While amino acids that have a large contribution to the formation of PC2 values are isoleucine, tyrosine, lysine and serine with a coefficient value of 0.522; 0.480; 0.396 and 0.375 respectively.

CONCLUSION

It can be concluded that PCA-combined HPLC could classify experimental vitamin C gummies made from bovine and porcine gelatin. Based on PCA analysis, it could be stated that commercial vitamin C gummy bought in Ciputat Market contain bovine gelatin.

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