International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 15, Special Issue 1, 2023

Original Article

AUTHENTICATION OF *RATTUS NORVEGICUS* FAT AND OTHER ANIMAL FATS USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) AND PRINCIPAL COMPONENT ANALYSIS (PCA)

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Received: 16 Nov 2022, Revised and Accepted: 05 Jan 2023

ABSTRACT

Objective: The objective of this study was to analyze fatty acids using Gas Chromatography-Mass Spectrometry (GC-MS) in combination with chemometric Principal Component Analysis (PCA) for the authentication of *Rattus norvegicus* fat from other animal fats.

Methods: Extraction of fat from raw meat of *Rattus norvegicus*, beef, chicken, pork, and dogs using the Bligh Dyer method, then derivatized with 0.2 N NaOCH3, precipitation of sodium glycerol was carried out by adding saturated NaCl to obtain methyl esters which were then injected into the GC-MS instrument. The GC-MS data were then processed using chemometric Principal Component Analysis (PCA) to group *Rattus norvegicus* fat with other animal fats (beef, chicken, pork, and dog).

Results: The results of the study revealed that fatty acids in *Rattus norvegicus* using GC-MS produced eleven types of fatty acids, namely: Lauric acid (1,1%), Myristic acid (1,15%), Palmitic acid (21,12%), Palmitoleic acid (2,06%), Stearic acid (8,23%), Vaccenic acid (2,43%), Oleic acid (26,51%), Linoleic acid (19,19%), Arachidic acid (0,09%), and Eucosatrienoic acid (0,39%). Chemometrics Principal Component Analysis (PCA) of *Rattus norvegicus* fat allows it to be classified with other animal fats.

Conclusion: The Gas Chromatography-Mass Spectrometry (GC-MS) method, in combination with chemometric Principal Component Analysis (PCA), offered effective tools for the authentication of fatty acid of *Rattus norvegicus*.

Keywords: Halal authentication, *Rattus norvegicus*, PCA, GC-MS

DOI: https://dx.doi.org/10.22159/ijap.2023.v15s1.47505 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Foods made from meat are very popular among Indonesian, but some producers with bad behavior have harmed consumers a lot by adulterating halal meat with non-halal meat [1, 2]. Bad behavior carried out by producers is not comforting, especially for countries with a majority Muslim population. to the community, especially Muslims [3]. The adulteration of meat by replacing or mixing halal meat with non-halal meat can cause various problems such as (1) religious and belief problems due to the substitution of halal meat, such as beef with non-halal meat such as rat meat, dog meat, wild boar meat and pork. [4-6], (2) health-related problems [3] and (3) allergenic reactions [7]. The nutrition and safety of meat are directly related to people's health and quality of life [8]. Authentication methods can be classified according to the areas where adulteration is most likely to occur, like meat processing treatment, meat substitution, meat origin, and non-meat ingredient addition. Accurate labeling is essential to inform consumers about the meat they eat [9].

Solid fat has a high melting point, and its fatty acid content is high in saturated fatty acids, while liquid fat has a high content of unsaturated fatty acids. The cause of the difference is due to the order of fatty acids, degree of saturation, and the constituent components of the fatty acids [10]. Several quantitative methods for analyzing rat meat have been developed. Fourier Transform Infrared Spectroscopy is the most commonly used technique. [11], Real-Time Polymerase Chain Reaction [12], multiplex PCR [13], and ELISA [14]. Another method that can be developed in analyzing adulteration containing animal meat, especially non-halal meat (rat meat), is to look at the fatty acid composition contained in it. This method works by converting the fatty acids into their ester derivatives which can then be analyzed using Gas Chromatography-Mass Spectrophotometry (GCMS) [15].

Fatty acids are natural components found in meats [16]. The animal fats, including rat (*Rattus norvegicus*), were mainly composed of free fatty acid, triacylglycerols (TAG), diacylglycerols (DAGs), and other minor components. As a result, the purpose of this study was to distinguish between rat (Rattus norvegicus) and other animal fats [17]. Analysis using Gas Chromatography-Mass Spectrophotometry (GC-MS) is an accurate and fast method for analyzing the fatty acid composition of meat or meat adulteration because it can analyze mixtures in small quantities and separate complex mixtures. However, the analysis using Gas Chromatography-Mass Spectrometry (GC-MS) to compare rat fat with fat from other animal meats using Bligh Dyer method extraction combined with Principal Component Analysis (PCA) has not been reported yet. Therefore, this research aims to know the types of fatty acids and classify fats from animal species using Gas Chromatography-Mass Spectrometry (GC-MS) combined with chemometrics principal Component Analysis (PCA).

MATERIALS AND METHODS

Materials

Samples of animal meats consisting of rat (*Rattus norvegicus*), beef (*Bos taurus*), chicken (*Gallus gallus*), pork (*Sus scrofa domesticus*), and dog meat (*Canis lupus familiaris*) were taken from Yogyakarta. HCl (Merck), dichloromethane (Merck), methanol (Merck), distilled water, *n*-hexane (Merck), 0.2 N $NaOCH_3$ solution, anhydrous Na_2SO_4 and saturated NaCl, Whatman filter paper, electric stove, analytical scale, vortex, oven, centrifuge, vacuum rotary evaporator, separatory funnel, and glassware. The reagents and solvents used were of proanalytical grade.

Sample preparation

The sample of rat meat (*Rattus norvegicus*) and other animal meat (beef, chicken, pork, and dog) was chopped using a commercial chopper before acid hydrolysis.

Acid hydrolysis

Twenty grams of rat meat and other animal meat (beef, chicken, pork, and dog) were hydrolyzed using 1 N Hydrochloric Acid. Then the sample was filtered using Whatman filter paper. Then samples were extracted using the Bligh-Dyer method [18, 19].

Fat extraction

Extraction using the Bligh-Dyer method was carried out with slight modifications. the steps in the extraction procedure follow the extraction method in the research that has been done by the previous author [3].

Derivatization

Each of the samples was 50 µl of fat from rat meat (*Rattus norvegicus*), and other animal fats (beef, chicken, pork, and dog) added to 1.0 ml of n-hexane and 200 L of 0.2 N $NaOCH_3$ solution, added in a water bath at a temperature of 90-100 °C for 10 min while shaking. A solution of 0.2 N $NaOCH_3$ was obtained by mixing 800 mg of solid NaOH in 100 ml of methanol, then enlarged in an air bath at a temperature of 90-100 °C for 10 min. Wait for the mixture to cool and add 1.5 ml of saturated NaCl to precipitate sodium glycerol, then vortex for 10 min. The supernatant containing fatty acid derivative methyl ester (FAME) was taken and injected into the Gas Chromatography-Mass Spectrometry (GC-MS) system [20].

Gas chromatography-mass spectrometry (GC-MS)

One µl of derivatized fatty acids were injected into the GC system with an autosampler (GC-MS Shimadzu Japan type GC-MS QP 2010). The separation was carried out in an SP™ 2560, 100m x 0.25 mm, 0.2 m column, with a stationary phase of diphenyl dimethyl polysiloxane type, injector temperature of 230 °C, column temperature of 70 °C, and increased to 300 °C in 10 °C increments per minute; the mobile phase flow rate is 1.15 ml per minute. The carrier gas is helium [21]. The detector for Mass spectrometry is a 70 MeV Electron Multifier Detector (EMD), and the analysis result was a mass spectrum compared to the WILLEY147 library contained in the GC-MS software.

Data analysis

Analysis using Chromatography-Mass Spectrometry (GC-MS). The methyl ester content in each sample (rat, beef, chicken, pork, and dog fat) was analyzed using PCA chemometric software using SIMCA software.

RESULTS AND DISCUSSION

Fat extraction

The Bligh-Dyer method for extracting and separating lipids from biological tissues using chloroform, methanol, and water has been used thousands of times. It is the "gold standard" for the analysis of extracted lipids [22]. In this study, extraction using the Bligh Dyer method was done with slight modifications, replacing chloroform with dichloromethane. Extraction of fat using the Bligh Dyer method because this method is simple, rapid, and efficient for extracting and purifying lipids from biological materials. Many foodstuffs used the Bligh-Dyer method to extract total lipids [23]. Before extraction using Bligh-Dyer method, the sample was hydrolyzed with 0,1 N HCL for improved extraction efficiency to release the bound lipids attached to protein and carbohydrate [3]. In the previous study, lipid extraction using an oven at 90 °C-100 °C for approximately an hour [24-27]. The results of each extraction were weighed to obtain the yield. Differences in yield results were made possible by differences in saturated and unsaturated TAG content. The different fat content can be caused by the extraction method, the part of the animal taken, the origin of the animal, and the food intake. The results of the yield of animal fat are obtained as shown in table 1.

Based on table 1, the fat content of rat extraction using the Bligh Dyer method is 2.27%, and beef fat is 2.33%, and the yield obtained in this study is relatively smaller than the fat content at another study reported, such as rat fat (Sprague Dawley rats) 36.77%, and beef fat 6.52% [24], the yield of rat fat (Rattus tanezumi) is 4.34% [25], and the yield rat fat (Wistar rat) 16.36% [26], from the extraction procedure using an oven at a temperature of 90-100 °C for 1-1.5 h. These differences are caused by different extraction methods, different sample sizes, and the possibility that the sample (part/adipose tissue) used does not contain too much fat [28].

Table 1: The yield of rat meat (*Rattus norvegicus*) and other animal meat (beef, chicken, pork, and dog)

Fatty acid	Sample weight (g)	Extraction yield (%)
Rat	20.0	2.27
Beef	20.0	2.33
Chicken	20.0	2.60
Pork	20.0	2.55
Dog	20.0	2.41

Table 2: The composition of methyl ester content in rat fat (*Rattus norvegicus*) and other animal fats (beef, chicken, pork, and dog)

Methyl ester	Percentage (%) of methyl ester				
	Rat	Beef	Chicken	Pork	Dog
Lauric acid (C12:0)	1.1	nd	0.05	nd	0.9
Myristic acid (C14:0)	1.15	0.95	0.62	0.92	2.31
Palmitic acid (C16:0)	21.12	18.29	18.98	20.95	20.63
Palmitoleic acid (C16:1)	2.06	2.39	4.38	1.4	2.75
Stearic acid (C18:0)	8.23	11.01	7.08	14.29	13.98
Vaccenic acid (C18:1n7)	2.43	2.88	nd	3.41	nd
Oleic acid (C18:1n9)	26.51	27.73	65.28	28.73	25.86
Linoleic acid (C18:2)	19.19	7.7	nd	18.23	14.69
Arachidic acid (C20:0)	0.09	0.84	0.1	0.17	0.21
Eucosatrienoic acid (C20:3)	0.39	nd	0.28	0.48	0.94

nd: not detected

Fatty acid analysis

Derivatization of fat is carried out because the fat produced from the extraction process is non-volatile; therefore, it is converted into the form of methyl esters to become volatile compounds [25]. Saturated and unsaturated fatty acids can turn into ester compounds through two processes: esterification and transesterification. The choice of solvent used is crucial because it can affect the content and quality of the fat obtained. One of the factors that can be used to select a suitable solvent for fat extraction is the degree or degree of polarity [28]. The lipid fraction is hydrolyzed to fatty acids first using NaOH in methanol. The derivatization was carried out using sodium

methoxide as a base catalyst. Fatty acid derivatization was carried out to obtain more volatile compounds. The addition of NaCl in the fatty acid derivatization process aims to precipitate glycerol from the breakdown of triglycerides. Sodium glycerolate is formed from the bond between sodium in the sodium chloride salt with glycerolate [29]. Fatty Acid Methyl Ester (FAME) has the lowest specific gravity, so the top phase as the supernatant is taken and injected into the GC-MS system. GC-MS analysis was carried out to determine the fatty acid composition. Fig. 1 shows the chromatogram results of fatty acids from rat (*Rattus norvegicus*).

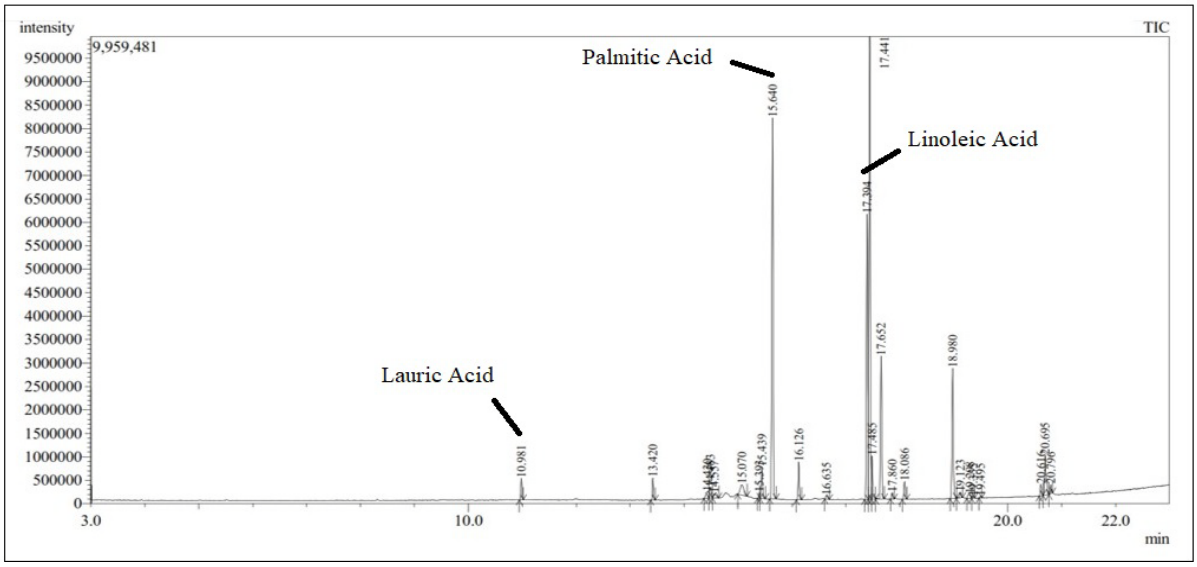

Fig. 1: Chromatogram of gas chromatography from rat fatty acid (*Rattus norvegicus*)

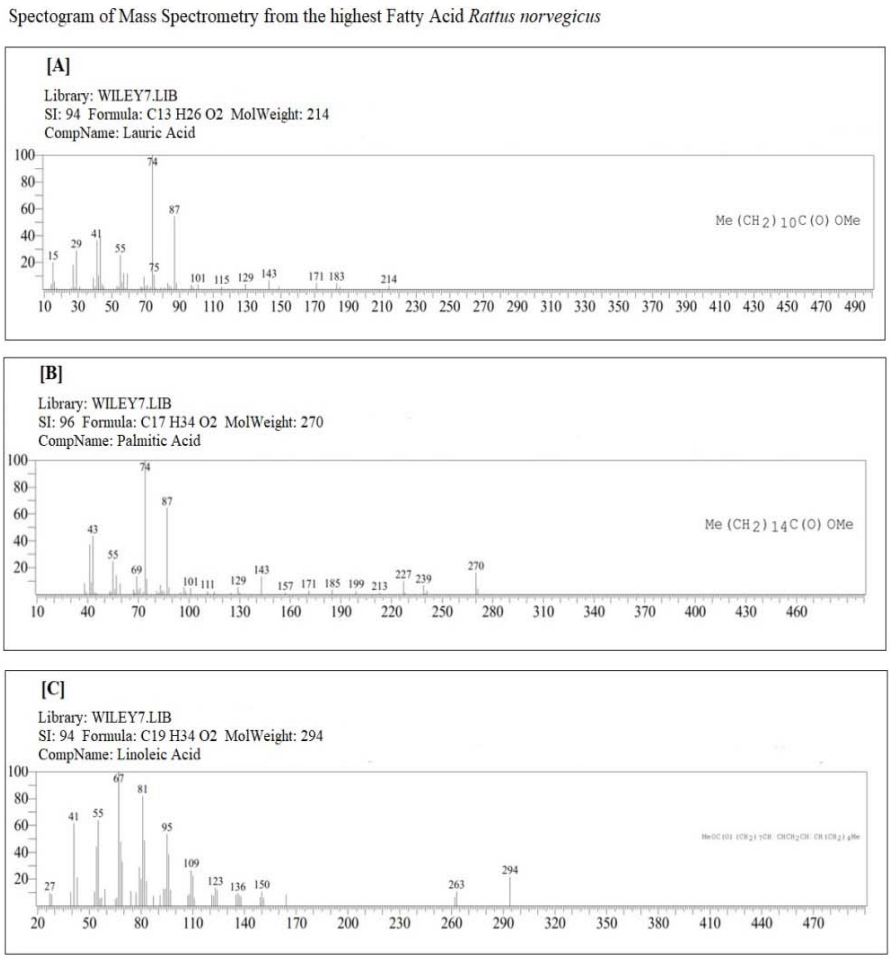

Fig. 2: Spectogram of mass spectrometry from rat fatty acid (*Rattus norvegicus*); [A] Lauric acid, [B] Palmitic acid, [C] Linoleic acid

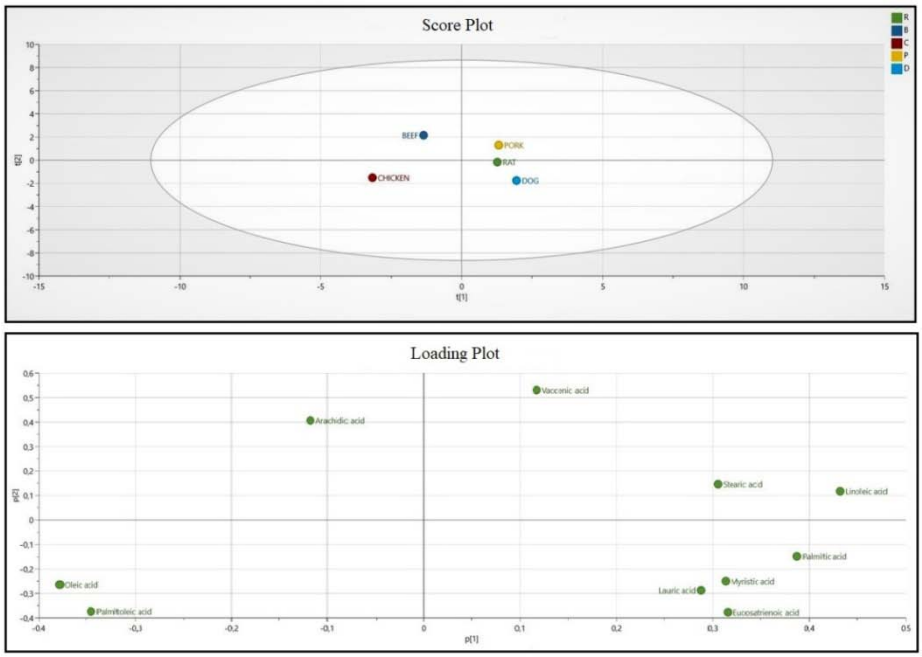

Fig. 3: PCA chemometric analysis of fatty acid profiles of rat fat (*Rattus norvegicus*) and other animal fats (beef, chicken, pork, and dog)

Comparison of fatty acids in the rat (*Rattus norvegicus*) and other animals (beef, chicken, pork, and dog)

Identification of the types of fatty acids in the rat (*Rattus norvegicus*) and other animals (beef, chicken, pork, and dog) fat was carried out by mass spectrometry based on the base peak and similarity index (SI) with the comparison of spectra from the library (WILLEY7) contained in the GC-MS software. If the SI value>90 is detected, it has a similar structure to the comparison data. The base peak is the peak with the greatest abundance in the mass spectrum and is assigned a value of 100%. The analysis results showed an SI value>90, indicating that the types of fatty acids in the sample matched or were similar to the comparison spectra. Fig. 2 shows the spectogram of Mass Spectrometry from rat fatty acid (*Rattus norvegicus*) with a similarity index value>90.

Derivatization results from extracted animal fats from rats (*Rattus norvegicus*), beef (*Bos taurus*), chicken (*Gallus gallus*), pork (*Sus scrofa domesticus*), and dog (*Canis lupus familiaris*). Their results were analyzed using GC-MS in methyl esters, show at table 2. The rat (*Rattus norvegicus*) has saturated and unsaturated fatty acids. Saturated fatty acids in the rat (*Rattus norvegicus*) are lauric acid, myristic acid, palmitic acid, stearic acid, and arachidic acid. While unsaturated fatty acids in the rat (*Rattus norvegicus*) are palmitoleic acid, vaccenic acid, oleic acid, linoleic acid, and eucosatrienoic acid. Therefore, if they are summed up and totaled, the high fatty acid in the rat (*Rattus norvegicus*) is an unsaturated fatty acid, and this is the same as a previous study about fatty acid *Sprague Dawley* rat [24]. Table 2 shows that the highest percentage of methyl esters in rats (*Rattus norvegicus*) is lauric acid 1.1% and palmitic acid 21.12% (saturated fatty acid), and also linoleic acid 19.19% (unsaturated fatty acid). In a previous study, the highest percentage of methyl ester in Linoleic acid in *Sprague Dawley* rats was 32.34% [24], linoleic acid in *Wistar rat* was 30.14% [26]. Then for palmitic acid, the highest methyl ester in *Rattus tanezumi*, with a percentage methyl ester of 27.65% [25]. The result is reported to show that fatty acids lauric acid and palmitic as characteristic of fatty acid of the rat. In this study *Rattus norvegicus* has the characteristic fatty acid are lauric acid, palmitic acid, and linoleic acid. Some factors that cause some fatty acids are not detected or become smaller or more content compared to some previous studies are differences in species, food, and animal habits [26].

Principal component analysis of rat (*Rattus norvegicus*) and other animals fat

Principal Component Analysis is an analytical method of interpreting data through data reduction. The number of variables in a matrix is reduced to produce new variables while retaining information from the data. The resulting new variable in the form of a score or main component [30]. PCA chemometrics aims to classify correlated variables and replace them through a new group called the principal component (PC). Although it reduces the number of initial variables, PCA maintains variability and initial information. The principal component analysis also helps provide pattern visualization and correlation analysis [31]. The PCA analysis used data on the types of fatty acids in various animals as variables. The matrix type is a correlation that connects the animal and fatty acids. PCA analysis can determine the similarities and differences in the composition and distribution of fatty acids in the fat of each animal. PCA results in the form of the main component (PC), representing the magnitude of the variation in the initial data, where PC1 contains the most significant variance. Objects with nearly the same PC value have similar Physico-chemical properties [31].

In the previous study, fat analysis of rats using Gas Chromatography-mass Spectrometry (GC-MS) combined with Principal Component Analysis (PCA) was conducted using Minitab 19 software [24-27]. For this study, the PCA analysis was conducted using SIMCA software. SIMCA's ability to determine whether a sample belongs to any predefined categories and if it does not belong to any class is a critical advantage. SIMCA class prediction produces: (i) the sample was divided into predefined categories, and (ii) the sample does not fit into any categories [32]. Fig. 2 shows the PCA scores plot generated by SIMCA. The results of the classification performance of the SIMCA model to distinguish between the rat (*Rattus norvegicus*) and other animals (beef, chicken, pork, and dog), a SIMCA model was constructed and optimized. The data in the same quadrant in the Score plot and Loading plot, especially if they are in a similar position, can be seen as a sample affected by the variable (fatty acids), the relationship between Score plot and Loading plot [33]. Principal Component Analysis (PCA) can classify fat rats (*Rattus norvegicus*) and other animal fats.

CONCLUSION

Fat extraction using the Bligh-Dyer method can be used for fat extraction in rats (*Rattus norvegicus*), beef (*Bos taurus*), chicken (*Gallus gallus*), pork (*Sus scrofa domesticus*), and dog (*Canis lupus familiaris*). Fatty acid analysis using GC-MS method combined with chemometrics. The highest fatty acids in rats (*Rattus norvegicus*) were lauric acid, palmitic acid, and linoleic acid. The total amount of unsaturated fatty acids in rats was 50.58%, which was higher than

that of saturated fatty acids, 31.69%. Chemometric Principal Component Analysis (PCA) can classify rats (*Rattus norvegicus*) and other animal fats.

ACKNOWLEDGMENT

Thank you to the pharmacy faculty at Universitas Andalas and the pharmacy faculty at Universitas Gadjah Mada.

AUTHORS CONTRIBUTIONS

All authors contributed equally to the research and agreed to the writing of the published manuscript.

CONFLICT OF INTERESTS

There is no conflict of interest in this research.

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