

HASIL CEK_ANY GUNTARTI, KARINA PRIMATYAS NINGRUM, IBNU GHOLIB GANDJAR, NINA SALAMAH

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Sprague Dawley Rats (rattus Norv*

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AUTHENTICATION OF *SPRAGUE DAWLEY* RATS (*RATTUS NORVEGICUS*) FAT WITH GC-MS
(GAS CHROMATOGRAPHY-MASS SPECTROMETRY) COMBINED WITH CHEMOMETRICS

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ABSTRACT

Objective: Misuse of rat meat as food worried people. Rat meat can come from research waste; one of the rats used in the research was the *Sprague Dawley*. Analysis of rat meat in food can be done using fat. The aim of this study was to authenticate rat fat with GC-MS combined with chemometrics.

Methods: The meat of *Sprague Dawley* rats, wild boar, goats, cow, and processed meatballs was put in the oven at 90-100 °C for one hour, then derivatized with BF₃ and NaOH in methanol to get the methyl ester for injected in GC-MS instrument. The results obtained were in the form of chromatograms and spectrograms. The data was processed using Principal Component Analysis (PCA) to grouping rat's meat with others (wild boars, goats, cows, and processed meatballs).

Results: Rat meat fatty acid analysis results with GC-MS were obtained oleate (43.32±1.43)%, linolenate (32.24±1.46)%, palmitate (19.75±0.09)%, palmitoleate (1.14±0.06)%, stearate (0.26±0.01)%, myristate (0.18±0.01)%, margarate (0.15±0.02)%, and pentadecanoate (0.14±0.01)%. The PCA chemometrics results showed that rat meats had scores that close to cows, which meant they had similar fatty acid composition.

Conclusion: The GC-MS method, combined with PCA chemometrics, tested rat fat with other animals and processed meatballs samples on the market.

Keyword: Chemometrics, Fatty acids, GC-MS, PCA, Rat, *Sprague dawley*

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INTRODUCTION

Food is one of the basic human needs. Lately, the safety of foods circulating in society is no longer guaranteed [1]. The production of meat-based food has a high susceptibility to counterfeiting, especially a mix of meat from wild animals [2]. Rat meat is classified as non-halal [3]. This has become attention to Indonesia; since the majority of the people are Muslim.

Based on the Foundation for Biomedical Research (FBR), 95% of all animals in the laboratory are rats [4]. White rats are often used as experimental animals because their genetic characteristics are unique. Also, they are easy to breed, cheap, and easy to be obtained. One of the rats' furrows used was the *Sprague Dawley* rat. They are often used for research on metabolism, diabetes, and nutrition. The 'abundant waste' from the research results can be misused as a substitute ingredient for other animals' meat; since they have similar characteristics. Food authentication analysis is an important issue to ensure that food quality is in accordance with its labeling [5]. Analytical methods to identify 'authenticity' can be based on detecting fat, protein, or DNA biomarker [6, 7]. Fat detection is a widely used method. The following are the examples of ways which have been successfully investigated by using Polymerase Chain Reaction (PCR) [8]; High Perform Liquid Chromatography (HPLC) [9], Gas Chromatography (GC) [10], Form Transform Infra-Red (FTIR) [11, 12], electronic nose [13], Liquid Chromatography-Mass Spectroscopy (LC-MS) [14], and Nuclear Magnetic Resonance (NMR) Spectroscopy [15].

Fat was chosen since it is a form of a sample that is easy to be obtained-only have to extract non-polar solvents [16]. Fat in animals is commonly found in adipose tissue [17]. The distribution of fat in meat can serve as a differentiator of meat from its animal (source). The composition of fat in meat is influenced by animal species, sex and types of food [18]. The grouping of fatty acid based on the types of animal can be done using PCA chemometrics analysis [12, 19]. Analysis using PCA shows that fat from cows, dogs, goats, pigs and chickens can be identified and distinguished [20].

The researches which have been done about "rats' fat in food preparations" were analyzed using FTIR combined with chemometrics [21]. However, this method has the disadvantage of not being able to identify the types and content of each fat component from a sample with certainty [22]. Therefore, GC-MS was used for the analysis; since it is able to find out the types of fatty acid from the sample components-combined with chemometrics PCA, which is able to classify fats from animal species.

MATERIALS AND METHODS

Tools and ingredients

Tools: Gas Chromatography-Mass Spectrometry instrument (GCMS Shimadzu Japan, type GCMS-QP2010 SE) with an auto-sampler machine, analytical scale, electric stove, oven, vortex, glassware.

Ingredients

The fat of *Sprague Dawley* rat was taken from Animal Test Rats (UD. Wistar), Yogyakarta, fat for wild boar from the forest in Central Java, fat for Goat from a goat satay seller in Yogyakarta, and fat for cows from an animal center in Yogyakarta. n-hexane, 0.2N NaOCH₃ solution (obtained from mixing solid NaOH in methanol), BF₃ solution, saturated NaCl, and anhydrous Na₂SO₄ [23].

Process of the research

Sample preparation

The fat of *Sprague Dawley* rats and other animals (boar, goat, and cow) were taken, washed, cut into small pieces, and stored in a freezer to keep the meat stable.

Fat extraction

Oil extraction is carried out by an extraction procedure using an oven at a temperature of 90-100 °C for 1-1.5 h. The filtered fat was then mixed with anhydrous Na₂SO₄, which was then centrifuged at 3000 rpm for 20 min. The oil layer was decanted, filtered with

Whatman paper, which was covered with anhydrous Na₂SO₄. The solution is stored in a refrigerator at <4 °C in an Eppendorf tube [24].

Derivatization

200 µl of oil/fat (from *Sprague Dawley* rats, chickens, goats, and cows) were added with 1.0 ml of n-hexane, and 200 ml of NaOH solution in methanol was heated for 10 min while shaken out. The mixture was then added with a BF₃ solution of 1.5 ml and heated for 10 min. After that, it was added with saturated NaCl of 1.5 ml and vortexed for 10 min. Supernatants that contained fatty acid methyl ester (FAME) derivatives were taken and injected into the gas chromatograph system [24].

Instrumentation system

The separation was conducted in a research DBI-MS column, 30 MX 0.25 mm ID, 0.25 µm; with a stationary phase of Polymethyl Ciloxan, injector temperature of 230 °C, column temperature of 70 °C (which then increased to 300 °C) with an increase of 10 °C/min and water rate of 1.15 ml/min. The mobile phase used was Helium gas. The MS

detector used was a 70 MeV Electron Multiplier Detector (EMD). The result of the analysis was in the form of a mass spectrum compared to the WILEY147 and NIST47 library-contained in the GCMS software.

Data analysis

The chromatogram data of fatty acid methyl ester content from each *Sprague Dawley* rats, boars, goats, and cows were grouped using chemometrics PCA with Minitab 19 [25].

RESULTS AND DISCUSSION

Fat extraction

The fat extraction was carried out using the dry rendering method, which was by using an oven at a temperature of 100 °C for 60-90 min. That method was chosen because it can prevent the loss of volatile oil components, have a simple implementation, and cheap since it did not use additional chemicals and yields a lot [19]. The yields are presented in table 1.

Table 1: The yield of fat extraction

Type of fat	Fat weight (gram)	Oil weight (gram)	Oil color	Oil physical form (Room temperature)	Yield %
<i>Sprague Dawley</i> rats	25.67	9.44	Clear White	Liquid	36.77
Boars	50.96	6.99	Clear yellow	Liquid	13.70
Goats	50.39	9.56	White	Solid	18.97
Cows	50.18	3.27	White	Solid	6.52

The yield percentage difference can be caused by the presence of both saturated and unsaturated triacylglycerides and non-triacylglyceride components found in each animal fat. The components of each oil can be affected by the extraction process, the animal parts/organs taken, animal species, as well as animal intake.

Fat derivatization

The extracted oil or fat cannot evaporate; therefore, the derivatization into the form of methyl ester should be done in order to become a volatile compound [23]. A volatile compound is a requirement of separating the mixture by gas chromatography. Derivatization of fatty acid can be done both through acid and base [24].

The derivatization was carried out using BF₃ in methanol as an acid catalyst. BF₃ was chosen because it has the advantage of

being able to methylate fatty acid quickly. Also, when combined with the saponification reaction, which in this case is the addition of NaOH in methanol, BF₃ can methylate fatty acid even quicker. NaOH was chosen because it can separate the phases properly [24].

In this research, it was added with NaCl, which functions as a precipitate of glycerolate from the breakdown of triglycerides into fatty acid. Sodium in NaCl salt will bind with glycerol and become sodium glycerolate [25]. The supernatant taken is the upper phase since Fatty Acid Methyl Ester (FAME) has the lowest specific gravity compared to other phases. The middle phase is a phase that contains methanol and water mixed with glycerol, while the lower phase is sodium glycerolate sediments. This Fatty Acid Methyl Ester (FAME) phase is injected into the GC-MS system (fig. 1).

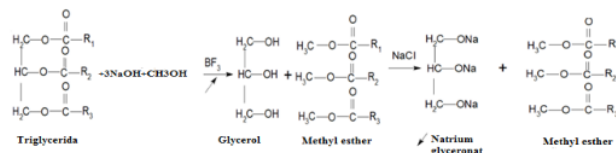


Fig. 1: FAME composing reaction

Profile of the *Sprague Dawley* rats' fatty acid

The analysis of the profile of *Sprague Dawley* rats' fatty acid was done using the methyl ester form. The parameters used are Time retention (Tr) and Similarity Index (SI); to determine the closeness of the

chemical structure of fatty acid types-which were compared between standard targets and fatty acid in spectra from the WILEY147 and NIST147 library found in the GC-MS software. If the SI result>90, it shows similarity to the target spectra [19]. Table II shows the *Sprague Dawley* rats' fatty acid in the form of methyl esters.

Table 2: Analysis of separation and identification of *Sprague Dawley* rat fat with GC-MS

No	Tr (min)	x±SD, (N=3)	CV	SI	MW	Component
1	19.23	0.18±0.01	8.18	95	242	Methyl Miristate (14:0)
2	19.87	0.14±0.01	7.14	95	256	6-Methyl Pentadecanoate (15:0)
3	20.59	1.14±0.06	5.80	95	268	Methyl Palmitoleate (16:1)
4	20.85	19.75±0.09	0.48	97	270	Methyl Palmitate (16:0)
5	24.21	0.15±0.015	9.96	95	284	Methyl Margarate (17:0)
6	25.63	32.24±1.46	4.54	91	294	Methyl Linoleate (18:2)
7	25.90	43.32±1.43	3.30	95	296	Methyl Oleate (18:1)
8	26.58	0.26±0.01	5.43	95	298	Methyl Stearate (18:0)

Tr (Time retensi); x= % area; SD (standard deviation); N= replication, CV= Coefficient of variation, SI (Similarity Index); MW (Molecular Weight)

The result of the GC-MS analysis is in table 2. Oleate is the highest constituent component of fatty acid in *Sprague Dawley* (SD) rats (43.32%), followed by linoleate (32.24%), palmitate (19.75%), palmitoleate (1.14%), stearate (0.26 %), myristate (0.18%), margarate (0.15%) and pentadecanoate (0.14%). Guntarti's research [26] stated that fatty acid compounds that appear with the

highest peak in 'house rats' are trans-9-octadecenoic or elaidic acid. Meanwhile, it stated that Wistar rats contain six types of methyl esters, the highest being oleate (40.48%), followed by linoleate (30.14%). Wistar and *Sprague Dawley* rats are both often used as experimental animals in research. Fig. 2 shows the comparison of the content of methyl esters in Wistar and *Sprague Dawley* rats.

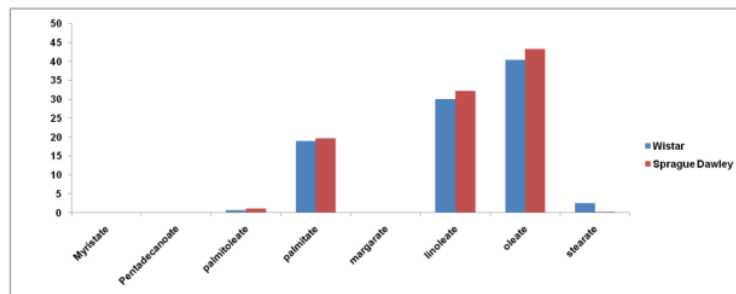


Fig. 2: Comparison of methyl ester of wistar and *Sprague Dawley* rat

Based on fig. 2, Wistar and *Sprague Dawley* rats have the same main content of fatty acids-namely palmitate, linoleate, and oleate. Palmitate is a saturated fatty acid, while linoleate and oleate are unsaturated fatty acids. Therefore, if they are summed up and totaled, the high fatty acid is an unsaturated fatty acid. The difference in fatty acid composition in each rat can be caused by the differences in species used or the influence of the animals' habitat and food.

The amount of unsaturated fatty acid content can affect the physical form in room temperature and fat stability: the higher the unsaturated fatty acid content, the lower the melting point will be [27]. It can be seen that *Sprague Dawley* rats have dominant unsaturated fatty acids-which means their melting point is lower. The result of the *Sprague Dawley* rats extraction showed that the physical form of the oil was liquid at room temperature.

Comparison of *Sprague Dawley* rats, boars, goats, and cows fatty acid

The process of taking fat and derivatization on boars, goats, and cows is similar to SD rats. Table 3 shows the result of methyl ester's acquisition of SD rats and other animals.

In table III, it can be seen that one of the characteristics of SD rats' fatty acid is that; they have Linoleate fatty acid (18: 2), which other animals do not have. The result of the analysis of fat profiles in boars, goats, and cows is a shift in the fatty acid content compared to previous research [26]. Guntarti's research [20] shows that boars' fatty acid has higher unsaturated fatty acids, while cows' fatty acid has higher saturated fatty acids. The differences in fatty acid constituents can be caused by the differences in animals' origin, food, or species (fig. 3).

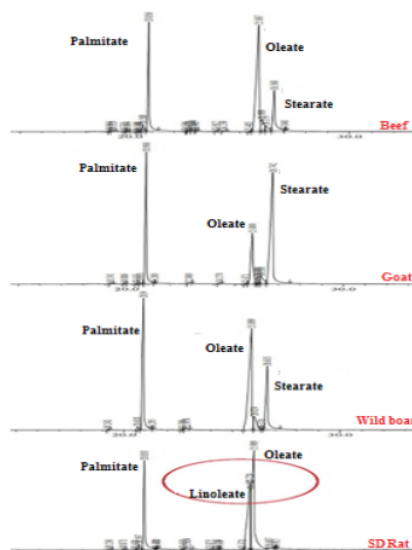


Fig. 3: Chromatogram of *Sprague Dawley* rat, wild boar, goat, and cow the x-axis was Tr (retention time), and the y-axis was total ion chromatogram (TIC)

Table 3: Percentage of methyl ester in *Sprague Dawley* rat fat, wild boar, goat, and cow

Methyl ester	Percentase (%) methyl ester (x±SD), N=3			
	<i>Sprague Dawley</i> rat	Wild boar	Goat	Beef
Myristate (14:0)	0.18±0.01	0.09±0.01	0.12±0.01	0.22±0.03
Pentadecanoate (15:0)	0.14±0.01	Nd	0.11±0.02	0.06±0.01
Palmitoleate (16:1)	1.14±0.06	0.72±0.01	0.37±0.04	1.66±0.12
Palmitate (16:0)	19.75±0.09	29.01±0.77	27.65±0.29	24.16±0.36
Margarate (17:0)	0.15±0.015	Nd	0.29±0.02	0.30±0.02
Linoleate (18:2)	32.24±1.46	Nd	Nd	Nd
Oleate (18:1)	43.32±1.43	43.70±0.28	3.99±0.20	50.05±0.67
Stearate (18:0)	0.26±0.01	19.88±1.40	45.38±1.19	13.97±0.01

*Nd: Not detected, SD=standard of deviation, N=Replication

Fig. 3 shows that chromatogram peak linoleate only appears in *Sprague Dawley* rats. The x-axis on the chromatogram in fig. 3 is time retention, while the y-axis is TIC (Total Ion Chromatogram). TIC is a chromatogram that is made by summing up the intensity of all mass

spectral peaks, which are included in the same scan [28]. When observed from the number of fatty acid constituents, it can be seen that *SD* rats have the most unsaturated fatty acid among the other animals (fig. 4).

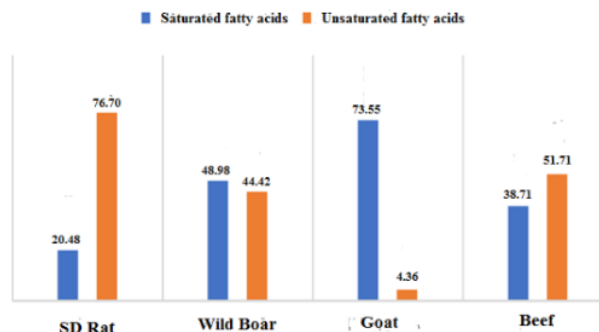


Fig. 4: Fatty acids in *Sprague Dawley* rat, wild boar, goat, and cow

Based on fig. 4, the highest constituent of *Sprague Dawley* rats' total fatty acid is 97.18%; boars 93.4%; goats 77.91%, and cows 90.42%. Most likely, this difference occurs from the extraction process, the process of separation in GC, food intake, as well as the animal parts/organs taken.

Principal component analysis (PCA) of methyl ester fatty acid in *Sprague Dawley* rats, boars, goats, cows, as well as meatball sample products

Principal Component Analysis (PCA) data interpretation is made by reducing data, where the number of variables in a matrix is reduced to produce new variables while maintaining the information owned by the data [29]. The sample was chosen: meatball. Meatball is one of the processed foods derived from animals, which is much in demand by society. The common trend of mixing meatball with rat meat has made people uneasy. The result of the analysis of methyl esters in processed food samples (meatball) is listed in table 4.

Table IV shows that the three samples of meatball have the same type of fatty acid profile, which consists of 7 types of fatty acids-where five are saturated fatty acids, and 2 are unsaturated fatty acids. Although the types of fatty acid which appear are similar; the amount of each fatty acid has a different value. The difference between the fat of *Sprague Dawley* rats and meatball (samples A, B, and C) is in the linoleate fatty acid. In the previous explanation with boars, goats, and cows, the difference is also in linoleate fatty acid content. This shows that linoleic acid is the typical fatty acid in rats, which are often used as research trials, namely Wistar and *Sprague Dawley* rats.

The PCA analysis variable is a type of fatty acid. The type of matrix chosen was the correlation that connected the types of a sample with fatty acid. The information obtained from PCA analysis showed the similarity between the fatty acid in *Sprague Dawley* rats, boars, goats, cows, as well as meatballs (samples A, B, and C).

Table 4: The result of the acquisition of methyl ester percentage in the fat of *Sprague Dawley* rats' and meatballs (Sample A, B, and C)

Methyl ester	Methyl ester (Percentage ((x±SD)%, N= 3)			
	<i>Sprague Dawley</i> rats	Meatball (sample A)	Meatball (sample B)	Meatball (sample C)
Myristate (14:0)	0.18±0.01	0.46±0.03	0.52±0.07	1.13±0.12
Pentadecanoate (15:0)	0.14±0.01	0.19±0.01	0.20±0.02	0.10±0.007
Palmitoleate (16:1)	1.14±0.06	1.82±0.10	1.08±0.19	0.98±0.14
Palmitate (16:0)	19.75±0.09	28.12±0.04	26.51±2.07	35.07±0.23
Margarate (17:0)	0.15±0.015	0.45±0.02	0.65±0.01	0.31±0.02
Linoleate (18:2)	32.24±1.46	Nd	Nd	Nd
Oleate (18:1)	43.32±1.43	32.3±0.53	31.35±2.09	23.58±0.41
Stearate (18:0)	0.26±0.01	17.72±0.18	27.28±0.60	27.92±1.59

*Nd: Not detected, SD= standard Deviation, N= Replication

Table 5. The report of PCA analysis of *Sprague Dawley* Rat, other animal and sample product at markets of its eigen analysis

Eigen analysis of the correlation matrix								
Eigenvalue	3.4722	2.1684	1.3563	0.7367	0.1907	0.0598	0.014	0.0016
Proportion	0.434	0.271	0.170	0.092	0.024	0.007	0.002	0.000
Cumulative	0.434	0.705	0.875	0.967	0.991	0.998	1.000	1.000
Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Myristate	0.297	0.297	0.366	-0.642	-0.205	-0.365	0.321	-0.014
Pentadecanoate	0.070	0.572	-0.424	-0.098	0.141	0.519	0.383	0.205
Palmitoleate	-0.189	0.502	0.386	0.266	0.608	-0.289	-0.132	0.150
Palmitate	0.448	-0.085	0.408	-0.213	0.185	0.567	-0.458	0.12-0.197
Margarate	0.266	0.535	-0.167	0.253	-0.486	-0.127	-0.510	0.352
Linoleate	-0.419	0.039	-0.330	-0.565	0.042	-0.133	-0.502	0.465
Oleate	-0.426	0.094	0.446	0.169	-0.544	0.247	0.092	0.736
stearate	0.493	-0.473	-0.188	0.266	-0.004	-0.312	0.046	

The PCA analysis conducted using minitab19 software had obtained 8 PCs. The selection of the number of PCs in PCA can be determined by observing the eigenvalue obtained from the PCA result. The number of PCs relevant to explaining the preliminary information from data is a PC with an eigenvalue >1. Below this limit, PCs are considered irrelevant. Table 5 shows that the PCA analysis produced 8 PCs. PC1, with an eigenvalue of 3.4722, can describe 43.4% of the total original

data variable. PC2, with an eigenvalue of 2.1684, can describe 27.1% of the total original variable. PC3, with an eigenvalue of 1.3563, is able to describe 17.0% of the total original variables. Therefore, only by using 3 PCs; can one already describe 87.5% of all original data variables and is relevant enough to explain the characteristics of the initial variables [29]. Fig. 5 shows the result of the fatty acid plot score: *Sprague Dawley* rats, boars, goats, cows, as well as meatball (sample A, B and C).

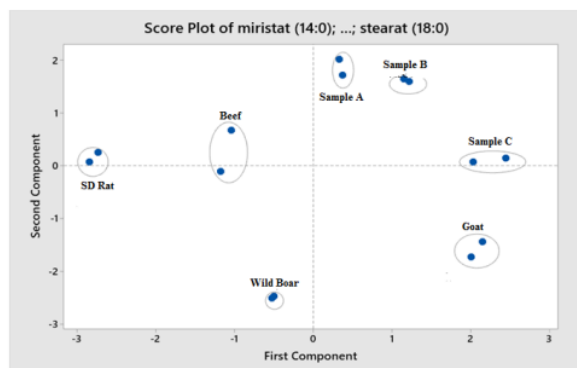


Fig. 5: Score plot of PCA analysis of SD rat, wild boar, goat, cow, and meatballs sample market: A, B, and C

Fig. 5 shows that PCA chemometrics is able to classify the fat of *Sprague Dawley* rats, boars, goats, cows, as well as meatballs (samples A, B, and C). The meatball samples' scores are very distant from the *Sprague Dawley* rats. Therefore, it can be concluded from the Plot Score that meatball (samples A, B, and C) do not have *Sprague Dawley* rats' fat content. The very distant score between *Sprague Dawley* rats with other animals and meatball (samples A, B, and C) is because linoleate fatty acid is only owned by *Sprague Dawley* rats-which means it cannot be found in the meatball samples.

CONCLUSION

The constituents of *Sprague Dawley* rats' (*Rattus norvegicus*) fat analyzed by gas chromatography-mass spectrometry (GC-MS) method consist of 8 types of fatty acid; namely oleate (43.32%), linoleate (32.24%), palmitate (19.75%), palmitoleate (1.14%), stearate (0.26%), myristate (0.18%), margarate (0.15%) and pentadecanoate (0.14%). The GC-MS method combined with PCA (Principal Component Analysis) chemometric is able to classify the fat of *Sprague Dawley* rats' (*Rattus norvegicus*), with other animals and samples of processed meatballs on the market.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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