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AUTHENTICATION OF WISTAR RAT FATS WITH GAS CHROMATOGRAPHY MASS SPECTOMETRY COMBINED BY CHEMOMETRICS

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ABSTRACT
Indonesia is a country with the largest Muslim population in the world, which is very concerned about halal food. The most problem that's very concerning nowadays was that food products were contaminated by unclean meat, such as rat meat. The purpose of this study was to authenticate rat fat using Gas Chromatography-Mass Spectrophotometry (GC-MS) combined with chemometrics. In this study, rat fat were heated in oven at 90 °C – 100 °C for approximately one hour until the oil came out. After that, the derivatization process was carried out to convert fat into methyl ester compounds using NaOCH3 and BF3. Methyl ester compound than injected into the GCMS instrument system. In addition to rat fat, other fat extraction were carried out, such as pigs, cows, chickens, wild boars, dogs, and goats. The combination of chemometrics Principal Component Analysis (PCA) was used to classify rat fat with other animal fat. Based on the results of the study showed that fatty acids in rats using GCMS produced 6 types of fatty acids, namely: myristat (0.15 ±0.09%), palmitoleate (0.73 ±0.54%), palmitate (19.08 ±3.54%), linoleate (30.14 ±16.90%), oleate (40.48 ±2.74%), and stearate (2.55 ±0.01%). Total content of rat fatty acids was 93.13%, with unsaturated fatty acids 71.35% and saturated fatty acids 21.78%. Chemometrics PCA from rat fat can be grouped with other animal fats.

Keywords: chemometrics; food; GC-MS; halal; PCA; Wistar rat fat

INTRODUCTION
Food is a basic human need, therefore food availability needs serious attention both in quality and quantity. Indonesia is a country with a Muslim majority of 207.2 million with a presentation of 87.18% in 2010 out of a total population of 237 million **(Muslim and Purwanto, 2013)**. In addition to food safety factors, the halal factor of a food product must also be of concern to the Muslim community. At present the awareness of the Muslim community to consume halal food increases along with the awareness of the Muslim community following Islamic laws **(Rohman et al., 2016)**. Along with the increase in people's income, the demand for meat consumption in various regions of Indonesia has increased. The price of basic ingredients which are quite expensive such as chicken meat, makes many producers mix it with meat which is relatively cheaper, one possibility is to use rat meat **(Guntarti and Prativi, 2017)**. Rat meat is a meat that is quite easy to obtain, even it can be obtained free of charge. Some media also reported the adulteration of beef meatballs with rat meat **(Lumakso et al., 2015)**. Examples of several cases on the market are forgery of chicken nuggets from pork, and nuggets from recycled materials **(Sari and Guntarti, 2018)**. Based on this, it is also feared that counterfeiting of processed chicken products using rat meat will also occur in Yogyakarta. Laboratory tests to determine fatty acid markers in the form of methyl esters in rats include using gas chromatography-gas spectrophotometry combined with chemometrics. This technique has been used in a variety of analyzes, such as food and pharmaceutical products **(Ronggo et al., 2007)**.

The chemometric method is one way to obtain important information about certain objects in the data by using statistical or mathematical techniques. The most commonly used types of chemometrics are (1) grouping techniques, such as Principle Component Analysis (PCA) and (2) quantitative analysis techniques with multivariate calibration, such as Partial Least Square (PLS).

Scientific hypothesis
The hypothesis in this study is that methyl esters from wistar rat animal fat can be analyzed using the Gas Chromatography Mass Spectrometry (GCMS) method. The methyl ester data combined with chemometrics is able to classify types of fat.

MATERIAL AND METHODOLOGY
Fat samples
Samples in the form of pork, beef, dog, goat, wild boar, chicken were obtained from the traditional market, Wistar white rats were obtained from other researchers' carcasses, the fat was taken. Materials used *n*-hexane (technical),

$NaOCH_3$ 0.2 N solution (E-Merck, pro-analysis quality), BF_3 solution (E-Merck, pro-analysis quality), saturated NaCl and anhydrous Na_2SO_4 (E-Merck, pro-analysis quality) **(Rohman and Che Man, 2011; Kumar et al., 2014)**.

Tools

GC-MS distributor from Ditek Jaya, Merck Shimadzu, Japan, type GCMS-QP2010 SE. The column used was Rtx-5ms, DB1-MS Restech, 30 m x 0.25 mm ID, 0.25 µm, stationary phase of polymethyl xiloxan, injector temperature of 230 °C, column temperature of 70 °C and increased to 300 °C with an increase of 10 °C.min^{-1}, and flow rate of 1.15 mL.min^{-1}. The mobile phase of Helium gas. MS Detector *Electron Multifier Detector* (EMD) 70 MeV. The mass spectrum was compared to the WILLEY147 & NIST47 library found in the GC-MS software. The methyl ester was injected as much as µL into the GC column in a manner of autosampler.

The Research Progress

Fat Extraction

Intake of fat was done by rendering at a temperature of 90 – 100 °C for approximately one hour in the oven. The resulting fat was then added with anhydrous Na_2SO_4 and then centrifuged at 3000 rpm for 20 minutes **(Rohman et al., 2012)**. The solution was stored in the refrigerator at -20 °C in a rolling test tube. The solution was used for the derivatization process.

Derivatization

Fat derivatization aims to convert fat into a form of fatty acid methyl ester by using NaOCH3 0.2 N and BF_3 solution. Derivative products containing fatty acid methyl ester derivatives (FAME) were taken and injected into the gas chromatograph system. A total of 1µL supernatant was injected into gas chromatography-mass spectrometry, replicated two times.

Statistical analysis

The results of the analysis in the form of a mass spectrum were compared with the WILLEY147 & NIST47 libraries contained in the GC-MS software. Data obtained from GC-MS was fatty acids in the form of methyl esters. The content of methyl esters of fatty acids from each animal fat was grouped using chemometrics PCA with minitab 16.

RESULTS AND DISCUSSION

Fat Extraction

Rendering extraction to obtain rat fat. The advantages of this method are that there are many extract yields, easy and inexpensive processing because it does not involve chemicals **(Rohman et al., 2016)**. The yield of 16.36%. The yields are influenced by the intake of food from the rats themselves, body fat taken, and the way the fat is extracted **(Lobb and Chow, 2007)**. The obtained lipid fraction was then carried out by the esterification process. The esterification reaction aims to convert the fatty acids into their methyl ester forms. The esterification process is carried out using BF_3 as a catalyst. BF_3 is an acidic compound **(Purbasari and Silviana, 2008)**. Figure 1 is a process of the esterification mechanism and the product of methyl ester is obtained.

Fatty Acid Composition in Wistar Rat.

Analysis of methyl esters from Wistar rat derivatization using gas chromatography-mass spectrometry (GC-MS) method. This instrument is a combination of gas chromatography with a mass spectrometer detector. Gas chromatography is used for the separation of fatty acid content in the form of methyl esters. In addition to the retention time (tR) of the separation results in gas chromatography, there is similarity index (SI) information to determine the proximity of the chemical structure of the type of fatty acids. The result of SI >90 shows the similarity of mass ion overflow to the target spectra/fat sample.

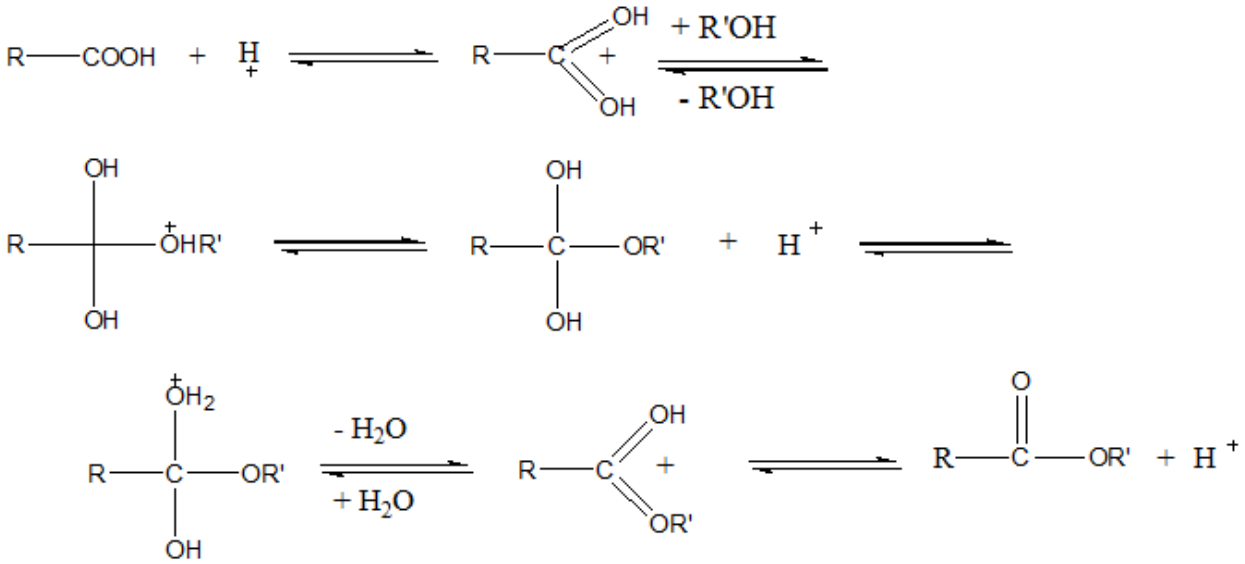

Figure 1 The Process of Esterification Reaction Mechanisms with Methanol and using Acid Catalysts (adapted from **Purbasari dan Silviana, 2008)**.

Table 1 Separation results and identification of compounds in Wistar rat fat with GC-MS.

Number	t_R (min)	% Area (x ±*SD*) (n = 2)	SI	MW	Compound Name
1	17.75	0.15 ±0.09	95	242	(C14:0) Methyl Myristate
2	20.34	0.73 ±0.54	96	268	(C16:1) Methyl Palmitoleate
3	20.81	19.08 ±3.54	96	270	(C16:0) Methyl Palmitate
4	25.60	30.14 ±16.90	86	284	(C18:2) Methyl oktadekadienoat/linoleate
5	25.93	40.48 ±2.74	88	296	(C18:1) Methyil Oleate
6	26.51	2.55 ±0.01	97	298	(C18:0) Methyl Stearate

Note: SI= *Similarity Index*, MW= Molecular weight.

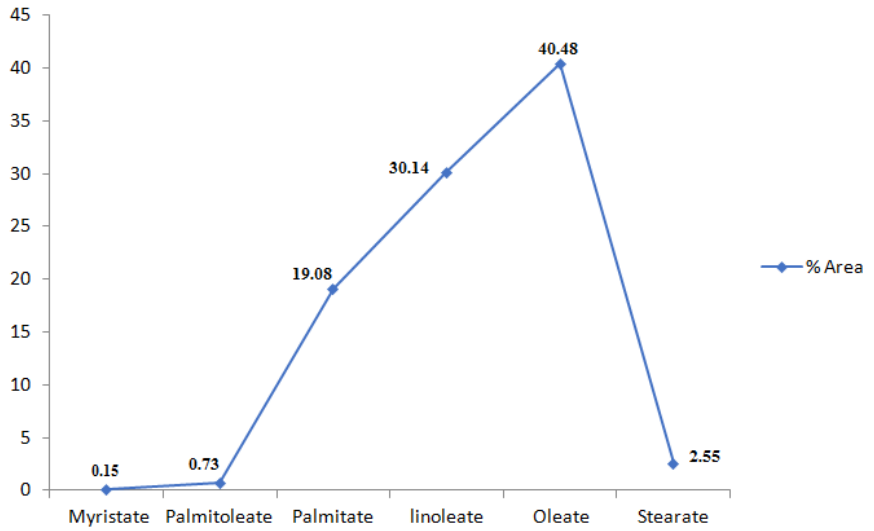

Figure 2 Line of the type of fatty acids in Wistar rat fat.

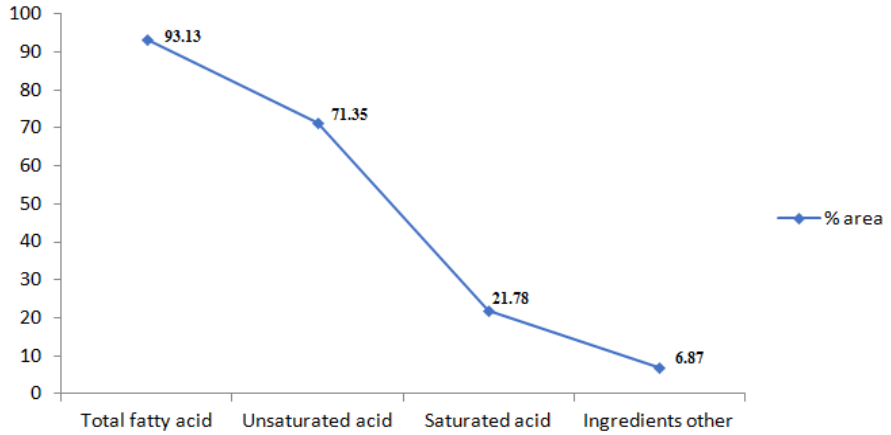

Figure 3 The Line of total fatty acid, saturated fatty acid, unsaturated fatty acid, and ingredients other content in Wistar rat.

Table 1 presents retention time (tR), % peak area, SI, molecular weight (MW) and estimated compounds and identification of white rat fat **(Guntarti and Amidin, 2018)**. The results of GC-MS analysis in Table I show the results of SI values >90 except oleic acid which is 88% and linoleic acid 86%. This shows that the target fatty acid type is suitable or similar to the comparison spectra. The fatty acid compound with a tR of 25.93 minutes and a SI value of 96% was similar to the comparison compound with the formula $C_{17}H_{36}O_2$ with m/z 296. The fatty acid was in the form of its methyl ester. Whereas if in the form of fatty acids, the compound formula is $C_{18}H_{33}O_2$.

Fat is an unstable component in the presence of light. The results of the analysis with GC-MS showed that oleic acid was the highest constituent component of fatty acids in rat fat with a percentage of 40.48%, followed by linoleic acid 30.14%, palmitic acid 19.08%, stearic acid 2.55%, palmitoleic acid 0.73%, and myristic acid 0.15%. The line of the types of fatty acids in Wistar rat fat is presented in Figure 2. unsaturated fatty acid with one double bond.

When viewed from unsaturated bonds, white rat fat contains many types of unsaturated fatty acids, namely palmitoleic acid (0.73%), linoleic acid (30.14%), and oleic acid (40.48%). Whereas saturated fatty acids are myristic acid (0.15%), palmitic acid (19.08%), and stearic acid (2.55%). If looked at the percentage of the content, then more unsaturated fatty acids is equal to 71.35%. Saturated fatty acids of 21.78% and 6.87% are ingredients other than methyl esters. Larger amount of unsaturated fatty acid content will affect the physical form of fat at room temperature and the stability of fat. Figure 3 presents a line of total fatty acid, saturated fatty acid, unsaturated fatty acid, and ingredients other content in Wistar rat.

Comparison of fat: Wistar rat, dog, wild boar, beef, pork, chick and goat.

Besides Wistar rat fat, other animals' fat used were: dog, beef, pork, chick, and goat. Fat retrieval is the same as done in white fat retrieval, which is by rendering with an oven at a temperature of 90 °C – 100 °C, for 30 – 60 minutes. The fat obtained is esterified to form its methyl ester with $NaOCH_3$ and BF_3 which are then injected into the GC-MS system. The results of analysis of dog, beef, pork, chick, goat, and wild boar fat are presented in Table II and Figure 4.

Based on Table II it can be seen that the fat of white rat and pork containing linoleic content (30.14%), and pork fat (21.49%). The highest oleic acid is in the content of pork (55.66%). Goat fat has the highest type of saturated fat, palmitate (23.55%) and stearic (47.13%).

In the results of previous studies **(Hermanto, Muawanah and Harahap, 2008)**, that pork and chicken fat contain margaric acid. Margaric acid content (C17: 0) in pork is 0.5%, and in chick 1.74% **(Hermanto, Muawanah and Harahap, 2008)**. **Guntarti (2018)** research results: beef fat has a high stearic acid (35.03%), while oleic acid is 14.90%. The results of this study, beef fat has a high oleic acid content (52.29%), while stearic acid is 12.59%. Except for goat fat, all animals contain the highest oleic acid; while goat fat, the highest is stearic acid. Figure 4 presents the content of saturated and unsaturated fat, and the total amount of fatty acids in various animals. Based on Figure 4, the saturated fatty acid content is high in goat fat (71.16%), wild boar fat (36.27%), beef fat (35.16%), dog fat (34.33%), lard/pork (27.78%), and the smallest is in chick fat (20.55%). The highest unsaturated fat content is in chick fat (73.2%), rat fat (71.35%), lard / pork fat (55.66%), dog fat (53.93%), wild boar fat (45.24%), and the smallest is in goat fat (19.19%). While the highest amount of total fat is in chick, followed by white rat.

Table 2 The results of the analysis of acid content in the fat of: Wistar rat, dog, wild boar, pork, chick, beef, and goat with GC-MS.

Methyl ester	Percentage of (%) methyl esters						
	Dog	Wild boar	Pork	Chick	Beef	Goat	White rat
Methyl myristat (C14:0)	0.33	nd	0.41	nd	0.29	0.25	0.15
Methyl pentadekanoate (C15:0)	nd	nd	nd	nd	0.36	0.23	nd
Methyl palmitoleic (C16:1)	0.34	nd	1.14	1.14	0.98	nd	0.73
Methyl palmitate (C16:0)	16.42	19.65	17.26	18.91	21.81	23.55	19.08
Methyl margarate (C17:0)	0.37	0.27	nd	nd	0.11	nd	nd
Methyl linoleate (C18:2)	nd	nd	25.75	21.40	nd	nd	30.14
Methyl oleate (C18:1)	53.59	45.24	55.66	50.66	52.29	19.19	40.48
Methyl stearate (C18:0)	17.21	14.37	10.11	1.64	12.59	47.13	2.55

Note: nd= not detected.

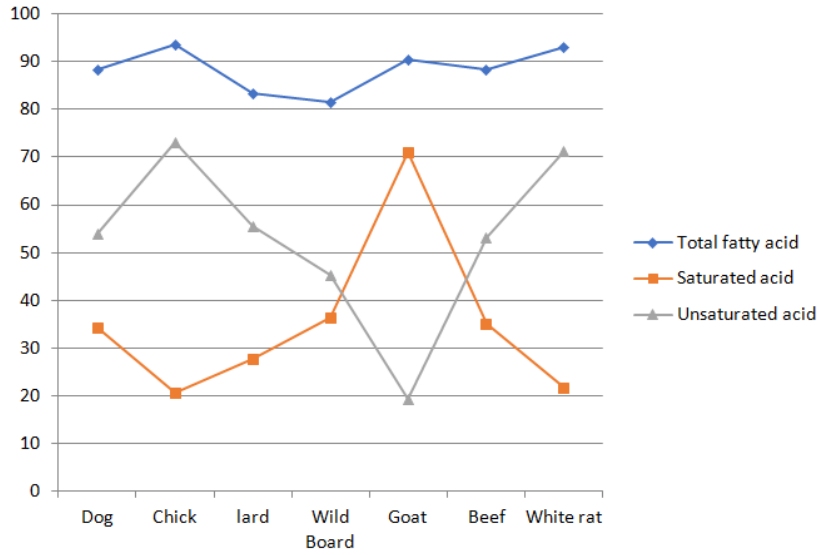

Figure 4 Amount of total fatty acid, saturated acid, and unsaturated fatty acids in the fat of: Wistar rat, dog, wild boar, pork, chick, beef and goat.

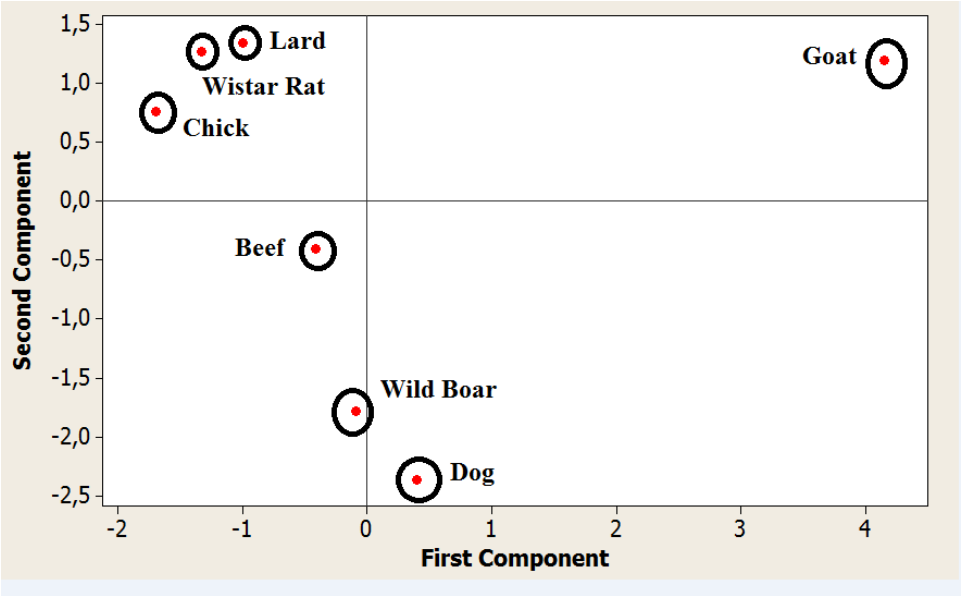

Figure 5 PCA Score Plot of fat from: Wistar rat. dog. chick. pork. wild boar. goat and beef by using fatty acids as a variable.

Table 3 The report of PCA analysis of sausage and some parameters of its Eigen analysis.

Eigenalysis of the Correlation Matrix								
Eigenvalue	3.8699	2.3938	1.0508	0.4382	0.1553	0.0920	-0.0000	-0.0000
Proportion	0.484	0.299	0.131	0.055	0.019	0.011	-0.000	-0.000
Comulative	0.484	0.783	0.914	0.969	0.989	1.000	1.000	1.000
Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Myristate	0.004	0.180	-0.932	0.054	0.237	0.011	0.122	-0.160
Pentadecanoate	0.473	0.219	0.123	0.001	0.003	-0.225	0.802	0.141
Palmitoleate	-0.373	0.346	-0.110	-0.503	-0.490	-0.532	-0.035	0.185
Palmitate	0.470	0.083	-0.145	-0.174	-0.705	0.397	-0.124	-.0224
Margarate	0.036	-0.586	-0.186	0.450	-0.400	-0.507	0.031	-0.028
Linoleate	-0.304	0.432	-0.013	0.634	-0.296	0.262	0.067	0.397
Oleate	-0.267	-0.513	-0.171	-0.328	-0.097	0.418	0.360	0.464
Stearate	0.502	0.003	-0.140	-0.035	0.151	-0.133	-0.437	0.705

Principal Component Analysis of wild boar and other animal fat.

PCA data interpretation is done by reducing data, in which the number of variables in a matrix is reduced to produce new variables while maintaining the information held by the data. The new variables generated are scores or main components **(Rohman and Man, 2012)**. PCA aims to group variables that are correlated with each other and replace them with new groups called main components (principal component) **(Coltro et al., 2005)**. PCA simplifies data by reducing a number of variables to a smaller number of orthogonal variables. This needs a correlation between variables. Although PCA reduces the number of initial variables, PCA retains variability and initial information. PCA also helps provide pattern visualization and correlation analysis **(Miller and Miller, 2010)**. PCA plot scores are presented in Figure 5. The results of replication greatly affect the location of the quadrants obtained, further showing that there are similarities in the physical chemical properties of the fatty acid content. Wistar rat fat is located between chick fat and lard. The results of replication measurements affect the proximity position of the grouping of animal fat.

The results of PCA analysis using Minitab resulted in 8 PCs presented in Figure 6. Each PC displays eigenvalue, proportion, and cumulative values. Eigenvalue variations can explain the data on each PC and show how much influence a variable on the formation of the characteristics of a matrix **(Miller and Miller, 2010).** In Table 3, PC1 with eigenvalue 3.8699 is able to describe 48.4% of the total original data variables while PC2 with eigenvalue 2.3938 is able to describe 21.90% of the total original variables, PC3 with eigenvalue 1.0508 is able to describe 13.10%. Thus, 3 PCs described the illustration data for discriminant analysis of 83.40%.

CONCLUSION

Mass spectroscopy gas chromatography method can be used to authenticate Wistar strain rat with fatty acids content.The content of that fatty acids in white Wistar strain rat is: myristate (0.15 ±0.09)%, palmitoleate (0.73 ±0.54)%, palmitate (19.08 ±3.54)%, linoleic (30.14 ±16.90)%, oleate (40.48 ±2.74)%, and stearic (2.55 ±0.01)%. Chemometrics PCA white rat, dog, wild boar, chick, pork, beef, and goat fat can be grouped.

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