Detection of Lard in Animal Fat Mixtures Using ATR-FTIR Fingerprint and SPME-GC/MS-Based Volatilomics

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Abstract: *This study aims to detect the presence of lard in several halal animal fats (beef, chicken, and goat fat) based on their infrared fingerprint and volatile compound profile (volatilomics). A mixture of fat samples obtained from halal animals and lard at different concentrations (0, 20, 40, 60, and 80%, v/v) were subjected to attenuated total reflection-Fourier transformed infrared spectroscopy (ATR-FTIR) and solid phase microextraction coupled to gas chromatography-mass spectrometry (SPME-GC/MS) analysis, respectively. The data was processed using orthogonal projection to the least square–discriminant analysis (OPLS-DA). The results showed that ATR-FTIR could only identify the presence of lard in chicken fat up to the lowest concentration used in this study (10%) but failed in other fat samples. SPME-GC/MS detected the presence of lard in all animal fats up to the lowest concentration added (10%). The results of this study revealed that the volatilomics technique had more potential to be developed as a basis for the rapid detection of halal and non-halal animal fat than the infrared fingerprint. This study also emphasized that markers of non-halal animal fats can be different when the same fats are added to different food products.*

Keywords: *halal authentication; lard; infrared fingerprinting; volatiles; chemometrics*

■ INTRODUCTION

Food manufacturers often combine lard with animal or vegetable oils to achieve a desired texture or physicochemical property [1]. For example, lard is a traditional material used to shorten and it contains essential fatty acids, fat-soluble vitamins, arachidonic acid, lipoproteins, and reduced levels of trans-fatty acids. Furthermore, lard contributes to a pleasant mouthfeel and melting in the mouth. It has a stable β′ crystalline structure, which is required to construct fine fat-based networks [2]. Replacement of lard with another type of animal or vegetable oil is usually required to reduce the product's calorie value and fulfill specific dietary restrictions. For example, food containing lard and its derivatives is prohibited from being consumed by Muslims. A quick and easy method for detecting lard in products containing animal fat is necessary to solve this problem. Until now, various methods have been used to differentiate and detect lard in halal fats or oils. Several existing methods are available, including FTIR. Previous studies reported that FTIR is an effective and sensitive analysis method to identify the presence of lard in vegetable oil [3] and in beef or chicken fats [4]. FTIR measurement involves mixing the samples with KBr and pressing them to form a fused disc before it can be placed into the spectrometer's IR light beam pathway. Several drawbacks in the use of KBr or any other salt in FTIR, such as moisture-interfering effects caused by the

hygroscopic properties of these salts, low reproducibility as a result of salt-to-sample matrix inhomogeneity and inconsistent ratio, lead to inconsistent path length and detector response [5]. Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was developed to overcome these limitations. ATR-FTIR can provide infrared fingerprints quickly and easily without using KBr grinding, thus eliminating the influence of difference-size particles and inhomogeneity of the KBr pellet [6]. Previously, ATR-FTIR was used to identify goat fat in pure ghee and lard in cow milk fat [7-8]. FTIR technique was also reported to be able to detect lard in five edible oils [3] and lard in chicken and beef fat [9]. However, the use of ATR-FTIR to detect the presence of lard in beef, chicken and goat fats has not yet been reported.

The analysis of products containing lard can also be done using gas chromatography hyphenated techniques like gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID). High precision and sensitivity are advantages of these methods. Examples of the application of these techniques to detect lard in various products have been reported. GC-FID was used to detect lard content in several edible oils [1,10], whereas GC-MS has been used to discriminate vegetable oils and to detect lard in butter [11]. Despite its many advantages, using GC-FID or GC-MS has disadvantages that require complicated sample preparation and expensive costs [12]. For example, fatty acids composition analysis using GC requires derivatization to provide more volatile and stable fatty acid derivatives [13]. The sample preparation process is prolonged, and the method complexity is increased by this derivatization step. Solid phase microextraction (SPME) is a solvent-free extraction method that is often coupled to the GC-MS. SPME's advantages include being non-destructive for extracting volatile compounds and not changing the original chemical composition of the volatiles [14]. No derivatization is required since tandem SPME–GC-MS targets volatile compounds present in the samples. Tandem SPME to GC-MS was used to study the effect of heating on the boar taint intensity of lard [15]. However, the technique has not been used for halal animal fat authentication. Therefore, in this study, we used FTIR-ATR and SPME coupled with GC-MS techniques to detect the presence of lard in halal animal fats (goat, chicken, and beef fats). The differences in the infrared spectrum and volatilome profile of the pure fat samples mentioned above and when the fats were mixed with lard in different concentrations (0, 10, 20, 40, 60, and 80%, v/v) were studied using principal component analysis (PCA) and orthogonal-projection to the least square analysis (OPLS-DA). Specific wavenumbers and volatile compounds predominantly found in each sample were determined using coefficient correlation and variables of importance to the projection (VIP) value. This is the first study to compare two spectroscopical techniques to detect the presence of lard in halal animal fats (goat, chicken, and beef fats). The result of this study is expected to provide a simpler and quick alternative method for halal fat authentication.

■ EXPERIMENTAL SECTION

Materials

LPPOM-MUI generously provided lard, beef fat, chicken fat, and goat fat. The chemicals used were anhydrous Na_2SO_4 (Merck. Darmstad, Germany) and a homologous series of an *n*-alkane solution (C10-40, Polyscience, Niles, IL, USA; 5 mg/L). All chemicals are of analytical grade.

Instrumentation

Infrared spectra were recorded using an FTIR-ATR spectrophotometer (Thermo Scientific Nicolet iS5) with fast recovery deuterated triglycine sulfate (DTGS) detector and OMNIC 9.0 software. Volatile compounds were concentrated using SPME with DVB/Car/PDMS 50–30 µm Supelco fiber (Sigma Aldrich, Bellefonte, USA). Volatile compounds of fat were analyzed using GC-MS QP2010 plus (Shimadzu, Japan). Multivariate data analysis was conducted using SIMCA ver. 13.0 (Sartorius-Umetric, Umeå, Sweden).

Procedure

Preparation of animal oil/fat samples for FTIR analysis

Animal oil sample preparation was carried out based on previous research by Lestari et al. [9] with a few

slight modifications to the heating time. The oil sample was heated in the oven at 90–100 °C for 30 min. The goat fat was sliced into small pieces and melted at 90–100 °C for 2 h in the oven. The oils were then filtered with three layers of muslin cloth and added by anhydrous Na_2SO_4. The resulting filtrate was centrifuged at a speed of 3,000 rpm for 20 min. A layer of fat formed after the centrifugation was removed, and the filtrate was filtered on a paper filter and added anhydrous Na_2SO_4.

ATR-FTIR analysis

ATR-FTIR (Thermo Scientific Nicolet iS5, equipped with OMNIC 9.0 software) analysis was carried out based on research by Pebriana et al. [16] with slight modifications because the sample was in liquid form. The filtered oil sample was dropped as much as 0.15 µL using a micropipette onto the ATR crystal in FTIR at a room temperature of around 25 °C. The spectrum was obtained in the wavenumber region between 450–4000 cm^{-1} using a spectrophotometer. The resulting spectrum was recorded as absorbance values with 32 scans at a resolution of 8 cm^{-1} with air as the reference spectrum. The spectrum was processed using OMNIC 9 software.

Extraction of volatile compounds using SPME

An oil sample (10 mL) was put into a 20 mL SPME vial. The SPME fibers were conditioned before use by heating them in a GC-MS injector at 250 °C for 10 min. The extraction process was conducted in a water bath; the fiber was placed in the headspace in the vial for 15 min at a water bath temperature of 50 °C. Each subsequent sample was given the same treatment and repeated twice for each sample [14].

Analysis of volatile compounds using GC-MS

Analysis with GC-MS (GC-MS QP20 10 plus, Shimadzu) began by inserting the SPME fiber containing volatile components into the GC-MS injection port. Sample injection was carried out at an injector temperature of 175 °C. Compound separation was performed on an RTX-5MS capillary column (l = 30 m, d = 0.25 mm, and layer thickness 0.25 µm, Agilent Technologies, California, USA). The oven temperature was maintained at 33 °C for 5 min, and then the temperature was programmed to increase at 5 °C/min until it reached 200 °C. The mass spectrometer was operated in electron ionization (EI) mode with the electron energy set at 70 eV and a scanning range of 35–450 *m/z*. Characterization of volatile compounds was done using the alkanes (C8-C20) standard and the NIST v.14 database.

Data analysis

Multivariate data analysis and modelling were conducted using SIMCA software (v.13.0, Sartorius-Umetric, Sweden). For ATR-FTIR data, the spectrum was converted into numeric data and then converted into an Excel file. The Excel file consists of wavelength number (450–4000 cm^{-1}) versus its corresponding relative peak area. For GC-MS data, we first conducted the identification of volatile compounds by calculating the LRI value of volatile compounds as explained below and matching the spectra data with the library NIST 14. The LRI was calculated according to the following formula (Eq. 1) [14]:

$$\text{LRI(compound)} = (100\times n) + (100\times z)\frac{t_r(\text{compound}) - t_r(n)}{t_r(N) - t_r(n)} \quad (1)$$

where LRI (compound) is the linear retention index value of the analyte compound, tr is the retention time of the analyte compound (min), n is the number of carbon atoms in the alkane that elute before the analyte elutes, N is the number of carbon atoms in the alkane that elute after the analyte elutes, and Z is the difference between the number of carbon atoms in the smaller and larger alkanes.

Next, an excel file consisting of the identified volatile compounds and their relative peak intensities was generated. The Excel files of ATR-FTIR and GC-MS data were further used as data for multivariate data analysis. The type of multivariate data analysis used is PCA and OPLS-DA. PCA was a preliminary analysis to see the general classification pattern between pure lard and its mixture with non-lard and fat. The PCA model is evaluated based on the variance values of the factors (Q^2). Infrared region or volatile compound markers were selected on OPLS-DA based on the VIP and coefficients correlation value. Requirements to be selected as marker compounds were having a positive

coefficient correlation value, the error bars of the compound in the coefficient plot not touching the x-axis and having a VIP value larger than 0.5.

■ RESULTS AND DISCUSSION

Infrared Fingerprinting Profile of the Oils

The representative infrared spectra of pure lard, chicken fat, beef oil, and goat fat are presented in Fig. S1. There are eleven main peaks (A to K), each of which indicates a different functional group. These spectra show that lard and non-pork animal fat have infrared absorption at similar wavelengths but with varying intensities. Lard had the highest absorbance peak intensity at the wavenumber of 1744.298 and 1159.009 cm^{-1}, which can be attributed to the carbonyl group (C=O) of triglyceride's ester bond and C–O group in ester, respectively. The goat fat had the highest absorbance peak intensity at the wavenumber of 2921.628 and 2853.167 cm^{-1}, indicating the presence of methylene (CH_2) groups in triglyceride's structure. The major absorption peaks of beef oil and chicken fat are less visible than the others. The samples' discriminant wave numbers and functional groupings cannot be seen visually in these spectra. Multivariate data analysis was needed to extract further information. The infrared absorption peaks and their attributed functional groups are shown in Table 1. Most of the peaks correspond to the characteristics of triacylglycerol functional groups.

Multivariate Data Analysis of FTIR Data

Multivariate data analysis makes the complex data easier to interpret and understand. The multivariate data analysis used in this study are PCA and OPLS-DA. The PCA model has an R^2X value of 0.987 and a Q^2 value of 0.972, indicating a reliable model [14]. The PCA score plot is shown in Fig. 1. On the upper part of the plot, pure chicken fats were slightly separated, whereas chicken fat containing lard was clustered closer to pure lard. This indicates that the PCA of infrared fingerprinting data can be traced to the presence of lard in chicken fat at the lowest concentration used in this study. However, pure beef fat and the mixture of lard-beef and lard-chicken fats, and the mixture of lard-goat fat with a higher percentage of lard (60 and 80%) were in the same cluster, whereas pure goat fat and the mixture of lard-goat fat with a lower percentage of lard (40 and 20%) were clustered together on the bottom part of the score plot. This result shows that ATR-FTIR failed to differentiate between lard, beef fat, and lard-beef fat mixture. ATR-FTIR, however, could recognize the presence of lard in goats at a percentage higher than 40%, as the last group of samples was in the same cluster as lard. In contrast, the PCA recognizes a mixture of lard at 60, 80, and 90% in goat fat still as "goat fat" since they are clustered on the bottom of the plot together with the pure goat fat (G1 and G2). From the loading bi plot (Fig. S1), this group is predominated by the peak at the wavenumber of 718–724 cm^{-1}, which

Table 1. The major wavenumber detected in animal oils and fats and the corresponding functional groups

Peak code	Wavenumber (cm^{-1})	Functional group [8,17]
A	721.2466	Overlapping of the out-of-plane vibration of cis-disubstituted olefin and the rocking vibration of methylene (CH_2)
B	965.1978	Trans- C–H out-of-plane bending vibration of olefins
C	1113.6900	Fatty acid C–H bending and C–H deformation vibrations
D	1159.0090	Stretch vibrations from the C–O group in esters
E	1235.1830	C–O ester
F	1375.9610	CH_3 group symmetric bending vibrations
G	1461.7780	CH_2 and CH_3 aliphatic groups' bending vibrations
H	1744.2980	Carbonyl group (C=O) from triacylglycerol's ester bond
I	2853.1670	Methylene (CH_2) group's asymmetric and symmetric stretching vibration
J	2921.6280	Methylene (CH_2) group's asymmetric and symmetric stretching vibration
K	3005.5160	*cis-* or *trans-* C–H stretch olefins

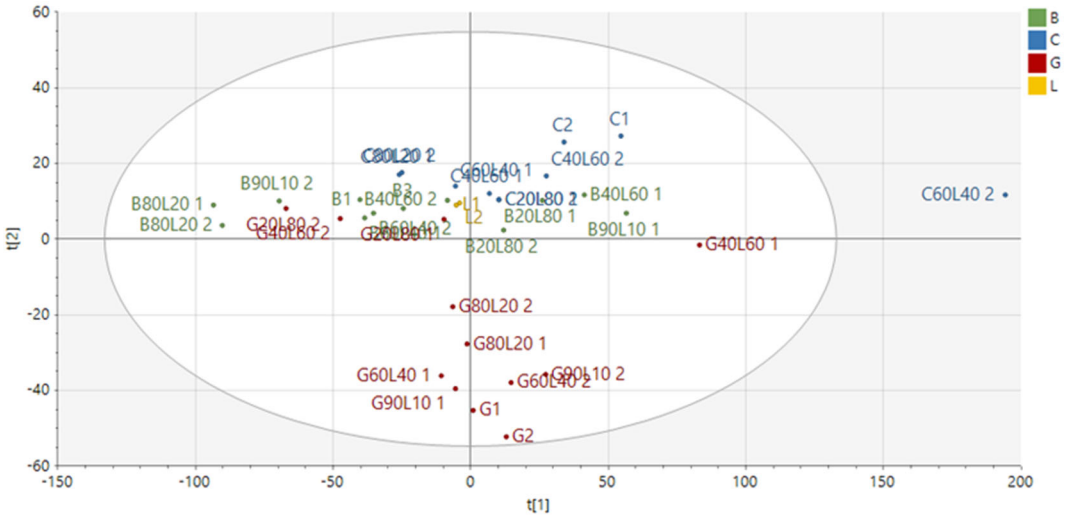

Fig 1. PCA Score plot of ATR-FTIR data of pure animal fat and a lard mixture with non-pork animal fat. (C: Chicken; B: Beef; G: goat; L: Lard; the numbers after the first and second letters show the percentage of animal oil in the mixture; the last number is the replication number)

corresponds to the overlapping peaks of the out-of-plane vibration of cis-disubstituted olefin and the rocking vibration of a methylene group.

ATR-FTIR can analyze various types of food and can provide an estimate of the amount of fat. However, ATR-FTIR is not a good option for identifying between saturated and unsaturated fatty acids [18]. This can be explained by the fact that the composition of major fatty acids in beef fat is similar to lard, except for C:10 fatty acid, which is present in beef fat but not identified in lard. On the contrary, the composition of major fatty acids in chicken fat differs from that of lard. Lard contains C:10, C:15, and C:17 fatty acids, which are absent in chicken fat [19].

Volatilome Profile

Untargeted volatile compounds analysis using SPME-GC-MS successfully detected 100 compounds in pure and mixed animal fat. Volatile compound identification was conducted by manually annotating chromatogram peaks with those of the NIST database. The LRI value of each metabolite was also calculated and matched with those of LRI in the literature as a confirmation. The representative chromatogram of pure animal oils and fat is presented in Fig. 2. Each sample had a different volatile compound composition. Table 2 summarizes all volatile compounds identified in pure animal oil and fats. They all fall into several groups, namely acids, aldehydes, alcohols, aliphatic hydrocarbons, cyclic hydrocarbons, esters, heterocyclics, ketones, organosulfurs, terpenes, alkanes, and ether.

Principal Component Analysis of Volatile Data

PCA analysis of volatilome data with Pareto scaling had an R^2X value of 0.884 and a Q^2 value of 0.557. Thus, the model is reliable [14]. The PCA score plot (Fig. 3) showed that all samples were clustered into three groups. All beef fat samples, pure or as a mixture with lard (green), were clustered on the upper part of the plot (group 1). Goat fat samples, pure or as a mixture with lard (red), were grouped on the left part of the plot (group 2). Pure lard (yellow) overlapping with pure chicken fat and a mixture of chicken fat and lard at all ratios were in the same group located on the right-bottom of the plot (group 3). This data indicated that lard, chicken fat, and their mixture had similar volatile profiles. An interesting pattern can also be seen in the PCA of volatile data. A mixture of lard–goat fat and lard–beef fat with a high percentage of lard (> 40%) was located close to the group of pure lard. It indicated that based on the volatile compounds, lard in goat or beef fat can only be detected at a percentage higher than 40%.

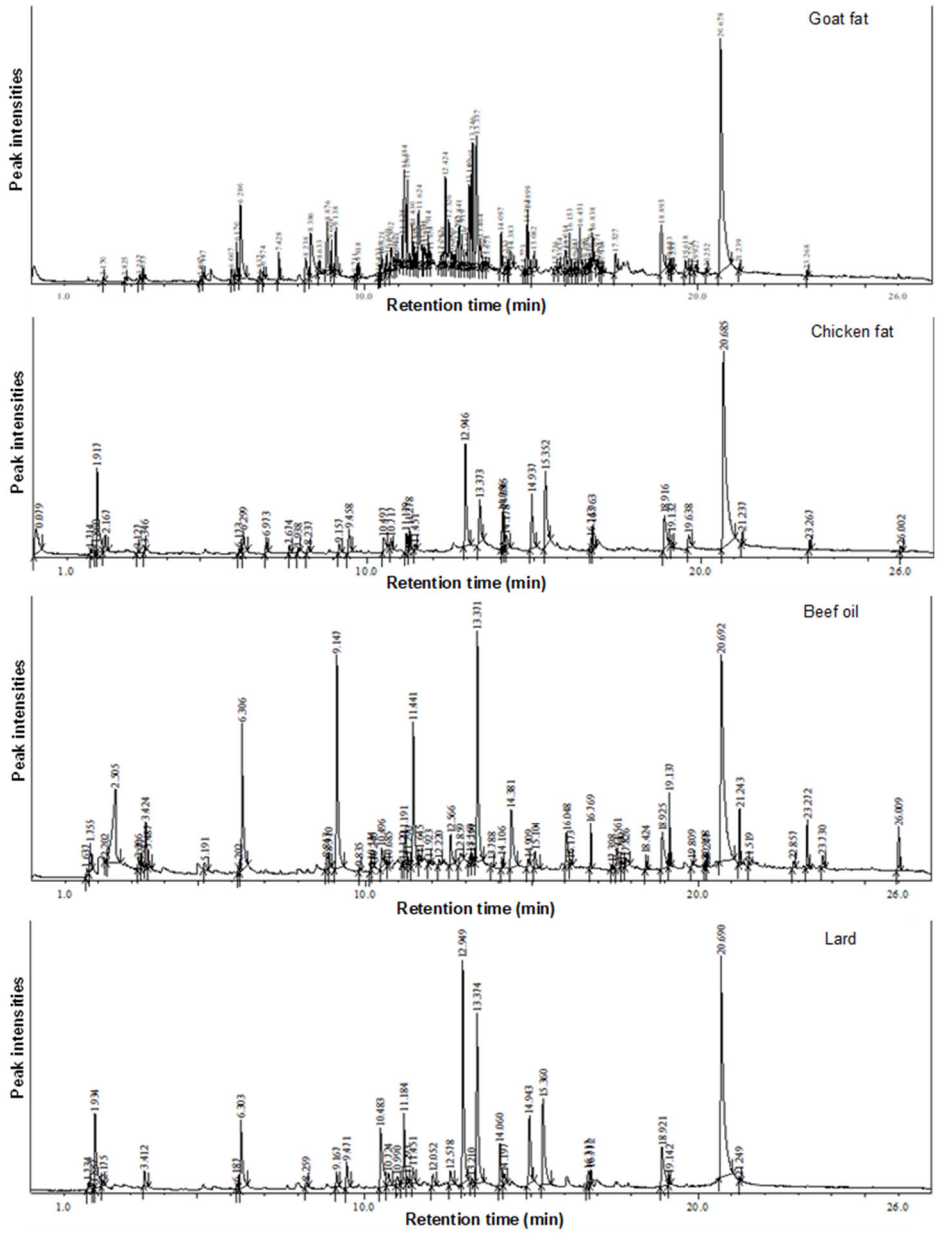

Fig 2. Representative chromatogram (total ion chromatogram) of pure animal oils and fats from SPME-GC/MS analysis

Table 2. List of volatile compounds identified in lard, chicken fat, beef oil, and goat fat

Compounds	LRI	Identification method[a]	CAS	Peak area relative (10^4) ± Standard deviation			
				Lard	Chicken fat	Beef oil	Goat fat
Acid							
Acetic acid	602	L	64-19-7	-	-	406.24±92.49	-
2-Ethylhexyl pentadecafluorooctanoate	993	M	-	-	-	-	28.39±21.14
Nonyl trichloroacetate	1085	M	65611-32-7	-	-	-	-
Methyl dodecanoate	1526	L	111-82-0	-	-	-	-
Aldehydes							
Pentanal	698	L	110-62-3	40.35±2.47	-	109.34±2.24	-
Hexanal	800	L	66-25-1	221.87±8.91	78.29±12.09	465.13±52.44	258.37±66.77

Compounds	LRI	Identification method[a]	CAS	Peak area relative (10^4) ± Standard deviation			
				Lard	Chicken fat	Beef oil	Goat fat
4-Heptenal	898	L	62238-34-0	-	20.83±2.98	-	-
Heptanal	901	L	111-71-7	36.35±2.27	28.97±1.26	565.44±119.08	77.55±99.62
2*E*-Heptenal	956	L	18829-55-5	245.68±27.55	74.59±20.41	55.13±20.61	-
Octanal	1001	L	124-13-0	43.38±1.31	-	342.41±94.51	35.89±11.96
2*E*-Octenal	1063	L	2548-87-0	43.08±8.62	-	68.13±41.03	-
Nonanal	1102	L	124-19-6	472.23±65.05	191.01±42.27	516.62±158.61	132.76±163.85
2*E*-Nonenal	1159	L	18829-56-6	-	-	219.35±80.51	-
Decanal	1195	L	112-31-2	-	-	38.91±17.97	42.98±16.23
2*E*-Decenal	1260	L	3913-81-3	-	-	95.49±46.48	-
2-Undecenal	1374	M	2463-77-6	-	-	55.47±35.44	-
Alcohol							
1-Methylcyclopropanemethanol	-	M	2746-14-7	-	-	-	-
2-Propen-1-ol	-	M	107-18-6	219.56 ± 22.65	336.77±55.03	-	-
1-Penten-3-ol	673	L	616-25-1	-	-	24.70±2.72	-
1-Hexanol	867	L	111-27-3	-	-	-	229.34±27.42
1-Heptanol	969	L	111-70-6	-	-	-	34.25±30.89
1-Octen-3-ol	986	L	3391-86-4	-	-	-	137.53±174.97
Aliphatic hydrocarbons							
Methylthiirane	606	L	1072-43-1	-	-	-	-
Octane	-	M	111-65-9	10.59±0.52	-	9.93±1.18	106.08±45.11
Decane	999	M	124-18-5	-	-	-	109.25±59.47
3,3-Dimethyloctane	1023	M	4110-44-5	-	-	-	-
3-Ethyl-2-methyl-1,3-hexadiene	1030	L	61142-36-7	32.74±5.30	-	-	-
2,3,6,7-tetramethyloctane	1108	M	52670-34-5	-	-	-	48.38±4.76
3-Vinyl-1,2-dithiacyclohex-4-ene	1191	L	62488-52-2	192.88±30.62	216.76±22.75	-	-
Dodecane	1199	M	112-40-3	-	-	-	-
4,6-Dimethyldodecane	1278	M	61141-72-8	-	-	-	108.78±10.53
3,3-Dimethylhexane	1278	M	563-16-6	-	-	-	-
Cyclic hydrocarbons							
2-Ethylfuran	702	L	3208-16-0	-	-	47.94±5.65	-
Toluene	773	L	108-88-3	-	-	20.58±4.49	27.02±5.02
o-Xylene	894	L	95-47-6	-	-	-	72.38±0.16
Styrene	890	L	100-42-5	-	-	34.16±6.48	-
1-Ethyl-3-methylbenzene	958	L	620-14-4	-	-	-	-
1,2,4-Trimethylbenzene	989	L	95-63-6	-	-	-	56.65±17.19
Propylcyclohexane	984	M	1678-92-8	-	-	-	18.06±10.47
Butylcyclopentane	987	M	13152-44-8	-	-	-	-
2-Pentylfuran	996	L	3777-69-3	-	54.70±5.71	83.27±23.71	-
Mesitylene	997	L	108-67-8	-	-	-	48.63±1.24
1,3-Bis(1,1-dimethylethyl)benzene	997	L	1014-60-4	-	-	-	8.75±0.41
1,3-Dichlorobenzene	1007	L	541-73-1	-	-	18.30±1.59	110.33±78.42
1,2,4,5-Tetramethylbenzene	1131	L	95-93-2	-	-	-	34.68±35.12
Esther							
Dimethyl phthalate	1466	L	131-11-3	179.83±0.88	166.53±23.82	183.20±9.23	136.31±39.02
Diethyl phthalate	1591	L	84-66-2	1143.83±4.07	1199.43±3.49	1232.90±5.84	954.24±123.86
Heterocyclics							
2-Vinyl-4*H*-1,3-dithiine	1217	L	80028-57-5	287.07±47.74	362.27±91.27	-	-
Ketones							
2-Hexanone	791	L	591-78-6	-	-	-	35.90±8.42
2-Heptanone	889	L	110-43-0	-	-	34.08±13.11	130.72±37.77

Compounds	LRI	Identification method[a]	CAS	Peak area relative (10^4) ± Standard deviation			
				Lard	Chicken fat	Beef oil	Goat fat
2-Octanone	992	L	111-13-7	-	-	-	211.49±102.66
Acetophenone	1066	L	98-86-2	-	-	-	-
2-Nonanone	1096	L	821-55-6	-	-	12.79±7.38	171.05±27.52
7-Pentadecanone	1292	M	6064-38-6	-	-	-	80.54±25.15
Organosulfurs							
Allyl methyl sulfide	-	M	10152-76-8	-	13.38±1.05	-	-
Diallyl sulfide	859	L	592-88-1	-	12.26±2.23	-	-
methyl 2-propenyl disulfide	919	L	2179-58-0	70.46±0.58	78.04±7.99	-	-
Dimethyl trisulfide	977	L	3658-80-8	48.67±10.99	48.48±25.84	-	-
Diallyl disulfide	1077	L	2179-57-9	562.97±42.10	363.72±44.45	-	-
Methyl 2-propenyl trisulfide	1132	M	34135-85-8	112.09±22.74	122.83±52.84	-	-
Di-2-propenyl trisulfide	1304	L	2050-87-5	32.61±8.55	31.59±1.64	-	-
Terpenes							
D-Limonene	102	M	5989-27-5	-	-	6.54±2.60	43.89±36.81
Eucalyptol	1046	L	470-82-6	-	-	-	30.57±12.81
Alkanes							
Heptane	-	M	142-82-5	-	-	-	3.66±0.94
Nonane	781	M	111-84-2	-	-	-	-
4-Methylheptane	-	M	589-53-7	-	-	-	12.15±5.09
2,4-Dimethylheptane	-	M	2213-23-2	-	-	-	11.95±6.42
2,4-Dimethyl-1-heptene	842	L	19549-87-2	-	-	-	65.77±27.54
3,3,5-Trimethylheptane	1007	M	7154-80-5	-	-	-	105.65±102.42
Undecane	1094	M	1120-21-4	-	-	-	226.18±152.61
3,7-Dimethyldecane	1125	L	17312-54-8	-	-	-	122.71±69.61
Others							
Borane, compd. with dimethylamine (1:1)	-	M	74-94-2	12.09±1.15	-	82.27±18.42	-
Pentedrone	-	M	879722-57-3	-	-	-	8.05±3.04
2,3-Dihydro-1,4-dioxin	-	M	543-75-9	-	-	-	10.95±4.34
Ether							
n-Butyl ether	788	M	142-96-1	-	-	-	34.27±10.19

Note. [a] Volatile compound identification verification method: L: The compound is identified by mass spectrum similarity analysis in the NIST 14 Library, and the compound has a similar LRI with those available in the data bank (odour.org.uk and NIST Chemistry Webbook); M: Compound identified by mass spectrum similarity analysis in NIST 14 Library, but LRI of the similar compound is not available in data bank (odour.org.uk and NIST Chemistry Webbook).

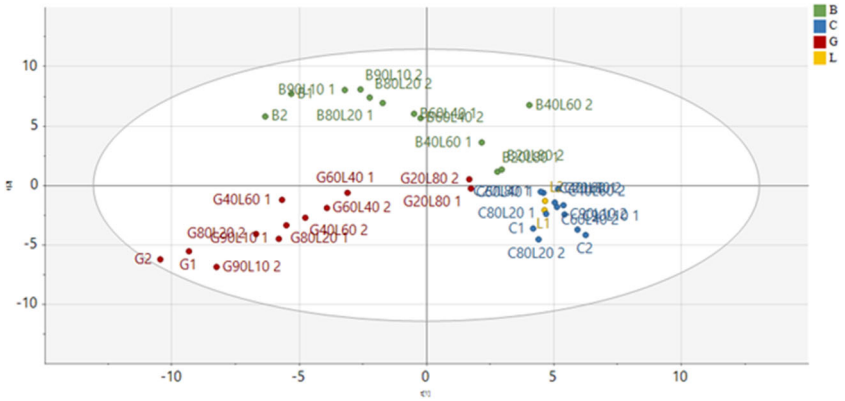

Fig 3. PCA score plot (C: chicken; B: beef; G: goat; L: lard; the number after the first and second letters show the percentage of animal oil in the mixture; the last numbers show the replication)

Less than 20%, PCA still classifies them in the same group with pure goat fat or pure beef fat, respectively.

Next, we constructed three classes OPLS-DA based on the grouping in the PCA (Fig. 4). The OPLS-DA score plot (Fig. 4(a)) showed a clear separation between the three classes, whereas the loading plot (Fig. 4(b)) showed compounds responsible for each class. While information on the volatile composition of animal oils is still limited, most of these compounds were previously reported in their respective meat. For example, heptanal, one of the discriminating volatiles for group 2, was detected in beef [20]. 1-Hexanol, one of the predominant volatiles in group 2, was identified as one of the major volatiles in fresh goat meat [21]. 2-Propen-1-ol, a discriminating factor for group 1, was reported as the major alcohol in cured pork [22].

The classification pattern we obtained after subjecting volatile data of all samples to PCA and OPLS-DA did not allow us to identify a specific marker for each pure animal oil since the models cannot differentiate between the pure oils and their mixture with lard. Using an X-variant plot belonging to each volatile of interest, we can observe the relative distribution of discriminating volatile compounds over the samples in groups 1 (green), 2 (blue), and 3 (red). These plots were generated using SIMCA software. Each identified volatile has its X-variant plots that can be used to see how the compound distributes (relatively) over the samples. On the X-axis, we have the name of the samples, whereas the Y-axis represents the intensity of the compound. The drawn line has green, blue, and red colors, which actually refer to the groups or classes we generated when conducting

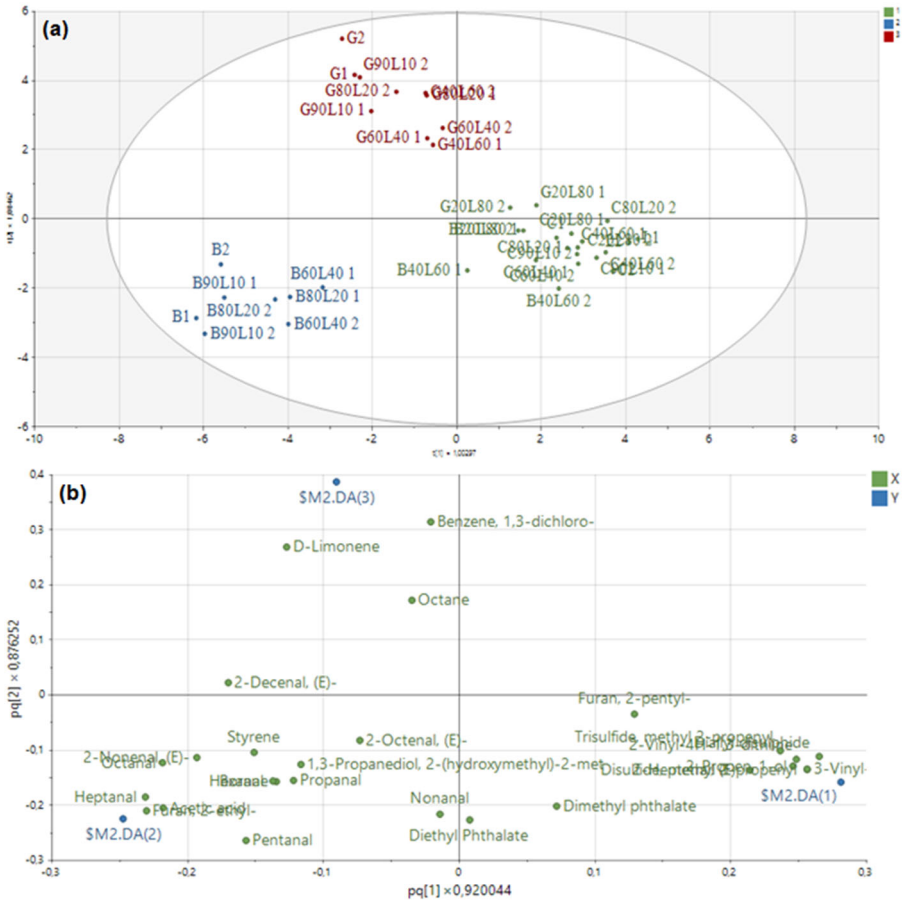

Fig 4. (a) The score plot and (b) loading plot of three classes OPLS-DA of volatile compounds of pure animal oils and fats and a mixture of lard with non-lard/fat at different ratios (C: chicken; B: beef; G: goat; L: lard; the number after the first and second letters show the percentage of animal oil in the mixture; the last numbers show the replication)

OPLS-DA volatile data analysis. By observing the trend of the line, including the color, we can get information on which class or group of particular compounds are the highest, the lowest, or the medium.

In the loading bi plot, compounds located close to the sample code indicate their relative abundance in the sample. As an example, the X-variant plots of 2-heptenal and diallyl disulfide, two discriminating volatiles of group 1 in OPLS-DA were chosen (Fig. 5). The highest relative concentration of 2-heptenal was found in lard, followed by chicken fat. The lowest was in beef oil, but it was not detected in goat fat. 2-Heptenal in chicken fat and beef oil increased after being spiked with lard. A similar pattern was observed in goat fat, particularly at a lard percentage higher than 60%. Diallyl disulfide is another discriminating volatile for group 1. Lard and chicken fat had the highest content of this compound, but it was absent in beef and goat fat. After spiking with lard, diallyl disulfide content in beef and goat fat gradually increased.

Pentanal is the discriminating volatile for group 2 (samples labeled in blue). It was the major volatile distributed in beef fat (Fig. 6), but a very low amount in lard was not detected in chicken fat and goat fat. However, the relative concentration of this compound in chicken fat and goat fat increased after being spiked with lard. Pentanal was reported as the major aldehyde in oxidized tallow [23] and at low amounts in the heated lard [24]. A small increase of pentanal in chicken fat and goat fat was probably also induced by the exposure to the heating during the SPME extraction process, besides the lard addition. The relative concentration of D-limonene

Fig 5. Distribution of (a) 2-heptenal and (b) diallyl disulfide, two discriminating volatiles of group 1 in OPLS-DA volatile data. (C: chicken; B: beef; G: goat; L: lard; the number after the first and second letters show the percentage of animal oil in the mixture; the last numbers show the replication). The color of the line (green, blue, or red, corresponds to the sample class made in OPLS-DA (Green = class 1, blue = class 2, red = class 3)

Fig 6. (a) Distribution of (a) pentanal and (b) D-limonene, two discriminating volatiles of group 2 and 3 in OPLS-DA volatilome data, respectively. (C: chicken; B: beef; G: goat; L: lard; the number after the first and second letters show the percentage of animal oil in the mixture; the last numbers show the replication). The color of the line (green, blue, or red, corresponds to the sample class made in OPLS-DA (Green = class 1, blue = class 2, red = class 3).

was the highest in goat fat, a small amount in beef, and not detected in lard and chicken fat (Fig. 7). Relative concentration of D-limonene in beef and goat gradually decreased after being spiked with lard. D-Limonene and other terpenes are not commonly found in animal fats; rather, they might originate from the feed, as reported elsewhere [25].

We created separate OPLS-DA models for volatile data of each pure oil/fat sample and their mixture with lard. All models showed significant separation among the three groups (pure oil/fat and their mixture with lard at all concentrations) (Fig. 7). Discriminating volatiles were selected from compounds with the largest VIP value, but only those with a positive correlation coefficient. Table 3 shows only the first three compounds with the highest VIP. Several compounds, such as 1,3-dichlorobenzene, diethyl phthalate, and dimethyl phthalate, were identified as significant markers in several classes, especially lard, goat fat, and their mixtures. These compounds were removed from Table 3 since they are not compounds naturally found in oil; their presence can originate from packaging (diethyl phthalate and dimethyl phthalate) [26] or fumigant/herbicide (1,3-dichlorobenzene) [27].

Most of the lard's volatile markers in all OPLS-DA models identified in this study were previously detected in lard, such as 2-heptanol, hexanal, nonanal, 2-propen-1-ol, diallyl disulfide [28], and 2-pentyl furan [29]. No reports on the presence of sulfur-containing volatiles, 3-vinyl-1,2-dithiacyclohex-4-ene, in lard. While there are very limited reports on goat fat, octane and nonanal,

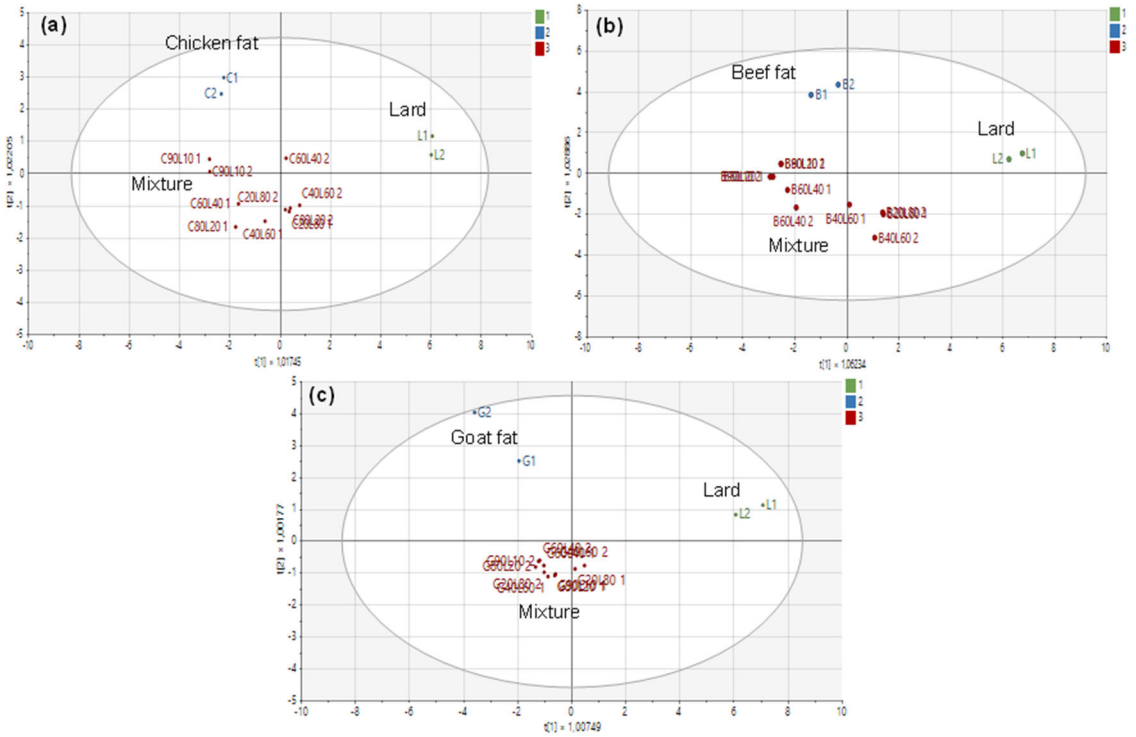

Fig 7. Score plot of separate OPLS-DA of volatile data (a) lard, chicken fat, and their mixture; (b) beef fat lard and their mixture; (c) lard, goat fat, and their mixture. All OPLS-DA models have Q^2 values of 0.501, 0.717, and 0.855

they were identified in small amounts in goat ham [25]. Previous reports on the three major volatile markers for chicken fat reported here could not be found, but other volatiles with lower VIP and not listed in Table 3 were detected in chicken broth [30]. The three volatile markers for beef oil were previously reported in the heated beef oil [31]. Interestingly, the markers for lard in the three OPLS-DA models are different. It can be explained that the three OPLS-DA models were built from the volatile profile of lard and the mixture of lard with different oils/fats: beef oil, chicken fat, and goat fat. When calculating markers for each relevant class, OPLS-DA compares the relative abundance of each variable (the volatiles) in an observation (e.g., lard) against other observations (e.g., beef oil and lard-beef oil mixture). Thus, the results might differ since these three oils/fats had different volatile compositions and relative abundances. Similar patterns were reported in our previous studies to identify volatile markers of wild boar in meatballs made from chicken, rat meat, and beef [14,32]. The results of our study highlighted that food products or ingredients are complex systems. When developing a method to identify markers for a specific adulterant (such as lard in beef fat), one has to consider that markers for the same adulterant can be different when it is used as an adulterant in a different food product (e.g., lard in chicken fat).

The study's findings showed that the volatilomics method—which uses tandem SPME and GC-MS—performs better at detecting lard in animal fats mixture than infrared fingerprinting with ATR-FTIR. PCA built from ATR-FTIR data failed to classify pure animal fats and animal fats spiked by lard, except for chicken fat. However, as previously mentioned, ATR-FTIR method is a rapid, non-destructive procedures that use no solvent and easy sample preparation, making this technique applicable for routine use. Thus, combining FTIR-ATR with multivariate analysis can still be used to detect the presence of lard in chicken fat. On the other hand, OPLS-DA built from the volatile data could recognize the presence of lard in all animal fats at the lowest concentrations used in this study (10%). Thus, the technique can be further developed as a basis for a rapid non-halal fat detection tool with minimal sample preparation applicable to mixture samples.

Table 3. Three selected volatile markers for each class of OPLS-DA with different oil samples

OPLS-DA Model 1: Lard, chicken, and lard-chicken mixture	
Compound	VIP
Class 1: Lard	
2-Heptenal	1.13228
Hexanal	1.14297
Nonanal	1.04939
Class 2: Chicken fat	
2-Propen-1-ol	1.07781
3-Vinyl-1,2-dithiacyclohex-4-ene	1.03407
Methyl-2-propenyl disulfide	1.03615
Class 3: Lard-chicken fat mixture	
Hexanal	1.14297
2-Vinyl-4H-1,3-dithiine	1.07902
Methyl-2-propenyl disulfide	1.03615
OPLS-DA Model 2: Lard, beef oil, and lard-beef oil mixture	
Class 1: Lard	
Diallyl disulfide	1.15934
3-Vinyl-1,2-dithiacyclohex-4-ene	1.15624
2-Propen-1-ol	1.15429
Class 2: Beef oil	
2-Octenal	1.11347
Octane	1.14089
Octanal	1.08102
Class 3: Lard – beef oil mixture	
Diallyl disulfide	1.15934
OPLS-DA Model 3 Lard, goat fat, lard-goat fat mixture	
Class 1: Lard	
Octane	1.51496
Methyl-2-propenyl trisulfide	1.02845
2-Pentyl-furan	1.02079
Class 2: Goat fat	
Octane	1.51496
Nonanal	0.941562
2-Propen-1-ol	0.919347
Class 3: Lard – goat fat mixture	
2-Pentyl-furan	1.02079
D-Limonene	0.989529
Diallyl disulfide	0.972793

■ CONCLUSION

ATR-FTIR can identify the existence of lard in chicken fat at the lowest concentration used in this study (10%) but in goat fat at a concentration higher than 40%. ATR-FTIR failed to differentiate lard from beef oil and lard-beef oil mixtures. SPME technique combined with GC-MS was able to detect lard in beef fat, chicken fat, and goat fat at all concentrations used in this study. OPLS-DA of volatiles data successfully identified markers for each animal oil and fat. Combined SPME techniques with GC-MS could detect the existence of lard in other animal oils in the lowest concentration in the study (10%). Further study is required to verify these results by absolute quantification of each volatile marker.

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■ CONFLICT OF INTEREST

The authors declared no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Silmiyah Putri, Heryani, Muhamad Fauzi Ramadhan, and Yane Regiyana conducted the experiment. Silmiyah Putri, Faleh Setia Budi, Sugeng Heri Suseno, and Nancy Dewi Yuliana wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

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