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Profiling of Volatile Compounds in Beef, Rat, and Wild Boar Meat using SPME-GC/MS

(Pemprofilan Sebatian Meruap dalam Daging Lembu, Tikus dan Babi Hutan menggunakan SPME-GC/MS)

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ABSTRACT

The high beef price triggers adulteration of beef and other *non-halal* animal meat, such as wild boar and rats. An appropriate and effective analytical method is needed to differentiate *halal* and *non-halal* animal meat. The SPME/GC-MS method could authenticate meat based on specific volatile compounds in each meat. The objective of this study was to characterize volatile compounds and determine the volatile marker in raw beef, rat, wild boar meat, and their mixtures using SPME/GC-MS. The chemometrics of principal component analysis (PCA) and orthogonal projection to latent structure discriminant analysis (OPLS-DA) classified raw beef, rat, wild boar, and their mixture. Correlation coefficients and VIP values were used to determine the volatile marker compounds for each meat in the OPLS-DA classes. The OPLS-DA results that the most robust marker in the beef class was dimethylfulvene, benzyl alcohol in the rat class, and 1,3,5-cycloheptatriene in the wild boar class. Furthermore, the most robust marker in the mixture of beef and rat class was benzaldehyde, 3-ethyl-, while 2,6-dimethyldecane was dominant in the mixture of beef and wild boar class. However, further study using larger number of samples which include commercial meat is required to confirm these results.

Keywords: Adulteration; chemometric; meat; OPLS-DA; volatile

ABSTRAK

Harga daging lembu yang tinggi mencetuskan pengadukan daging lembu dan daging haiwan lain yang tidak halal, seperti babi hutan dan tikus. Kaedah analisis yang sesuai dan berkesan diperlukan untuk membezakan daging haiwan yang halal dan tidak halal. Kaedah SPME/GC-MS boleh mengesahkan daging berdasarkan sebatian meruap tertentu dalam setiap daging. Objektif kajian ini adalah untuk mencirikan sebatian meruap dan menentukan penanda meruap dalam daging lembu mentah, tikus, daging babi hutan dan campurannya menggunakan SPME/GC-MS. Kemometrik analisis komponen utama (PCA) dan unjuran ortogon kepada analisis struktur-diskriminasi terpendam (OPLS-DA) mengelaskan daging mentah, tikus, babi hutan dan campurannya. Pekali korelasi dan nilai VIP digunakan untuk menentukan sebatian penanda yang tidak menentu bagi setiap daging dalam kelas OPLS-DA. Keputusan OPLS-DA bahawa penanda paling teguh dalam kelas daging lembu ialah dimetilfulvene, alkohol benzil dalam kelas tikus dan 1,3,5-cycloheptatriene dalam kelas babi hutan. Tambahan pula, penanda yang paling kukuh dalam campuran kelas daging lembu dan tikus ialah benzaldehid, 3-etil-, manakala 2,6-dimetildekana adalah dominan dalam campuran daging lembu dan kelas babi hutan. Walau bagaimanapun, kajian lanjut menggunakan bilangan sampel yang lebih besar termasuk daging komersial diperlukan untuk mengesahkan keputusan ini.

Kata kunci: Daging; kemometrik; meruap; OPLS-DA; pengadukan

INTRODUCTION

The high beef price triggers adulteration of beef and other non-halal animal meat, such as wild boars and rats. *Halal* meat is meat that is allowed to be eaten and slaughtered according to Islamic sharia (Muhamad et al. 2018). The current authentication method is able to distinguish types of *halal* and *non-halal* animal meat (Nakyinsige et al. 2012).

Wild boar is used for food and sports hunting all over the world. The prospect of breeding wild boars has sparked interest in this species as a meat producer (Sales & Kotrba 2013). Wild boar meat is much cheaper than beef, thus triggering adulteration of beef. In addition, beef adulteration using rat meat, such as in meatballs, possibly occurs as the increase of rat population in the field. In Indonesia, several cases of beef adulteration with rat meat were reported in mass media. The reason of beef adulteration was due to the high cost of beef in Indonesia that triggered unethical producers to substitute illegally beef with rat meat (Rahmania et al. 2015). For this reason, an appropriate and effective analytical method is needed to authenticate *halal* and *non-halal* animal meat.

Molecular biology-based and enzyme-linked immunological approaches are used to identify meat adulteration (Tian et al. 2013). In the past two decades, molecular-based technologies became an invaluable option for detecting food authenticity and integrity (Ellis et al. 2016). Moreover, metabolomics is emerging as a powerful tool for studying, identifying crucial biomarkers, and showing metabolic mechanisms (Peng et al. 2015). The main technique in metabolomics is metabolic fingerprinting, a non-targeted technology that considers all measurable peaks or signals, including those from unknown analytes for sample classification (Garcia & Barbas 2011). Volatilomics is a branch of metabolomics that studies volatile metabolites detection, characterization, and quantification in biological systems. Its contribution is critical in food-related domains, including food safety, quality, and authenticity (Lytou et al. 2019).

In volatilomics, the synergistic combination of gas chromatography (GC) and Mass Spectrometry (MS) is a primary analytical workhorse. The GC separation process chromatographically resolves analytes based on their volatility and polarity. MS, on the other hand, detects ions and creates a mass spectrum for each analyte, which aids in identification. (Yip & Chan 2013). Therefore, the GC-MS method could authenticate meat. Various chromatographic techniques using GC provide the possibility for fast and reliable separation in determining

non-halal components. One requirement for separating mixtures by GC is that the analyte must be volatile. The chromatographic technique is suitable for identifying volatile compounds in meat because each type of meat has a distinctive aroma related to its volatile components (Chen et al. 2019; Shahidi et al. 1986).

Solid-phase microextraction (SPME) could be used to facilitate sample preparation on GC-MS instrumentation. SPME is frequently used in analytical practice because of its simplicity and solvent-free operation, short extraction time, and the possibility of automation. In addition, the approach is popular because of its simple connection to GC and reasonably good results in the isolation of trace analytes (Balasubramanian & Panigrahi 2011; Spietelun et al. 2010). SPME could be used as a routine sample preparation technique because of its simple operation, good repeatability, and low cost (Cavalli et al. 2003; Kataoka 2002). The sample is put into a vial, mixed with or without solvent, heated to a specific temperature, and the target analyte is absorbed into the SPME fibre layer. After reaching equilibrium, the sample is injected into GC for further analysis (Soncin et al. 2007).

Studies have been conducted on identifying volatile compounds using SPME-GC-MS in several raw animal meat species. They included identification of volatile compounds to authenticate fresh pork, duck, and goose (Soncin et al. 2007). Fresh and cooked Iberian and Lean pigs produced different volatile compounds (Estévez et al. 2003). Ramírez et al. (2004) examined pork fried in four different oils, producing different volatile compounds according to the oil used. Mini-roast pork analysis identified 86 volatile compounds (Xie et al. 2008). Similarly, four different breeds of cattle have different volatile compounds (Chen et al. 2019). In line with this, Pavlidis et al. (2019) identified volatile compounds from fresh pork and beef.

This study determined volatile compounds presence in raw meat of beef, rat, wild boar, and their mixtures using SPME-GC/MS. The discriminating volatile compounds for each group of samples were determined using multivariate data analysis. Soft independent modelling of class analogy (SIMCA) as an established method for multivariate classification used for volatile compound data analysis of raw meat (Bylesjö et al. 2006). The chemometrics of Principle Component Analysis (PCA), an unsupervised feature of multivariate data analysis, was used as a first-pass method to identify differences in volatile compounds of beef, rat, wild boar and their mixtures (Nejadgholi & Bolic 2015). The combined use of PCA, and Orthogonal Projection to

Latent Structure - Discriminant Analysis (OPLS-DA) in data processing provide valuable insights into general spectral trends and predictive spectral features of the group of the meat type under study (Worley & Powers 2016). PCA was used to classify volatile compound in raw meat according to the animal type. PCA aims to reduce the dimensionality of multivariate data while preserving relevant information. OPLS-DA was then used to refine the classification pattern obtained from PCA (Eriksson et al. 2013). Cross-validation and response permutation tests were then used to test the reliability of the resulted PCA and OPLS-DA models. Discriminating volatile compounds for each type of meat was selected based on the correlation coefficient and Variable Influence on Projection (VIP) values.

MATERIALS AND METHODS

MATERIALS

The rat sample (*Rattus argentiventer*) was selected from 200 female rats weighing 80-200 g from the rice field study center of Subang, West Java, Indonesia. The raw rat meat was separated from the bones, then ground and mixed. The wild boar (*Sus scrofa*) meat samples were selected from three female wild boars weighing 50-60 kg from Banyu Asin Forest, South Sumatera, Indonesia. The frozen wild boar was wrapped in sack and transported to Bogor. Beef sample was Brahman cross beef (*Bos taurus*) weighing 400-550 kg, which is obtained from a halal slaughterhouse in Bogor. All meat samples were stored in the freezer (-33 °C) before use.

METHODS

Raw meat sample preparation

Frozen raw meat samples were cut, minced, and weighed to 8 g with the formula according to Table 1. The total sample used in this study was 25 samples of raw meat. The minced raw meat samples were put into a 22 mL glass vial with PTFE/Silicone septa (Agilent) to extract the volatile compound. The raw beef, rat, and wild boar samples were made in three replicates. The raw meat was mixed with the composition following the common adulteration practice in Indonesia, such as beef mixed rat or beef mixed with wild boar, but no mixture of rat and wild boar meats. The ratios of the raw meat mixtures were 20:80, 40:60, 60:40, and 80:20. The mixture of raw meat samples were made in two replicates.

Headspace SPME method

The headspace solid-phase micro-extraction method was

used to isolate the volatile chemicals in meat samples. The volatiles were absorbed using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 2 mL fiber (Supelco, Bellefonte, PA, USA). Before use, the fiber is heated in a GC-MS injector at 250 °C for 15 min to remove contaminants. For meat analysis, the conditions of SPME sampling used were 8 g of minced meat added into a 22 mL glass vial with PTFE/Silicone septa (Agilent). The vial was closed hermetically, and the contents were put in a water bath for 60 min at 40 °C to extract volatile compounds, and the extracted fiber was injected into GC-MS. Desorption of volatile compounds occurs in the injection port of GC MS for 5 min.

GC-MS method

An Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, USA) and an Agilent 5973C XL EI/CI MSD mass spectrometer was used in this study. Helium gas was used as a carrier at a constant flow rate of 1 mL/min. The injection port was equipped with a 0.75 mm i.d., Agilent liner suitable for SPME. GC-MS analysis was conducted by inserting the fibre previously exposed to the samples into the injection port. The sample was injected in the splitless mode at 250 °C. The compounds were separated in a capillary DB-WAX column with 30 × 0.25 mm dimensions and a film thickness of 0.25 µm (Agilent Technologies, Santa Clara, USA). The temperature of oven was maintained at 40 °C for 5 min and then increased at 4 °C per minute to 150 °C. The temperature was further raised to 250 °C at 30 °C per min and held for 5 min, while the interface temperature was set at 280 °C. The mass spectrometer was operated in the electron ionization mode with the electron energy set at 70 eV, a scanning range of 29-550 m/z, a speed of 1.37 scans/s, and a gain factor of 1. The ion source and quadrupole analyzer temperatures were set at 230 °C and 150 °C, respectively. This method has been used previously by Pavlidis et al. (2019) and Pranata et al. (2021) with slight modifications. Pavlidis used the HP-5MS column, while this study used the DB-WAX column.

DATA ANALYSIS AND COMPOUND IDENTIFICATION

The Agilent GC-MS was used to process the collected raw data, including peak area integration and normalization. This process obtained a data matrix containing sample information and relative intensities of the compounds. GC-MS data was manually annotated based on metabolites mass spectra comparisons between the Chemstation E. 02.02.1431 output and the NIST14 Mass

Spectral Library. Each annotated metabolite's linear retention index (LRI) was calculated by comparing their retention time on the DB-WAX column to the retention time of the alkane solution (C8-40, Sigma Aldrich, Germany; 5mg/L). LRI was used to support the volatile compound identification produced by NIST mass spectral library (Zellner et al. 2008). SIMCA-P software (v.16.0, Sartorius-Umetric, Umea, Sweden) was used to conduct multivariate data analysis (PCA and OPLS-DA). Cross-validation and response permutation tests were then used to validate the PCA and OPLS-DA models.

Cross validation indicator represented by Q^2 values of at least 0.4 are considered acceptable. Permutation test with 100 permutations was performed. In permutation test, a credible model should have a higher Q^2 value than Q^2 values generated by random models utilizing the same data set (Worley & Powers 2016). Significant discriminating compounds for each group were selected based on the VIP and coefficient correlation value (Pranata et al. 2021). The higher the VIP score (greater than 1), the more significant the volatile compound as a marker of each meat or based on the type of animal (Trivedi & Iles 2012).

TABLE 1. Raw meat preparation

Types of raw meat			Code	Replication
Rat (%)	Beef (%)	Wild boar (%)		
100	0	0	R	3
0	100	0	B	3
0	0	100	WB	3
80	20	0	B2R8	2
60	40	0	B4R6	2
40	60	0	B6R4	2
20	80	0	B8R2	2
0	20	80	B2W8	2
0	40	60	B4W6	2
0	60	40	B6W4	2
0	80	20	B8W2	2

RESULTS AND DISCUSSION

VOLATILE PROFILE OF EACH MEAT

The SPME GC-MS method identified volatile compounds in raw beef, rat, wild boar, and their mixtures. Using GC-MS, volatile identification is usually achieved by library search based on comparing the experimental mass spectra with those stored in a suitable library (e.g., via the National Institute of Standards and Technologies, NIST). However, ambiguous identifications can be obtained, especially in the case of structurally related compounds that give similar spectra, thus, reducing the possibility to obtain a complete characterization of the

compounds under investigation (Bianchi et al. 2007). Different methods must be used to validate compound identification (Zellner et al. 2008). Therefore, in this study we used NIST library and also LRI to confirm the compound identification.

The volatile compounds in the raw meat samples are shown in Table 2. Overall, 153 volatile compounds were found in beef, 215 in rats, 151 in wild boar, 517 in a mixture of beef and rat, and 413 in a mixture of beef and wild boar. The five dominant group of volatiles in beef were alcohol, aromatic hydrocarbons, aldehydes, alkanes, and carboxylic acids (Figure 1). The rat was dominated by aromatic hydrocarbons, alcohols, terpenoids, ketones, and aldehydes. Similarly, wild boar contains many

aromatic hydrocarbons, alcohol, ketones, terpenoids, and aldehydes. The mixture of beef and rats contains a lot of volatile compounds of alcohols, aromatic hydrocarbons, terpenoids, ketones, and alkanes. Furthermore, beef and wild boar mixture contain much alcohol, aromatic hydrocarbons, terpenoids, alkanes, and aldehydes. In comparison, alcohol is dominant in beef, while aromatic hydrocarbons are robust in wild boar. Similarly, alcohol is dominant in beef and rats, while wild boar is dominated by aromatic hydrocarbons.

Volatile compounds of meat consisted mainly of alcohols, aldehydes, and ketones, as well as esters, hydrocarbons, and miscellaneous compounds (Pavlidis et al. 2019). However, most of the volatile groups measured by this method is in agreement with the beef

volatiles reported in previous studies. The volatile compounds identified in beef include alcohol, aldehydes, hydrocarbon, ketones, and acids (Bueno et al. 2019; Kilgannon et al. 2020; Wang et al. 2017). Meanwhile, wild boar contained hydrocarbons, aldehydes, ketones, and alcohols (Argemí-Armengol et al. 2019; Lammers et al. 2009). However, carboxylic acids, ketones, alcohols, terpenes, furans, as well as nitrogen, phenolic, and sulfur compounds were not identified in the headspace of raw meat (Ramírez et al. 2004).

In beef, pork, and mixed meat, alcohols, aldehydes, ketones, esters, hydrocarbons, and terpenes were found (Pavlidis et al. 2019). The volatile compounds identified from decaying mice were alkane, aldehyde, alcohol, ketone, sulfur compounds, and aromatic heterocycle (Paczkowski et al. 2012).

1 TABLE 2. Volatile compounds identified in raw beef, rat, wild boar, and their mixtures using SPME/GC MS

Compounds	RT	LRI	Method identification ^a	Peak area ($\times 10^4$)				
				B	R	WB	B/R	B/WB
<i>Aldehydes</i>								
Hexanal	6.3248	1076	L	482.35	194.59	291.99	1596.11	361.56
2-Heptenal, (Z)-	14.8751	1320	L	147.74	44.62	94.31	175.58	219.08
Nonanal	17.2832	1387	L	117.49	99.64	35.67	179.70	43.43
2,4-Heptadien-1-al	20.5474	1478	L	33.18	23.15	-	33.06	50.61
Benzaldehyde	21.3562	1520	L	27.97	76.41	35.16	37.62	43.20
Heptadecanal	34.0440	2008	M	393.92	58.27	531.13	101.61	49.20
n-Octanal	13.5551	1278	L	125.67	-	-	44.42	43.46
2-Nonenal, (E)-	21.8496	1527	L	-	92.68	-	205.77	-
Benzaldehyde, 3-ethyl-	26.7669	1689	M	-	106.50	-	436.88	-
2-Octenal, (E)-	18.3950	1408	L	-	46.62	-	60.46	-
n-Decanal	17.6639	1484	L	-	-	36.33	-	36.95
Benzaldehyde, 2-nitro-, diaminomethylidenehydrazone	11.8963	1231	M	-	-	-	-	143.17
<i>Alkanes</i>								
4,6-Dimethylundecane	6.0096	1066	M	269.52	-	-	148.24	328.84
Undecane	6.7054	1097	L	167.34	-	92.23	-	153.90
Undecane, 4,7-dimethyl-	13.3649	1271	M	59.00	94.75	-	-	46.95
Tetradecane	17.5270	1398	L	73.18	116.26	-	212.53	41.65
Pentadecane	20.7853	1497	L	633.81	130.12	-	193.69	-
5-Ethyl-2-methyl octane	5.5640	1051	M	664.75	-	-	-	438.60
2,6-Dimethyldecane	7.0859	1101	M	285.13	-	-	126.86	257.88
Tridecane	13.7870	1293	L	-	2461.90	-	5378.71	-
Hexadecane	24.0020	1598	L	-	48.22	-	25.81	15.50
Dodecane	10.0171	1198	L	-	-	81.83	256.41	63.20
Decane, 2,3,5-trimethyl-	9.5653	1168	M	-	-	-	497.65	-

Compounds	RT	LRI	Method identification ^a	Peak area ($\times 10^4$)				
				B	R	WB	B/R	B/WB
<i>Alkenes</i>								
Indene	19.6734	1459	M	56.52	65.17	31.06	273.06	74.13
Azulene	27.7006	1723	M	-	-	-	76.27	-
Dimethylfulvene	7.6983	1117	M	3466.34	52.44	187.31	364.85	597.65
1-Cyclohexene, 1-ethynyl-	9.2621	1160	M	1962.09	658.38	220.39	296.83	293.80
4-Ethylcyclohexene	11.1113	1210	M	66.99	51.12	80.00	144.14	89.99
1,3-Hexadiene, 2,5-dimethyl-	25.9763	1662	M	-	39.93	-	-	-
5-Methyl-1-heptene	13.9949	1289	M	-	-	-	21.09	-
1-Undecene, 5-methyl-	16.5342	1361	M	-	-	-	-	57.60
Cyclopentene, 1-ethenyl-3-methylene-	9.4764	1165	M	-	-	-	-	173.01
<i>Alcohols</i>								
1-Heptanol, 2-propyl-	6.1405	1070	M	129.34	-	-	-	314.14
1-Octanol, 2-butyl-	6.8837	1094	M	553.43	-	-	335.58	622.38
2-Pentanol	7.4545	1113	L	8612.74	55.45	331.58	913.26	1167.62
4-Methyl-2-heptanol	11.5810	1223	M	62.40	152.49	76.14	81.09	132.16
3-Methyl-3-butenol	13.0199	1251	L	66.57	53.00	37.95	82.94	52.77
2-Dodecanol	14.2090	1294	M	1211.54	1033.20	-	2359.90	1994.48
1-Undecanol	15.3804	1328	M	34.73	29.43	20.28	62.78	71.49
1-Hexanol	16.3259	1360	L	64.40	197.07	85.30	220.58	42.24
5-Hepten-2-ol, 6-methyl-	20.0659	1464	L	22.68	43.03	25.82	47.93	44.32
1-Octanol	23.0627	1553	L	125.71	184.77	60.97	141.97	44.13
3-Methylbutanol	11.2897	1212	L	105.10	68.40	126.12	67.29	128.65
1-Pentanol	12.8832	1255	L	140.56	32.46	-	120.80	68.99
1-Octen-3-ol	19.6378	1452	L	129.23	411.18	56.60	929.37	192.38
2-Butoxyethanol	18.0146	1402	L	33.23	-	550.77	25.53	486.97
1-Hexanol, 2-ethyl-	20.8850	1488	L	726.75	48.50	-	-	-
Cyclooctyl alcohol	24.7573	1700	L	-	83.59	-	133.25	21.49
Benzyl alcohol	31.7676	1865	L	-	504.48	-	75.59	-
2-Pentanol, 4-methyl-	11.8128	1124	L	-	83.01	-	120.44	-
2-Hepten-1-ol, (E)-	18.1038	1407	M	-	33.05	-	20.82	124.44
1-Heptanol	19.8401	1450	L	-	-	34.29	-	-
3-Phenyl-2-butanol	8.1324	1129	M	-	-	-	196.72	331.52
2,5-Hexanediol, 2,5-dimethyl-	29.3118	1781	M	-	-	-	33.61	-
1,2-Cyclohexanediol, (E)-	20.6961	1487	M	-	-	-	57.72	-
n-Tridecanol	15.0119	1317	M	-	-	-	199.84	-
Ethanol, 2-(dodecyloxy)-	19.2335	1442	M	-	-	-	34.89	-
1-Pentanol, 4-methyl-	16.4746	1360	M	-	-	-	111.67	-
<i>Carboxylic acids</i>								
3-Hydroxybutyric acid	8.9053	1150	M	772.58	110.69	30.32	158.13	97.67
Acetic acid	19.5963	1450	L	139.88	-	60.50	-	41.30
4-Hydroxybutyric acid	24.5492	1581	L	38.76	-	39.03	37.86	29.70

Compounds	RT	LRI	Method identification ^a	Peak area ($\times 10^4$)				
				B	R	WB	B/R	B/WB
2-Amino-6-methylbenzoic acid	30.2870	1818	M	1056.48	3798.28	729.54	2000.87	558.62
2-Amino-5-methyl benzoic acid	30.4951	1826	M	1200.00	2717.26	253.22	1008.46	329.28
<i>Esters</i>								
Isoamyl formate	13.1863	1266	M	63.98	80.40	62.95	352.28	27.63
Methyl caprate	23.7999	1590	L	-	104.46	-	38.75	-
Methyl palmitate	35.4896	2204	L	-	-	-	29.69	-
<i>Eter</i>								
2-Ethoxyethyl ether	18.4664	1418	M	-	-	70.48	58.40	171.45
<i>Heterocyclics</i>								
2-Methylthiophene	7.8886	1123	L	7419.98	129.65	649.76	827.07	888.12
Acridine, 9-methyl-	21.7249	1520	M	501.33	547.30	423.58	409.97	251.08
Furan, 2-pentyl-	12.5382	1249	L	-	48.74	-	28.28	48.07
Indole	36.5957	2441	L	-	-	-	421.85	-
<i>Aromatic Hydrocarbons</i>								
Toluene	4.3448	1018	L	2104.82	1348.89	1685.73	5520.54	1696.66
Styrene	12.1697	1241	L	60.46	41.29	91.72	77.95	99.81
.psi.-Cumene	14.6670	1289	L	37.70	41.01	-	46.16	57.83
p-Cymenene	18.7102	1437	L	85.06	185.84	163.63	94.60	249.15
Naphthalene	27.6173	1718	L	148.81	321.21	138.52	623.70	173.21
m-Ethylphenol	34.4492	2150	L	17.57	28.18	11.88	14.88	-
p-Cresol	34.6336	2073	L	18.07	49.00	14.35	95.03	-
o-Ethylphenol	35.2046	2028	L	19.84	49.23	-	-	-
p-Vinylguaiacol	35.5909	2220	L	109.42	146.72	107.90	44.79	17.73
2,4-Di-tert-butyl-phenol	36.0249	2321	L	194.49	197.41	165.03	73.94	143.31
o-Xylene	10.1004	1183	L	639.08	-	-	181.21	113.03
Benzene, 1,2,3,5-tetramethyl-	19.0254	1430	L	35.97	37.36	25.35	29.86	317.35
Benzene, 1,3,5-trimethyl-	12.7463	1251	L	-	218.57	39.88	123.07	40.69
Anethole	25.8038	1656	M	-	20.11	-	24.02	-
o-Guaiacol	31.1316	1878	L	-	576.76	-	-	-
o-Xylenol	34.5990	2066	M	-	26.97	-	-	-
m-Cresol	34.6400	2115	L	-	24.90	-	-	-
Eugenol	35.1570	2180	L	-	11.92	-	-	-
Isobutenylbenzene	18.4251	1417	M	-	39.79	-	-	-
Benzene, 1,2,4,5-tetramethyl-	17.8363	1419	L	-	20.70	-	-	-
Ethylbenzene	8.6140	1136	L	-	-	175.32	120.75	311.97
m-Xylene	8.7922	1147	L	-	-	246.90	166.11	174.71
Butylhydroxytoluene	32.6416	1899	L	-	-	-	32.32	22.44
p-Xylene	8.6557	1142	L	-	-	-	-	318.06
<i>Ketones</i>								
5-Hepten-2-one, 6-methyl-	15.4577	1337	L	65.06	87.53	23.31	120.46	42.18

Compounds	RT	LRI	Method identification ^a	Peak area ($\times 10^4$)				
				B	R	WB	B/R	B/WB
2,3-Dimethyl-2-cyclopentenone	22.0517	1530	M	41.81	68.76	39.52	227.80	21.17
Acetophenone	25.2508	1645	L	22.55	31.52	23.32	40.48	14.10
2-Heptanone	9.7199	1170	L	289.48	348.61	128.34	425.71	197.47
Cyclobutanone, 2,2-dimethyl-	11.4681	1219	M	77.13	-	116.87	42.97	70.31
2-Heptanone, 6-methyl-	12.2290	1240	L	69.80	138.88	-	58.36	25.32
Acetoin	13.9118	1287	L	-	375.59	877.03	863.35	615.53
3-Octanone, 2-methyl-	15.2497	1322	L	-	82.68	-	124.66	-
2,3-Octanedione	15.8502	1344	L	-	82.03	-	53.77	33.56
2-Nonanone	17.2180	1375	L	-	184.01	34.75	71.57	1949.13
3-Tridecanone	29.0323	1771	M	-	33.46	-	12.38	-
11-Dodecen-2-one	23.8773	1590	M	-	21.60	-	18.56	-
5-Methyl-2-hexanone	9.8628	1155	L	-	-	-	-	84.45
<i>Nitrogen compounds</i>								
1,2,4-Triazol-4-amine, 5-ethyl-3-(3-methyl-5-phenylpyrazol-1-yl)-	22.5157	1546	M	332.07	447.43	283.00	391.82	1483.91
Diethyltoluamide	35.8525	2280	M	177.67	255.77	83.79	99.40	117.31
Hydrazine, 1,2-dimethyl-	19.6680	1455	M	-	83.73	-	-	-
Benzyl nitrile	32.7546	1912	L	-	57.19	-	61.15	-
<i>Sulfur compounds</i>								
Bis(dimethylthiocarbamyl) sulfide	33.9230	1997	M	-	-	-	-	42.86
Methyl diethyldithiocarbamate	33.9854	1999	M	55.13	74.64	486.30	1050.95	517.48
Dimethyl trisulfide	15.0297	1329	L	101.13	88.43	-	502.87	31.91
Di-n-decylsulfone	15.4044	1329	M	-	-	32.28	-	-
<i>Terpenoids</i>								
Limonene	9.5416	1166	L	621.20	497.77	210.63	180.47	178.87
2-Carene	12.6570	1134	L	66.57	55.27	-	47.59	94.76
7-Hydroxycitronellal	20.2917	174	M	19.12	168.88	-	29.05	20.97
1-Terpinenol	23.4611	1573	L	36.04	170.25	-	97.95	41.92
L-Camphor	21.1540	1511	L	86.11	-	56.04	204.74	690.38
Terpinene-4-ol	24.0795	1591	L	29.11	61.68	31.94	29.43	25.97
α -Terpinolene	13.8348	1282	L	107.74	-	-	-	-
d-2-Bornanone	20.9400	1491	L	107.19	363.45	442.06	163.75	434.89
Fenchol	23.6275	1574	L	-	168.68	36.53	79.07	37.94
β -Terpineol	25.0723	1646	L	-	107.57	-	41.13	-
dl-Menthol	25.3280	1630	L	-	44.57	23.50	47.13	17.86
1,3,8-p-Menthatriene	19.9827	1411	L	-	52.21	-	100.95	34.72

^aReliability of identification (L: MS data and RI in agreement with those of authentic compounds; M: MS data in close agreement with the NIST14 Mass Spectral Library)

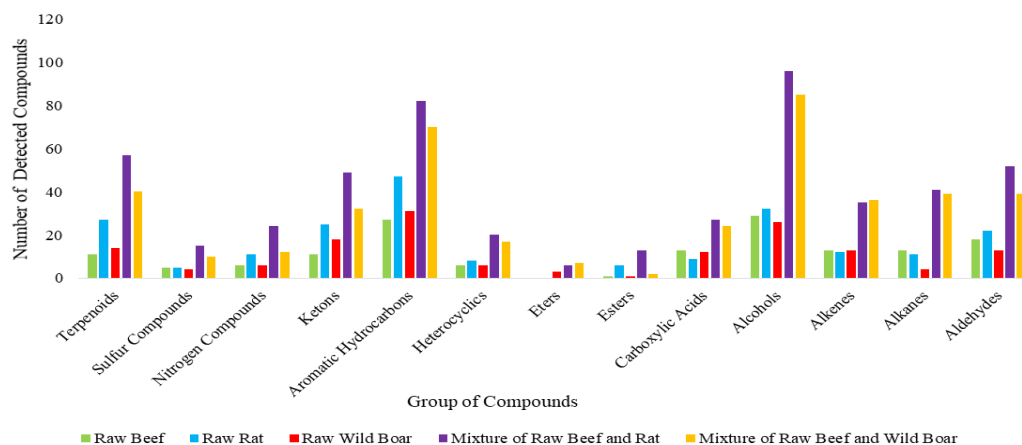


FIGURE 1. Composition of the volatile compounds detected in each type of raw meat and their mixtures

PCA ANALYSIS OF MEAT

Unsupervised PCA evaluates the quality of the market value added (MVA) model conducting supervised OPLS-DA. Unsupervised PCA was performed on the GC-MS data using unit-variance (UV) scaling to assess the meat classification pattern based on volatile compound. Furthermore, multiplicative signal correction (MSC) filtering was applied to remove artifacts and interference unrelated to the target analytes (Eriksson et al. 2013). UV transformation reduces the masking effect in the scaling section and identifies differential metabolites more efficiently (Yang et al. 2015). The PCA model resulted in 0.54 and 0.40 cumulative R^2X and Q^2 , respectively. According to Worley and Power (2013), the validation PCA model's indicator represented by $Q^2 \geq 0.4$ was considered acceptable. Figure 2 illustrates a score plot showing that the separation of meat class samples is apparent. The score plot showed five distinct groups, including raw beef (B1-B3), rat (R1-R3), wild boar (W1-W3), the mixture of beef and rat (BR 1-BR8), and the mixture of beef and wild boar (BW1-BW8) (Figure 2). The mixture of beef and rat cluster was between beef and rat samples, while the mixture of beef and wild boar samples was closer to the wild boar samples. This is probably because, in the mixture of beef and wild boar, the volatile compounds of wild boar were more dominant than beef.

Almost a thousand volatile molecules have been identified in meat or model systems including meat components. For the creation of these many compounds, a variety of reaction processes have been proposed. Vitamin degradation, notably thiamine degradation, heat degradation of carbohydrates and amines, and the

Maillard reaction, including Strecker degradation, are the most common (Bailey 1994).

To observed discriminating volatile compounds for each group, PCA loading biplot is used (Figure 3). Loading biplot shows information about samples classification and at the same time discriminating compounds for each group. Volatile compounds located nearby the particular group were the predominant compounds in that group. Limonene, 1-octen-3-ol, and 1-cyclohexene, 1-ethynyl are among the predominant volatiles in rat group. Heptadecanal, toluene, and indole are dominant in beef group. Heptadecanal, p-cymenene, and indole are the representative major compounds in wild boar group. For mixture samples, there are apparently different marker which depends on the percentage of each meat. For example, BR1, 20% beef and 80% rat, hexanal is found as the one of discriminating compound. In BR3, 40% beef and 60% rat is 3-Octane, 2-methyl. In BR5 (Beef 60% and rat 40%) is toluena.

OPLS-DA ANALYSIS OF RAW MEAT

The orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA) model could classify the raw meat types successfully. Figure 4 shows that the OPLS-DA score plot has better results than PCA. Also, the OPLS-DA model with five classes (class 1 to 5 for beef, rat, wild boar, mixed beef and rat, and mixed beef and wild boar, respectively), showed good separation among samples. The model has a cumulative explanation variance of $R^2X = 0.795$, $R^2Y = 0.947$, and $Q^2 = 0.723$. This indicates the reliability of our OPLS-DA model. According to Zhang et al. (2015), R^2X and R^2Y represent the fraction of the original X and Y data matrixes used to build

the OPLS-DA model and provide an estimation of how well the model fits the X and Y data, respectively. Q^2 represents the predictive accuracy of the model. Worley

and Power (2013) reported a good fitness and acceptable predictability of the OPLS-DA model represented by $Q^2 \geq 0.4$.

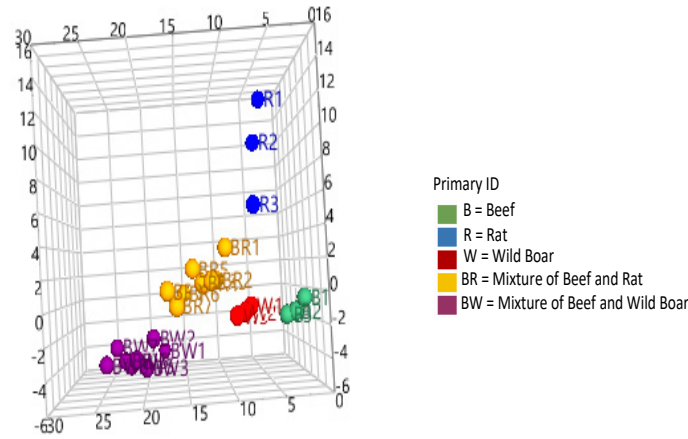


FIGURE 2. PCA score plot of meat volatile data (R^2X 0.54, Q^2 0.40)

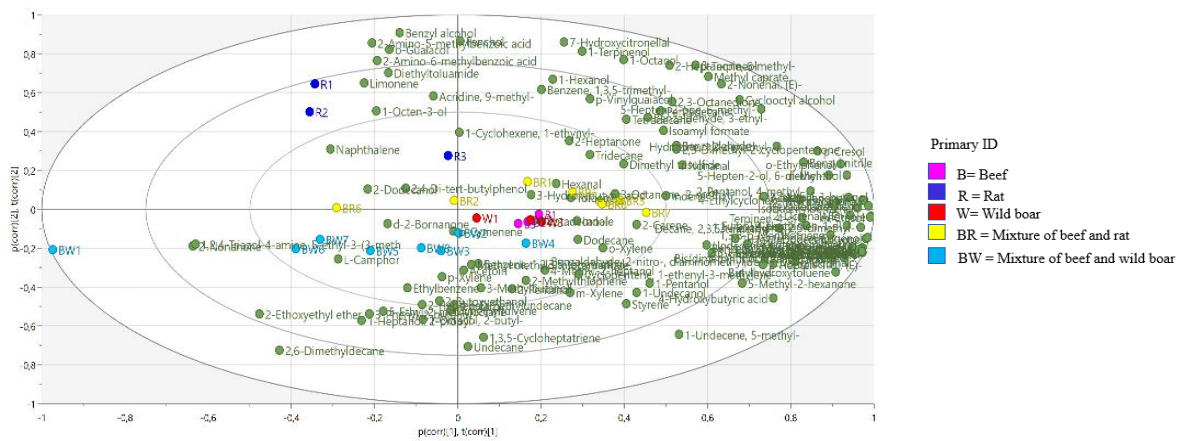


FIGURE 3. PCA loading biplot, showing samples classification and volatile compounds responsible for the grouping. Volatile compounds located nearby the particular group were the predominant compounds in that group

Validation was carried out using 100 random permutations. R^2Y and Q^2Y from the permuted analysis in the bottom-left corner were lower than the associated beginning values in the top-right corner (Figure 5). This suggests that the model is stable and that there is no overfitting (Song et al. 2021). In addition, the cross-validated analysis of variance (CV-ANOVA) p-value was

less than 0.05 (0.0012), indicating that the model was valid (Eriksson et al. 2008).

POTENTIAL VOLATILE MARKER RAW MEAT OF BEEF, RAT, WILD BOAR, AND THEIR MIXTURE
Correlation coefficients and VIP values were used to determine the volatile marker compounds for each type

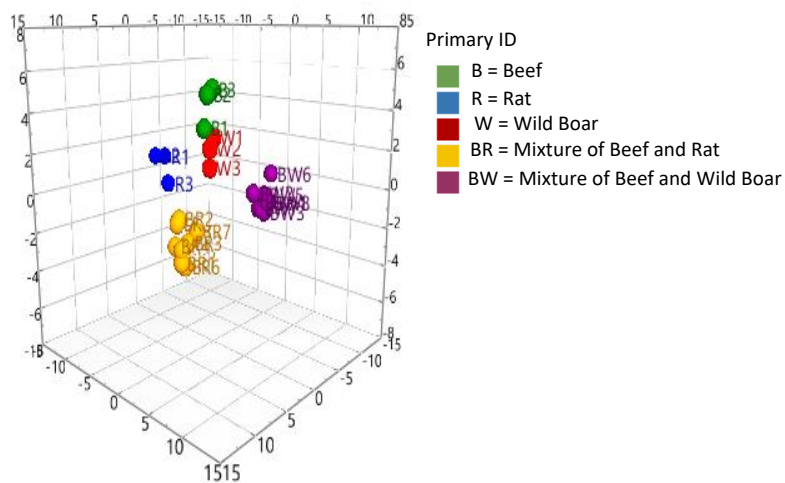


FIGURE 4. OPLS-DA score plot of meat volatile data (R^2X 0,795, R^2Y = 0,947, Q^2 0,723)

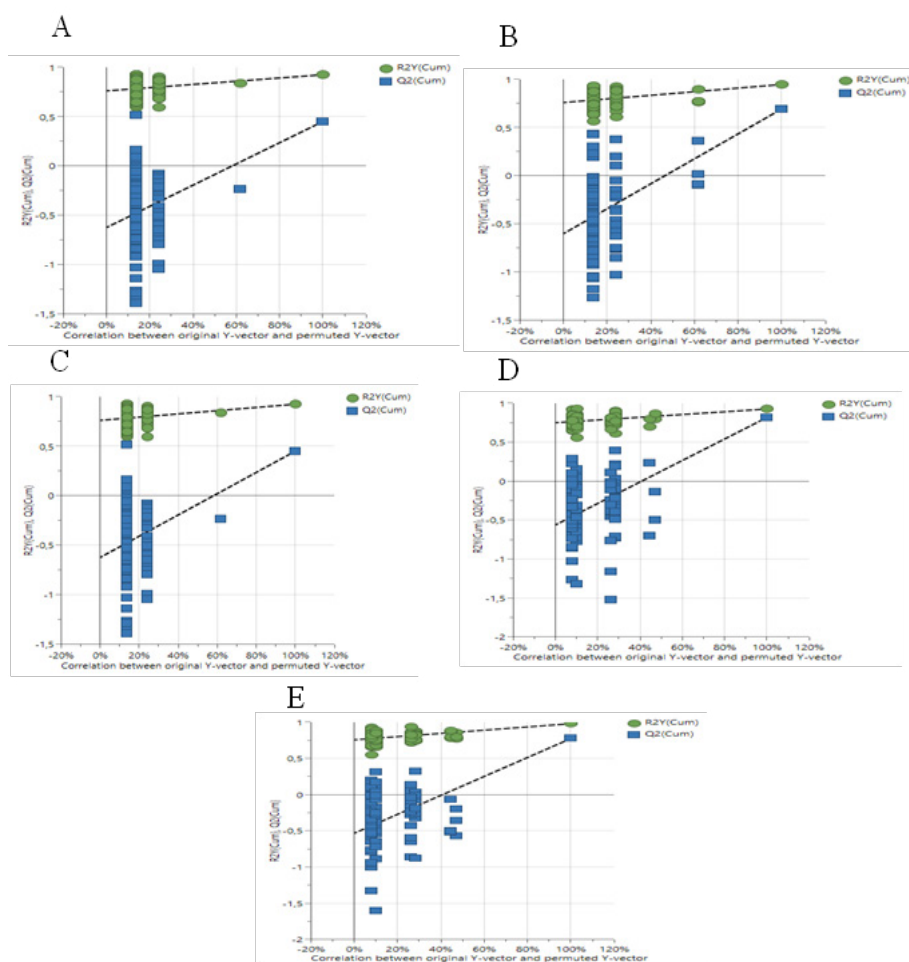


FIGURE 5. Permutation test for the OPLS-DA model for raw meat (A) Beef, (B) Rat, (C) Wild boar, (D) Mixture of beef and rat, (E) Mixture of beef and wild boar

of meat in the OPLS-DA class. The compound positively correlated with the groupings could be determined using the coefficient, while the VIP value has only a positive value. Moreover, ten volatile compounds with higher positive correlation and the VIP values bigger than one were selected from each OPLS-DA class as a marker for each meat class, as shown in Table 3.

Table 3 shows that the marker with the highest VIP value in the raw beef was dimethylfulvene. Other markers include 2-methylthiophene, 2-pentanol, 3-hydroxybutyric acid, 1-cyclohexene, 1-ethynyl-, 1-hexanol, 2-ethyl-, n-octanal, and o-xylene. A previous study stated that octanal, 1-pentanol, and pentadecene were found in beef (Ba et al. 2010). Also, pentanal and octanal have been identified in beef meat (Pavlidis et al. 2019).

The volatile marker with the highest VIP value in the wild boar class was 1,3,5-cycloheptatriene. Other markers were p-vinyl guaiacol, heptadecanal, acetoin, acridine,9-methyl-, 2,4-di-tert-butyl-phenol, 1-heptanol, 4-hydroxybutyric acid and cyclobutanone, 2,2-dimethyl-. A previous study stated that 1-heptanol, heptanal, decanal were detected in wild boar (Lammers et al. 2009). Also, the stale pork was found to have 2,3-butanedione, 3-methyl butyraldehyde, 3-methyl-1-butanol, and acetoin (Sun et al. 2017). The acetoin identified in bacon flavour frequently occurs in food aromas and provides a buttery note to cooked meat (Shahidi et al. 1986). Ramirez et al. (2004) stated that the most abundant volatiles isolated in raw wild boar was carboxylic acids. This study also found 3-hydroxybutyric acid as a marker of wild boar.

The most robust discriminator in the rat class was benzyl alcohol. Other markers were 2-amino-5-methyl benzoic acid, 2-amino-6-methyl benzoic

acid, 1-terpinenol, 7-hydroxycitronellal, 2-heptanone, 6-methyl-, fenchol, methyl caprate, β -terpineol, and limonene. There is no study which examined volatile compounds in the raw rat (*Rattus argentiventer*). The volatile compound 3-hydroxybutyric acid was found in mice with colitis induced by dextran sulfate sodium (Shiomi et al. 2011). Furthermore, decaying mice (*M. glareolus*) were found to have dimethyl disulfide, heptanal, 3-methyl butane-1-ol, octan-3-one, hexan-1-ol, dimethyl trisulfide, nonanal, 1-octen-3-ol, phenol, and indole (Paczkowski et al. 2012).

The mixture of beef and rat class was dominated by benzaldehyde, 3-ethyl-. Other markers were 1-octen-3-ol, hexanal, tetradecane, and p-cresol. There is no study which identified volatile compounds in beef and rat mixture, though 1-octen-3-ol found in decaying mice. The strongest volatile compound mixture of beef and wild boar was 2,6-dimethyldecane, while other markers were methyl diethyldithiocarbamate, 3-phenyl-2-butanol, and 1-octanol, 2-butyl-. A previous study showed that beef, pork, and their mixture contained were 1-octanol, heptanal, heptanal, octanal, 2-butanone, 2-heptanone, decane, undecane. Also, benzaldehyde and decanal were found in beef and pork but absent in their mixture (Pavlidis et al. 2019).

Raw meat has a mild flavour but contains a large number of chemicals that act as precursors of volatile compound. Amino acids, reducing sugars, lipids, and thiamine are the key flavour precursors in meat, and they provide the distinctive scent after heat treatment. Volatile compound production is a two-way process. This is due to Maillard reaction, lipid oxidation, interactions between Maillard reaction products and lipid oxidation products, and thiamine degradation processes (Kosowska et al. 2017).

TABLE 3. Ten compounds with positive coefficient values and the VIP value were selected from each OPLS-DA class of raw meat

Name of volatile compound	VIP	Chemical group
<i>Raw Beef</i>		
2-Methylthiophene	1,62829	Heterocyclics
2-Pentanol	1,55029	Alcohols
3-Hydroxybutyric acid	1,27587	Carboxylic acids
1-Ethynyl-1-cyclohexene	1,17534	Alkenes
2-Ethyl-1-hexanol	1,09697	Alcohols
n-Octanal	1,09452	Aldehydes
o-Xylene	1,03471	Aromatic hydrocarbons

Name of volatile compound	VIP	Chemical group
<i>Raw Rat</i>		
Benzyl alcohol	1,14358	Alcohols
1-2-Amino-5-methyl benzoic acid	1,09352	Carboxylic acids
2-Amino-6-methyl benzoic acid	1,08388	Carboxylic acids
1-Terpinenol	1,07861	Terpenoids
7-Hydroxycitronellal	1,07775	Terpenoids
2-Heptanone, 6-methyl-	1,04034	Ketones
Fenchol	1,07288	Terpenoids
Methyl caprate	1,03776	Esters
β -Terpineol	1,03721	Terpenoids
Limonene	1,02129	Terpenoids
<i>Raw Wild Boar</i>		
1,3,5-Cycloheptatriene	1,439	Alkenes
p-Vinylguaiacol	1,35352	Aromatic hydrocarbons
Heptadecanal	1,28748	Aldehydes
Acetoin	1,24457	Ketones
Acridine, 9-methyl-	1,2101	Esters
2,4-Di-tert-butyl-phenol	1,19468	Aromatic hydrocarbons
1-Heptanol	1,18448	Alcohols
4-Hydroxybutyric acid	1,17281	Carboxylic acids
Cyclobutanone, 2,2-dimethyl-	1,08341	Ketones
<i>A mixture of Beef and Rat</i>		
Benzaldehyde, 3-ethyl-	1,16846	Aldehydes
1-33-Octen-3-ol	1,13275	Alcohols
Hexanal	1,07347	Aldehydes
Tetradecane	1,05026	Alkanes
p-Cresol	1,05753	Aromatic hydrocarbons
Indole	0,973409	Heterocyclics
Dimethyl trisulfide	0,971652	Sulfur compounds
Naphthalene	0,924809	Aromatic hydrocarbon
2-Dodecanol	0,899621	Alcohols
Tridecane	0,840406	Alkanes
<i>A mixture of Beef and Wild Boar</i>		
2,6-Dimethyldecane	1,14107	Alkanes
Methyl diethyldithiocarbamate	1,11832	Sulfur compounds
3-Phenyl-2-butanol	1,06843	Alcohols
1-Octanol, 2-butyl-	1,00244	Alcohols
5-Ethyl-2-methyloctane	0,992474	Alkanes
1-Heptanol, 2-propyl-	0,942047	Alcohols
4,6-Dimethylundecane	0,931248	Alkanes
1,2,3,5-tetramethyl-benzene	0,767247	Aromatic hydrocarbons
2-Carene	0,729666	Terpenoids
(Z)-2-Heptenal	0,690706	Aldehydes

CONCLUSION

Each type of animal meat has a unique volatile compound. This study identified the volatile compound markers in raw beef, rat, wild boar, and their mixtures. The OPLS-DA model showed that the most robust marker in raw beef was dimethylfulvene. Furthermore, the rat class was dominated by benzyl alcohol, while the wild boar contained more 1,3,5-cycloheptatriene. The beef and rat class mixture contained more benzaldehyde, 3-ethyl- while the mixture of beef and wild boar was dominated by 2,6-dimethyldecane. The results of this study showed that SPME-GC/MS could be used for profiling of volatile compounds in beef, rat, wild boar meat, and their mixtures. Further research using larger amount of samples including meat samples bought from the market are required to validate this study. The use of internal standard to allow quantification of volatile compounds which were identified as markers for each group is also necessary to confirm the results of this study.

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