Volatile compounds, texture, and color characterization of meatballs made from beef, rat, wild boar, and their mixtures

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ABSTRACT

The purpose of this research was to characterize the volatile compounds, texture, and color profile of meatballs made from beef, rat, wild boar, and their combinations. Volatile compounds were analyzed using SPME/GC-MS and multivariate data analysis (PCA, PLS-DA). Additionally, several textural features such as hardness, gumminess, chewiness, cohesiveness, and colour (L, a*, b*, C, and h) were also analyzed. The findings revealed that texture and color characteristics can only be used to differentiate meatballs based on their raw meat materials when meat adulterants are used in high concentrations (≥50%). PLS-DA analysis of volatile data revealed distinct groupings among various types of meatballs, including meatballs adulterated with rat or wild boar at the lowest percentage used in this study (20%). By using VIP and correlation coefficient, the strongest markers in beef, rat, and wild boar meatballs were identified as (Z)-2-amino-5-methyl-benzoic acid, 2-heptenal, and cyclobutanol, respectively. Nonanal was consistently found as a significant marker in the meatballs made from a mixture of beef-rat and beef-wild boar at different ratios. This study demonstrated that the volatile profile of meat is more reliable than physicochemical profiles for developing an analytical tool for quickly identifying undesired meat in meat-derived products.

1. Introduction

Meatballs are one of Indonesia's most popular street food. The high price of beef triggers the adulteration of beef with other meats with cheaper price, such as pork, horses, wild boars, and even rat meat. Meat adulteration cases frequently occur in Indonesia. The case is very sensitive when the adulterant is from non-halal animals, since Indonesia is one of the largest Muslim populations in the world. Wild boar is used for food and sport hunting all over the world [1, 2]. The recent increase in natural populations and the potential of farming wild boars have stimulated interest in this species as a meat source [1]. Wild boar is frequently used as a meat adulterant because the price is significantly cheaper than beef. Even worse, in Indonesia, the beef was also found to be adulterated with rat [3]. This is because of the vast population of rats as pest in the paddy fields. Wild boar and rats are haram animals, which means they are strictly prohibited from being consumed by Muslim. This adulteration practice is not only important for halal-haram issue but also for ethical violation in general. Once being processed into meat-derived products such as...
meatball, these different types of meats are difficult to differentiate. Therefore, research providing information on the physical and chemical difference between different type of product made from different meats are highly required. The data then can be used as a basis of development of new authentication tool for meat products - adulteration detection.

Several techniques for meat authentication have been developed. For example molecular biology-based technique using enzyme-linked immunological methods [4] and DNA-based markers [5]. Other techniques include various spectroscopy and chromatography methods which targeting numerous primary and secondary metabolites present in different meat and meat processed products [6, 7, 8, 9, 10]. Metabolomics is one of emerging tool for such a purpose. The main technique in metabolomics is metabolic fingerprinting, which is a non-targeted technology that considers all detectable peaks or signals, including those from unknown analytes, for sample classification [11]. Metabolomics is viewed as potential tool to be applied for significantly reduce food fraud and its negative impacts [12]. Volatilomics is a metabolomics field that detects, identifies, and quantifies volatile metabolites in a biological system. Its contribution is highly important in several food areas, such as safety, quality, and authenticity [13]. GC is a suitable for identifying volatile compounds in meat and its processed product because each type of meat has a distinctive aroma related to its volatile components [14, 15]. SPME could be used to facilitate sample preparation on GC-MS instrumentation. SPME is widely applied in analytical practice because of its simplicity, solvent-free operation, short extraction time, and the possibility of automation. Also, the technique is favored due to its straightforward link up with GC and relatively good results in the isolation of trace analytes [16, 17]. SPME technique coupled to GC-MS was reported as a powerful tool to differentiate various type of samples based on their volatiles profile differences, e.g. fresh raw beef quality with different lipids composition rate [18], four different pig breeds [14], and cooked beef with different period of aging [19], and meatballs made from beef, chicken and wild boar [10]. In addition to meat authentication based on volatiles profile, some studies used meat physicochemical properties for the detection of meat species and their processed products [20, 21, 22].

The objectives of the above-mentioned studies on SPME-GCMS-based volatile compounds and physicochemical property characterization of meat were mostly related to meat quality in general, with the effect of different processing methods, storage conditions, and species or breeds being studied. A similar method can be used to distinguish between meatballs made from halal and non-halal animals. This study determined the volatile compounds and physicochemical profile of meatballs made from halal (beef), and non-halal (rats and wild boar) animals, as well as their combinations at various compositions using SPME-GC/MS. The discriminating volatile compounds for each group of samples were determined using multivariate data analysis. PCA, an unsupervised feature of multivariate data analysis, was used as a first-pass method to identify differences in volatile compounds of meatballs [23]. The combined use of PCA and PLS-DA in data processing provide valuable insights into general spectral trends and predictive spectral features of the group of the meat type under study [24]. The classification pattern produced by PCA was then refined using PLS-DA [25]. Cross-validation and response permutation tests were then used to test the reliability of the resulted PCA and PLS-DA. Discriminating volatile compounds for each type of meatballs were selected based on the correlation coefficient and VIP values. Additionally, their physical properties which include texture and color, were also measured and analyzed using PCA to observe typical texture and color features for each type of meatballs.

2. Materials and methods

2.1. Materials

2.1.1. Raw meat sample collection

All the meat used in this research was purchased from the market. Samples of rats (Rattus argentiventer) were taken at random from a trader in Subang, West Java, Indonesia. Then, 400 female rats were selected with a bodyweight of 80–200 g. The rat meat was separated from the bones, then mixed, ground and homogenized. The wild boar (Sus scrofa) samples were selected from six female wild boars weighing 50–60 kg from the market in Banyu Asin Forest, South Sumatera, Indonesia. The frozen wild boars were wrapped in sack and transported to Bogor city. The ham, belly, and loin parts were taken from each wild boar in equal amounts, mixed and put in a sealed bag. The silverside of six Brahman cross cows (Bos taurus), weighing 400–550 kg, was obtained from the market in Bogor, West Java, Indonesia. The meat was purchased 36 h after the animals were slaughtered in halal slaughterhouse (RPH Bubulak, Bogor, West Java, Indonesia). The meat from different individual wild boars and cows was kept separate until they were processed into meatballs. Before use, all meat samples were kept in the freezer (−33 °C). The meat was thawed for 12 h before processed into meatballs. The meat was ground and homogenized before being processed into meatballs.

2.1.2. Meatball sample preparation

Meatballs are made according to a recipe that is usually made in Indonesia. The meatballs formulation used in this study was summarized in Table S1 (Supplementary Data). The meatball was prepared only using raw meat, tapioca 5% and ice cube 20%. No garlic, pepper, and sodium tri polyphosphate were used to avoid masking effects to the volatile profile of the samples. Meats were cut into small pieces (2 × 2 × 2 cm), then mixed and ground together with tapioca and ice cube. The dough was then rounded manually with diameter of 2.5 cm and weight 10 g, approximately. The raw meatballs were then put in boiling water for 10 min, then drained and cooled at room temperature for 15 min.

Pure beef and wild boar meatballs (MB, MW) were prepared in 3 separate batches using meat from different individual animal, except rat (MR). In case of mixed meatballs preparation, all meat from individual beef, or from individual wild boar, were mixed and homogenized. Meatballs made from a mixture of beef and rat at 4 ratios (2:8, 4:6, 6:4, and 8:2) were prepared separately as individual batches (MB2R, MB4R6, MB6R4, MB8R2, respectively) with 2 replications each, as well as meatballs made from a mixture of beef and wild boar (MB2W, MB4W6, MB6W4, MB8W2). All cooking utensils were carefully washed and drained before preparing the new batch. There were in total 25 batches, resulting in 250 meatballs. Twenty five meatballs were randomly selected for volatile analysis using SPME-GC/MS. For color and texture analysis, meatballs were prepared in similar way. The meatballs were made in 3 independent batches. Five meatballs were taken from each batch.

2.2. Methods of texture, color, and volatile analysis

2.2.1. Texture profile analysis

Meatballs were heated at 80 °C for 5 min and cooled at the room temperature. Then, they were cut off two sides to get 2 of 10 mm depth strips. The texture profile analyses (TPA) of meatballs were determined by using a texture analyser (Model TA-XT2 Texture Analysis, England) and equipped with a 25 kg load cell and the spherical probe (p/0.5 s, 1.2 cm diameter ball probe). The texture analyser's conditions were as follows: pre-test speed of 2.0 mm s⁻¹; post-test speed of 5.0 mm s⁻¹; test time 5.0 s; trigger type auto; and trigger force of 10 g. TPA measurements were done per strip for a total of five meatballs for each batch. The measurements were taken for hardness, springiness, cohesiveness, gumminess, and chewiness [26].

2.2.2. Color

Color values of meatball were determined using chromometer CR-400 (Minolta, Japan) set to the L*, a*, b* color space and illuminant D65, observer angle of 2°, aperture size of 10 mm. The instrument was calibrated using a white standard plate before color readings were performed. The color values were measured following exposure to air for 15
2.2.3. SPME procedure

The volatiles were absorbed using a DVB/CAR/PDMS 2 ml fiber SPME apparatus (Supelco, Bellefonte, PA, USA). Before use, the fiber was heated in a GC-MS injector at 250 °C for 15 min to remove contaminants. Next, 8 g of minced meatball was 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 80 min at 45 °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. To remove volatile contaminants, the fiber was exposed to the GC injection port for 15 min before the analysis [10].

2.2.4. GC-MS protocol

An Agilent 7890A GC (Agilent Technologies, Santa Clara, USA) and an Agilent 5973C XL EI/CI MSD MS were 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 fiber previously exposed to the samples into the injection port. The sample was injected in the splitless mode (250 °C). The oven was set at 40 °C for 5 min and then increased to 150 °C (4 °C min⁻¹). The temperature was further raised to 250 °C (30 °C min⁻¹), and held for 5 min. The interface temperature was maintained at 280 °C. The MS was operated in the electron ionization (70 eV), a scanning range of 29–550 m/z, a speed of 4.37 scans s⁻¹, and a gain factor of 1. The ion source and quadrupole analyzer temperatures were set at 230 °C and 150 °C, respectively [10, 27].

2.2.5. Statistical analysis and multivariate data analysis for texture and colour measurement data

The data of texture and colour were analysed using PCA (SIMCA-P software v. 16.0, Sartorius-Umctic, Umea, Sweden). The data obtained from beef-rat and beef-wild boar meatballs at 40:50 ratios were also analysed using a nested design with the type of meatball as a fixed effect while the individual as a random effect. Duncan’s new multiple range tests were also used to resolve the difference among treatment means. A value of P < 0.05 was used to indicate a significant difference.

2.2.6. Data processing and multivariate data analysis for volatile compounds

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 also 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; 5 mg/L). The identified volatile compounds were input as raw data for PCA and PLS-DA (SIMCA-P software v. 16.0, Sartorius-Umctic, Umea, Sweden). Pareto scaling was used to remove noise caused by instrument error or other possible causes before the data was analyzed using multivariate data analysis. Cross-validation and response permutation tests were used to validate the PCA and PLS-DA models. The validation indicator represented by Q² values of at least 0.4 are considered acceptable. A credible model should have a higher Q² value in permutation testing than Q² values generated by random models utilizing the same data set [24]. Significant discriminating compounds for each group were selected based on the VIP and coefficient correlation value [10].

3. Results and discussion

3.1. Texture and colour profile of beef, rat, wild boar, and the mixture meatballs

3.1.1. PCA analysis

PCA with 4 components for texture and colour measurement data was first conducted with only meatballs made from pure beef, rat and wild boar (PCA1). The PCA explained 92.7% of total variation (R²X = 0.927) with Q² = 0.59, indicating model reliability [24]. The score plot and loading plot of the first two components was shown in Figure 1A (i) and (ii), respectively. A clear grouping pattern between the three types of meatballs was observed. Next, PCA was also separately conducted for texture and colour measurement data of pure beef meatballs, pure rat meatballs, pure wild boar meatballs, and a mixture of beef-wild boar meatballs (PCA2) as can be seen in Figure 1B (i) and (ii) (five components, R²X = 0.936 and Q² = 0.483). Lastly, PCA was conducted for pure beef meatballs, pure rat meatballs, pure wild boar meatballs, and a mixture of beef-rat meatballs (PCA3), presented in Figure 1C (i) and (ii) (three components R²X = 0.801, and Q² = 0.581). In the PCA2 score plot of the first two components as shown in Figure 1B (i), meatballs made from a mixture of beef and wild boar at different compositions were scattered between pure beef and pure wild boar meatballs. Interestingly, their positions reflect the percentage of the meat type; for example, meatballs made up of 80% beef and 20% wild boar (MW28B at all replications) were closely clustered around pure beef beef meatballs, which were followed by their counterparts (MW48B, MW68B, and MW88B). The last group, which made up of 80% wild boar and 20% beef (MW88B), was the closest to the pure wild boar meatballs. A similar pattern was observed in PCA3 score plot (Figure 1C (i)), where meatballs made from the mixture of beef and rat at different compositions were scattered between pure beef and pure rat meatballs. In the loading part of all PCAs (as presented in Figure 1A (ii), B (ii), and C (ii)) a similar pattern of textural and colour features unique to each cluster was observed. Beef meatballs were characterized by high cohesiveness, hardness, chewiness, and gumminess, while wild boar and rat meatballs were the opposite. Wild boar had a typical high score of C, L, and b* values. Rat meatballs were characterized by a high redness (a*) score.

The texture and color of mixture meatballs are determined by the percentage of raw meat ingredients used. For example, meatballs made up of 20% wild boar and 80% beef were highly influenced by cohesiveness, hardness, chewiness, and gumminess, similar to those of pure meatballs. Only when meatballs were composed of at least 60% wild boar and 40% beef, did the texture and color of wild boar meatballs resembled pure wild boar meatballs. A similar pattern was observed in beef and rat meatballs. These patterns indicated that when wild boar or rat meats were used to adulterate beef in meatball products at a percentage less than 40%, the texture and color properties could not be distinguished from pure beef meatballs.

3.1.2. Two way ANOVA of texture and color data of MR6B4 and MW6B4

For mixture meatballs, those with ratios of beef and non-beef of 40:60 (MR6B4 and MW6B4) were chosen to be separately analyzed using two way ANOVA and Duncan’s test (Table 1). Based on the interview with the trader in the market, this ratio was the most frequently used in adulteration practice. In their respective PCA biplots, these mixtures were located between their counterparts made from pure meats. Separate statistical analysis between beef, rat, wild boar, MR6B4 and MW6B4 revealed that there was no consistent pattern in the measured texture features among different type of meatballs, as shown in Table 1. It is noteworthy that, except for cohesiveness, all texture features of MR6B4 and MW6B4 were significantly different as compared with pure beef meatballs (P < 0.05). For color analysis, only MW6B4 was significantly different in all color features as compared to meatballs. Rat meatballs and MR6B4 was significantly different
from beef meatballs and wild boar in $L^*$ value, indicating that meatballs made with rat as an adulterant might have the darkest color than those of beef and wild boar meatballs.

The results of the textural and color characterization of meatballs as mentioned in Table 1 supported the previous results that it could be useful to distinguish meatballs based on their raw meat materials only when the adulterant meat is used in relatively higher percentage ($\geq 50\%$).

This is in accordance with previous study which measured similar texture and color features of meatballs made from beef, rat, pork, dog, and their mixtures [28]. Currently, there are not so many studies reporting the physical characteristics of meatballs made of different types of meat.

### 3.2. Volatiles profile of meatball samples

Overall, 404 volatile compounds were identified in beef meatballs, 371 in rat meatballs, 283 in wild boar meatballs, 956 in meatballs made from beef and rat mixture, and 885 in meatballs made from beef and wild boar mixture (Table 2).
meat contains a significant amount of nonvolatile chemicals, which act as precursors to volatiles responsible for the flavor of diverse meat products. Amino acids, peptides, saccharides, inorganic salts, and inorganic acid are among the precursors, with amino acids, peptides, and reducing sugars being the most important [29]. It was recently reported that different fresh meats, such as beef, pork, lamb, chicken, and turkey, contained qualitatively and quantitatively different types of amino acids [30]. For example, serine was only identified in pork leg, turkey leg, and chicken breast, but not in lamb and beef legs. Fat and fatty acids are also particularly important in species-specific flavor imparting volatiles formation. A previous report showed that different animal species had different fatty acids profile [31]. Lauric acid, for example, is found in beef and pork but not in lamb or chicken. Some species may contain the same amino acids or fatty acids in different concentrations. Meat thermal processing produces a large number of volatiles as a result of various reactions such as Maillard reactions, lipid oxidation, interactions between Maillard reaction products and lipid oxidation, Strecker degradation, and carbohydrate breakdown [29, 32]. It is understandable that the resulting volatile compounds will differ when the precursors used in these reactions differ qualitatively and quantitatively. In other words, when meat from different species are mixed and heated, the volatiles produced are more diverse than those produced by single meat because more diverse precursors involve in their formation. The data presented above can explain why meatballs made from a meat mixture contained more volatiles than their single counterparts, as found in our study (Figure 2). These findings are consistent with recent research by Leng et al. (2020), who discovered that the total volatile basic nitrogen (TVB-N) content of a mixture of minced beef and pork was significantly higher than the TVB-N content of minced beef alone or minced pork alone [33].

3.3. PCA of volatile data of all meatball samples

Unsupervised PCA was then used to study the meatballs classification pattern based on the volatile compound composition. Exponentially Weighted Moving Average (EWMA) filtering was applied to remove the signal noise. The resulted PCA explained 82.7% of total variation (R² = 0.827) and Q² = 0.661, indicating the reliability of the model [24]. The PCA 3D score plot of the first three components showed that beef meatballs (MB, green bullets), rat meatballs (MR, blue bullets), and wild boar meatballs (MW, black bullets) were well-separated (Figure 3). The meatballs made from a mixture of beef and rat (MBR, red bullet) and a mixture of beef and wild boar (MBW, yellow bullets) were scattered between the beef meatballs, the rat meatballs, and wild boar meatballs. These patterns indicated that the data can be further analyzed using a supervised multivariate data analysis PLS-DA to fine tune the discriminating volatiles for each group.

3.4. PLS-DA of meatballs made from pure beef, rat, wild boar meatballs, and their combinations

In this study, beef meatball adulteration was simulated by combining beef with wild boar or rat meat. Because there has been no report of beef meatballs being contaminated with rat and wild boar meat, this mixture was not used in this study. The discriminating compounds in various types of meatballs were determined using PLS-DA. The analysis was divided into two steps to obtain a clearer classification. PLS-DA was first performed on pure beef meatballs, and pure rat meatballs (Figure 4).

Next, a different PLS-DA model was created for pure beef meatballs, pure wild boar meatballs, and meatballs from a mixture thereof (Figure 5). Each PLS-DA score plot showed a distinct clustering between different groups of samples (Figures 4 and 5).

Validation with 100 random permutations was performed to assess the reliability of the PLS-DA model constructed from the volatile data of pure beef meatballs, pure rat meatballs, and meatballs made from their mixtures (Supplementary Figure S1). R²Y and Q²Y values (green circles and blue squares in the bottom-left corner) of the permuted models were lower than the associated initial values (green circles and blue squares in the top-right corner of the plot). This indicates the model's stability and reliability [34]. Additionally, the p-value for the cross-validated analysis of variance (CV-ANOVA) was 0.000570626, less than 0.005, demonstrating good model validity [35]. Similar permutation test and CV-ANOVA were also used to validate the PLS-DA model created from volatile data of pure beef meatballs, pure wild boar meatballs, and meatballs made from their mixture (Supplementary Figure S2). All the validation data indicated a good reliability of the PLS-DA model. Selection of volatile marker compounds for each PLS-DA class were done based on the coefficient correlation and VIP value. Only volatile compounds with positive coefficient correlation and the VIP values >1 were selected from PLS-DA of meatballs volatiles data.
Table 2. Volatile compounds identified in beef, rat, wild boar meatball and their mixtures using SPME/GC MS.

| Compound          | RT    | LRI  | Method | Peak Area (x104) | B | R | WB | B/R | B/WB |
|-------------------|-------|------|--------|------------------|---|---|----|-----|------|
| **Aldehydes**     |       |      |        |                  |   |   |    |     |      |
| Butanal, 3-methyl | 3.6909| 914  | L      | 829.36           |   |   |    |     |      |
| Pentanal          | 3.7026| 935  | L      | 2210.06          |   |   |    |     |      |
| Glutaraldehyde    | 6.1880| 1072 | M      | 1396.36          |   |   |    |     |      |
| Hexanal           | 6.3545| 1076 | L      | 7228.30          |   |   |    |     |      |
| Heptanal          | 9.4750| 1174 | L      | 7219.52          |   |   |    |     |      |
| n-Octanal         | 13.5789|1280 | L      | 1311.44          |   |   |    |     |      |
| 2,4-Heptadien-1-ol|20.3454|1478 | L      | 1860.34          |   |   |    |     |      |
| Decanal           | 20.8568|1494 | L      | 1130.44          |   |   |    |     |      |
| Benzaldehyde      | 21.1362|1513 | L      | 1497.65          |   |   |    |     |      |
| 2,4-Decadienal, (E)-|29.8771|1808 | L      | 987.45           |   |   |    |     |      |
| Alkanes           |       |      |        |                  |   |   |    |     |      |
| 5-Ethyl-2-methyloctane|6.2343|1053 | M      | 1336.53          |   |   |    |     |      |
| Decane            | 34.0211|2005 | M      | 265.79           |   |   |    |     |      |
| 17-Octadecan      | 34.2055|2025 | M      | 123.68           |   |   |    |     |      |
| Octadecan         | 34.9606|2108 | M      | 138.26           |   |   |    |     |      |
| 2,4-Decadienal    | 28.7054|1777 | L      | 8.12             |   |   |    |     |      |
| 2-Tridecanal, (E)-|21.7309|1520 | M      | -               |   |   |    |     |      |
| 2-Decenal, (E)    | 25.2270|1640 | L      | -               |   |   |    |     |      |
| 2-Undecenal       | 27.8849|1712 | L      | -               |   |   |    |     |      |
| 2,4-Decadienal, (E)-|29.8771|1808 | L      | -               |   |   |    |     |      |
| Alkanes           |       |      |        |                  |   |   |    |     |      |
| 5-Ethyl-2-methyloctane|6.2343|1053 | M      | 1336.53          |   |   |    |     |      |
| Undecane, 5,7-dimethyl | 5.9861 | 1065 | M      | 386.79           |   |   |    |     |      |
| Nonane, 3-methyl  | 6.8899| 1095 | M      | 886.24           |   |   |    |     |      |
| Undecane, 3-methyl| 7.1039| 1101 | M      | 840.20           |   |   |    |     |      |
| 2,4-Dimethylhexane| 7.2228|1105 | M      | 537.14           |   |   |    |     |      |
| Undecane, 5-methyl| 8.1086| 1128 | M      | 1044.21          |   |   |    |     |      |
| Undecane, 3,4-dimethyl|8.3405|1135 | M      | 549.18           |   |   |    |     |      |
| 3,5-Dimethylheptane|10.5110|1193 | M      | 56.41            |   |   |    |     |      |
| 2,4,6-Trimethylcyclohexane|11.1769|1211 | M      | 140.72           |   |   |    |     |      |
| 3,6-Dimethylundecane|11.9617|1233 | M      | 327.33           |   |   |    |     |      |
| 3,7-Dimethyloctane| 12.7170|1254 | M      | 52.02            |   |   |    |     |      |
| Undecane,4,7-dimethyl | 13.3946|1272 | M      | 200.04           |   |   |    |     |      |
| Undecane,3,7-dimethyl|16.0586|1348 | M      | 279.34           |   |   |    |     |      |
| Tetradecane       | 17.8184|1398 | L      | 149.89           |   |   |    |     |      |
| 2-Methyldecane    | 19.7687|1457 | M      | 156.48           |   |   |    |     |      |
| Pentadecane       | 20.9046|1497 | L      | 24.92            |   |   |    |     |      |
| Hexadecane        | 24.0560|1596 | L      | 192.28           |   |   |    |     |      |
| Cyclopropane, nonyl|25.8514|1658 | M      | 84.49            |   |   |    |     |      |
| Isopropylcyclohexane|22.6584|1550 | M      | 22.27            |   |   |    |     |      |
| 3,8-Dimethyldecane| 11.3493|1215 | M      | 46.78            |   |   |    |     |      |
| Tridecane         | 11.1769|1211 | M      | 140.72           |   |   |    |     |      |
| Cyclopentane, nonyl|19.4298|1448 | M      | -               |   |   |    |     |      |
| Dodecane          | 9.4641| 1198 | L      | -               |   |   |    |     |      |
| Cyclooctane, methyl | 14.2034|1294 | M      | -               |   |   |    |     |      |

(continued on next page)
Table 2 (continued)

| Compound | RT (min) | LRI | Method | Peak Area (x104) |
|----------|---------|-----|--------|-----------------|
|          | B       | R   | WB     | B/R  | B/WB |
| Alkenes  |         |     |        |      |      |
| 6-Octene, (Z)- | 18.7697 | 1450 | L     | 76.79 | -    | 37.31 |
| Cyclopentene, 1-ethenyl-3-methylene- | 20.6309 | 1483 | M     | 119.68 | 112.14 | 94.39 |
| 4-Ethylcyclohexene | 22.3732 | 1541 | M     | 1076.22 | 601.37 | 270.08 |
| 1-Hexene, 3,5,5-trimethyl- | 20.6309 | 1483 | M     | 119.68 | 112.14 | 94.39 |
| 1,3-Hexadiene, 3-ethyl-2-methyl-, (Z)- | 17.7411 | 1396 | M     | 52.40 | 851.07 | 99.76 |
| 3,5-Dimethyl-1-hexene | 23.0508 | 1563 | M     | - | 694.98 | - |
| Methyl ethyl cyclopentene | 28.5925 | 1755 | M     | - | 41.82 | 5.03 |
| 1-Undecene, 8-methyl-, (Z)- | 22.3732 | 1541 | M     | 1076.22 | 601.37 | 270.08 |
| 1-Octene, 3,7-dimethyl- | 17.6519 | 1394 | M     | 195.67 | 278.74 | 50.35 |
| 1-Tetradecene | 19.5308 | 1445 | M     | - | 179.34 | 50.58 |
| 1-Hexene, 3-ethyl-2-methyl-, (Z)- | 17.7411 | 1396 | M     | 52.40 | 851.07 | 99.76 |
| 3-Methylbutanol | 11.3873 | 1212 | L     | 145.76 | - | 147.32 |
| 2-Methyl-1-indanol | 19.3408 | 1445 | M     | - | 179.34 | 50.58 |
| 7-Octen-2-ol, 2,6-dimethyl- | 20.1968 | 1473 | L     | 29.55 | 228.64 | 53.90 |
| 1-Heptanol, 2,4-dimethyl- | 19.8400 | 1460 | L     | 550.28 | 742.95 | 60.89 |
| 2-Butyloxyethanol | 18.0148 | 1402 | L     | 387.20 | 761.47 | - |
| 2-Hepten-1-ol, (E)- | 18.1575 | 1410 | M     | 149.07 | 1039.53 | 72.56 |
| 2-Octen-1-ol, (E)- | 24.5552 | 1647 | M     | - | - | - |
| 2-Nonen-1-ol, (E)- | 24.7157 | 1691 | L     | 169.05 | 1289.62 | 678.82 |
| 1-Dodecanol | 33.6941 | 1978 | L     | 504.40 | 224.90 | 117.01 |
| 3-Methylbutanol | 11.3873 | 1212 | L     | 145.76 | - | 147.32 |
| 2-Methyl-1-indanol | 25.4292 | 1643 | M     | 93.08 | 230.07 | 24.00 |
| 1-Octan-3-ol | 19.5308 | 1452 | L     | 1998.06 | 9242.54 | 7612.31 |
| 1-Heptanol | 19.8400 | 1460 | L     | 550.28 | 742.95 | 60.89 |
| 5-Hepten-2-ol, 6-methyl- | 20.0778 | 1464 | L     | 762.42 | 465.73 | 371.99 |
| 7-Octen-2-ol, 2,6-dimethyl- | 20.1968 | 1473 | L     | 29.55 | 228.64 | 53.90 |
| 1-Octanol | 19.3408 | 1445 | M     | - | 179.34 | 50.58 |
| 1-Octan-3-ol | 19.5308 | 1452 | L     | 1998.06 | 9242.54 | 7612.31 |
| 2-Methyldecanoic acid | 23.2173 | 1569 | M     | 89.62 | 31.30 | - |
| 2-Amino-6-methyl benzoic acid | 30.0017 | 1807 | M     | 9686.90 | 4607.38 | 1837.35 |
| 2-Amino-5-methyl benzoic acid | 30.4060 | 1822 | M     | 8434.68 | 3812.24 | 292.22 |
| Caproic acid | 31.0186 | 1829 | L     | - | 2869.94 | - |
| Lauric acid | 36.6733 | 2487 | L     | - | 1597.67 | - |
| Acetic acid | 19.5192 | 1450 | L     | - | - | 41.13 |

(continued on next page)
| Compound | RT | LRI | Method | Peak Area (×10^4) |
|----------|----|-----|--------|------------------|
|          |    |     |        | B               |
|          |    |     |        | R               |
|          |    |     |        | WB              |
|          |    |     |        | B/R             |
|          |    |     |        | B/WB            |

**Esters**

| Compound | RT | LRI | Method | Peak Area (×10^4) |
|----------|----|-----|--------|------------------|
|          |    |     |        | B               |
|          |    |     |        | R               |
|          |    |     |        | WB              |
|          |    |     |        | B/R             |
|          |    |     |        | B/WB            |

**Eter**

| Compound | RT | LRI | Method | Peak Area (×10^4) |
|----------|----|-----|--------|------------------|
|          |    |     |        | B               |
|          |    |     |        | R               |
|          |    |     |        | WB              |
|          |    |     |        | B/R             |
|          |    |     |        | B/WB            |

**Heterocyclics**

| Compound | RT | LRI | Method | Peak Area (×10^4) |
|----------|----|-----|--------|------------------|
|          |    |     |        | B               |
|          |    |     |        | R               |
|          |    |     |        | WB              |
|          |    |     |        | B/R             |
|          |    |     |        | B/WB            |

**Aromatic Hydrocarbons**

| Compound | RT | LRI | Method | Peak Area (×10^4) |
|----------|----|-----|--------|------------------|
|          |    |     |        | B               |
|          |    |     |        | R               |
|          |    |     |        | WB              |
|          |    |     |        | B/R             |
|          |    |     |        | B/WB            |

(continued on next page)
Cooked meat such as meatball contains a complex mixture of volatile compounds which may derive from lipid and water-soluble precursors. These compounds provide roast, boiled, fatty, species-related flavors and the characteristic aroma of cooked meats [36]. Moreover, lipid thermal degradation produces chemicals that give cooked meat its fatty odors and those that determine the flavors of various species [37]. However, many volatiles found in meat species may have contradictory results in the literature. Several compounds might be transmitted directly from ingested feeds into animal tissue, while others resulted from alteration of feed molecules by ruminal bacteria [27].

Table 3 summarizes the ten most significant volatile compounds positively associated with pure beef, pure rat, and beef-rat mixture meatballs. The complete list of volatiles with their coefficient and VIP value is available as supplementary data (Table S2). It is shown that 2-amino-5-methyl benzoic acid was identified as the most robust discriminator in the pure beef meatball class. Other volatile compounds markers for this group were 2-amino-6-methyl benzoic acid, heptanal, benzaldehyde, 5-ethyl-m-xylene, 2,3-octanedione, ethylbenzene (Z)-8-methyl-2-undecene, 5-ethyl-2-methyloctane, and 3-methyl-undecane. A previous study found that benzaldehyde, heptanal, and undecane were present in cooked beef [38]. Additionally, benzaldehyde, heptanal, 2,5-cotane-dione, and undecane were also identified in roasted beef [39]. The most robust discriminator in the rat meatball class was (Z)-2-heptenal, followed by 3-methyl-3-butenol, caproic acid, pentanal, 2-tert-butyl-cyclohexanol, 3-methyl- butanal, 6-methyl-2-heptanol, and 3,6-dimethylundecane. Nonanal was the most significant discriminator in the beef and rat meatball mixture. Other volatile markers for this class also included 1-pentanol, cyclobutanol, 1-octen-3-ol, 1-octanol, indole, dimethyl trisulphide, benzene, 1,3,5-trimethyl-, 2-ethylnitrobenzene, naphthalene. A previous study found benzaldehyde, 1-octen-3-ol, hexanal dimethyl trisulphide in cooked beef [15]. In this previous report, 1-octen-3-ol is an alcohol group compound with a mildew-like odor found in fresh meat stew. It has an important role in the stew's flavor [15]. Recent studies used FTIR spectroscopy and multivariate data analysis to detect rat adulteration in raw beef and beef meatballs based on typical functional group profiles [3, 40].

### Table 3 (continued)

| Compound                             | RT   | LRI | Method* | Peak Area (x104) |
|--------------------------------------|------|-----|---------|-----------------|
|                                      |      |     |         | B | R | WB | B/R | B/WB   |
| Acetophenone                         | 25.0427 | 1627 | L | 84.74 | 254.58 | 24.60 | 257.42 | 135.60 |
| 3-Tridecanone                        | 29.0265 | 1797 | L | 12.12 | 142.29 | 18.69 | 208.37 | 65.31 |
| 2-Nonenol                            | 17.2596 | 1375 | L | 331.88 | 747.17 | - | 543.74 | 20.67 |
| 11-Dodecen-2-one                     | 23.7882 | 1587 | M | 27.04 | 66.15 | 65.32 | 51.19 | 78.54 |
| γ-Nonalactone                        | 34.1403 | 2026 | L | 50.03 | 44.87 | 44.04 | 23.55 | 132.11 |
| Nona-3,5-dien-2-one                  | 32.0411 | 1885 | M | 31.81 | 542.32 | - | 245.73 | 202.31 |
| 2-Undecanone                         | 23.8597 | 1593 | L | - | 134.67 | - | 17.66 | 39.95 |
| 2-Methyl-3-octanone                  | 15.1189 | 1322 | L | - | 355.34 | 239.85 | 294.43 | 267.15 |
| 5-Hepten-2-one, 6-methylene           | 15.5292 | 1332 | L | - | 191.23 | 128.78 | 184.62 | 232.52 |
| 2,6-Dimethylcyclohexanone            | 16.3797 | 1322 | L | - | 96.03 | - | 529.02 | 328.85 |
| 3,5-Octadien-2-one                   | 21.2849 | 1500 | L | - | 46.43 | - | 358.36 | - |
| Tetrahydrothiopyran-4-one             | 33.9738 | 2000 | M | - | - | 1.60 | 142.78 | 326.32 |
| 4-Nonenone                           | 21.9688 | 1528 | M | - | - | - | 47.75 | - |
| Nitrogen compounds                   |      |     |         | B | R | WB | B/R | B/WB   |
| 1,2,4-Triazol-4-amine, 5-ethyl-3-(3-methyl-5-phenylpyrazol-1-yl)- | 22.5216 | 1546 | M | 243.01 | 392.52 | 584.08 | 495.45 | 730.46 |
| Benzyl nitrile                       | 32.7309 | 1918 | L | 108.25 | 146.33 | - | 220.32 | 20.25 |
| Diethyltoluamide                     | 35.8406 | 2278 | M | - | 72.48 | 475.31 | 219.65 | 31.70 |
| Sulfur compounds                     |      |     |         | B | R | WB | B/R | B/WB   |
| Disulfide, dimethyl                  | 6.0812 | 1071 | L | 1061.06 | 1533.49 | 439.25 | 1270.85 | 897.32 |
| Disulfide, di-tert-dodecyl           | 14.9645 | 1316 | M | 181.91 | - | - | 60.44 | - |
| Dimethyl trisulfide                  | 15.0297 | 1329 | L | 210.67 | 198.81 | 746.64 | 2532.62 | 6007.90 |
| Terpenoids                           |      |     |         | B | R | WB | B/R | B/WB   |
| Limonene                             | 9.4524 | 1166 | L | 743.87 | 493.53 | 712.20 | 660.02 | 1287.35 |
| α-Terpinolene                        | 13.8524 | 1282 | L | 660.16 | 638.50 | 579.36 | 862.62 | 187.97 |
| 1,3,8-p-Menthatriene                 | 19.9827 | 1411 | L | 119.00 | - | - | 540.66 | - |
| d-2-Bornanone                        | 20.7379 | 1491 | L | 113.38 | 948.28 | 61.85 | 334.72 | 489.97 |
| Fenchol                              | 23.5681 | 1574 | L | 154.38 | 235.87 | 20.85 | 362.13 | 14.46 |
| Terpinen-4-ol                        | 23.8833 | 1591 | L | 53.22 | 48.43 | 31.73 | 173.08 | 184.92 |
| β-p-Menthol                          | 25.1141 | 1612 | L | 104.33 | 147.46 | 45.52 | 169.79 | - |
| Isoborneol                           | 25.7444 | 1659 | L | 9.10 | 108.75 | 14.81 | 103.88 | 76.87 |
| 1-Terpinenol                         | 23.3481 | 1573 | L | - | 279.60 | 49.66 | 140.56 | - |
| Camphor                              | 20.7676 | 1491 | L | - | 73.46 | - | - | - |
| β-Terpineol                          | 24.9060 | 1646 | L | - | 49.50 | 48.54 | 315.59 | 293.23 |
| dl-Menthol                           | 25.3344 | 1630 | L | - | 48.98 | 47.32 | - | - |
| L-Camphor                            | 21.0176 | 1511 | L | - | 45.83 | - | 67.15 | 40.72 |
| α-Curcumene                          | 28.6282 | 1773 | L | - | - | - | 135.76 | 23.81 |

* Reliability 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.
classify rat's meatball and beef meatballs including samples obtained from the market [3], and successfully classify lipid components extracted from beef meatballs and rat's meatballs using three different lipid extraction methods with 100% accuracy [40]. However, because studies on volatile compound characterization in rat raw meat or rat meatballs are uncommon, it is difficult to compare the volatiles data obtained in the present study with other reports.

The ten most significant positive discriminating compounds in PLS-DA of pure beef meatballs, pure rat meatballs, and meatballs made from their mixtures were summarized in Table 4. The complete list of volatiles with their coefficient and VIP value is available as supplementary data (Table S3). The beef discriminating volatile profile in this PLS-DA was nearly identical to that of the PLS-DA of beef, rats, and beef-rat mixture meatballs shown in Table 3, except that 6-methyl-5-hepten-2-ol and glutaraldehyde were not identified as significant markers here. Cyclobutanol was identified as the strongest discriminator in the wild boar meatballs class. Other volatile compounds were also identified which included undecane, 3-methyl-, 2-methyl docane, 1-hexanol, lauric...
acid, benzene, 1,2,4,5-tetramethyl-, and diethyltoluamide. A previous study reported that benzaldehyde, heptanal, 2,5-octanedione, and undecane were detected in roasted pork [39]. Hexanal compounds were also discovered in pork cheeks cooked at various temperatures [41]. The strongest discriminator in the mixture of beef and wild boar meatball was nonanal. Other markers included 1-pentanol, heptanal, 1-octene, 3,7-dimethyl-, dimethyl trisulfide, 1-heptanol, caproic acid, and 2,4,6-trimethylolethane. It was reported that key volatiles of cooked beef and pork were as follow; octanal, nonanal (E,E)-2,4-decadienal, methanethiol, methional, 2-furfurylthiol, 2-methyl-3-furanthiol, 3-mercapto-2-pentanone, and 4-hydroxy-2,5-dimethyl-3-(2H)-furanone [29]. A previous study also showed that nonanal, heptanal, 1-pentanol and 1-heptanol, and dimethyl trisulfide were the volatile compounds in boiled pork [42] and in cooked beef [38]. There are currently few reports on volatile compounds in wild boar meatballs [10], whereas the presence of volatiles in fried wild boar meat was recently summarized [1, 43]. Aldehydes dominated the volatile profile of fried wild boar meat, with nonanal, 2(E)-decenal, hexanal, and octanal being among the most significant. Octanol was also identified, but to a lesser extent. Recently, several quality parameters for wild boar carcass were reported, including a number of volatiles [44], the most abundant of which were hexanal, 2,3-butanedione, 3-methyl-2(5H)-furanone, furan, and 1-octen-3-ol. These findings differed slightly from those of our current study, in which some of the mentioned compounds were not detected (e.g., 3-methyl-2(5H)-furanone), while others were identified but not as significant markers (e.g., furan, 1-octen-3-ol, hexanal, and 2,3-butanedione) (Table S4).

The data in Tables 3 and 4 show that volatile markers for mixture meatballs differed from those found in single meatballs. This is explained by the fact that Pranata and colleagues included pure chicken meatballs in their multivariate analysis alongside pure beef and pure wild boar meatballs, which may affect the distribution of x-variables in overall multivariate models. Aldehydes dominated the volatile profile of fried wild boar meat, with nonanal, 2(E)-decenal, hexanal, and octanal being among the most significant. Octanol was also identified, but to a lesser extent. Recently, several quality parameters for wild boar carcass were reported, including a number of volatiles [44], the most abundant of which were hexanal, 2,3-butanedione, 3-methyl-2(5H)-furanone, furan, and 1-octen-3-ol. These findings differed slightly from those of our current study, in which some of the mentioned compounds were not detected (e.g., 3-methyl-2(5H)-furanone), while others were identified but not as significant markers (e.g., furan, 1-octen-3-ol, hexanal, and 2,3-butanedione) (Table S4).

The data in Tables 3 and 4 show that volatile markers for mixture meatballs differed from those found in single meatballs. This is explained in the same way that Figure 2 is explained. It has previously been discussed that when meat from different species is mixed and heated, the volatiles produced are more diverse than those produced by single meat.
because different volatile precursors are mixed and subjected through the thermal process, resulting in the formation of more diverse volatiles than when meatballs are made from a single type of meat. Marker selection in each group of meatballs was done based on VIP value. VIP indicates the relative importance of each variable (X variables) in the model [25]. Since in mixture meatballs we have now more volatile compounds, the relative importance of each variable (X variables, volatile compounds) in each group of meatballs were done based on VIP value. VIP indicates the relative importance of each variable (X variables, volatile compounds) in the model [25]. Since in mixture meatballs we have now more volatile compounds, the relative importance of each variable (X variables, volatile compounds) in each group of meatballs were done based on VIP value.

4. Conclusions

The texture and color characteristics of meatballs measured in this study were found to be inconsistent and could not be used to identify meatballs based on raw meat compositions except at higher concentrations (50%). Volatile compound data obtained from SPME-GCMS and multivariate data analysis, on the other hand, was able to show a clear classification among meatballs made from different types of raw meat. The PLS-DA model showed that the strongest markers in beef, rat, and wild boar meatballs were 2-amino-5-methyl benzoic acid (Z)-2-heptenal, and cyclobutanol, respectively. Additionally, nonanal was consistently found as a dominant marker in meatballs made from a mixture of beef-rat and a mixture of beef wild boar. The results of this study revealed that the volatile profiles of different types of meat can be used as a basis to develop a quick analytical sensor which can detect the presence of undesired types of meat based on their volatile profiles. Further research on the verification of the volatile compounds identified as markers for each meatball group in this study is required. Verification can be accomplished by quantifying each compound using an internal standard. Because the target compounds to be quantified are already known, this future study takes a different approach than the current -omics-based research. Different analytical methods can be used, though using the same instrument (GC-MS) is the preferred method for volatiles analysis. Method validation, which includes detection limit, quantification limit, curve linearity, working range and linear range, accuracy, and recovery, must be performed prior to quantification to ensure the method fits the target compounds well.

Declarations

Author contribution statement

Lia Amalia: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Nancy Dewi Yuliana: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Putriwaningsih Sugita: Conceived and designed the experiments; Wrote the paper.

Desi Aroah: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Utami Dyah Salfitr, Anjar Windarsih, Dachryinus, Nor Kartini Abu Bakar: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Feri Kusnandar: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data included in article/support material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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