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Review

The Metabolomics Approaches Based on LC-MS/MS for Analysis of Non-Halal Meats in Food Products: A Review

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Abstract: Halal meats are meats that are allowed to be consumed by Muslim societies according to Islamic law (Syariah). Due to the development of food technology, non-halal meats such as pork or canine meat are added to food products to reduce the production costs. Non-halal meats also include meats from animals which are not slaughtered according to Syariah law; therefore, the availability of a standardized analytical method capable of detecting the presence of non-halal meats with high sensitivity is very urgent. The metabolomics technique, either targeted or untargeted approaches based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) measurements is an emerging analytical method applied to the identification of non-halal meats in food products. The LC-MS/MS measurements provide an enormous metabolomics data, therefore, sophisticated data analysis tools such as chemometrics is required. Among the chemometrics techniques, exploratory data analysis for supervised and unsupervised pattern recognition, including principal component analysis (PCA), hierarchical cluster analysis (HCA), and linear-discriminant analysis (LDA), are the most-used. This review focused on the recent application of LC-MS/MS in combination with chemometrics for the detection and identification (qualitative analysis) of non-halal meats in food products. The selection criteria used for the papers in this review were studies on the application of metabolomics using LC-MS/MS and chemometrics for the halal authentication of meat products between 2005 and 2022. The results showed that potential biomarkers of non-halal meats could be found using chemometrics analysis. Therefore, it can be concluded that a combination of LC-MS/MS and chemometrics is promising for development as a standard analytical method for the analysis of non-halal meats in food products.

Keywords: non-halal meats; metabolomics; chemometrics; biomarkers; halal authentication; LC-MS/MS

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1. Introduction

Meats and meats-based food products are known as good sources of proteins, which are needed for human development and growth, because they also contain essential amino acids, minerals, vitamins, and micronutrients. Muslims have become increasingly concerned about the meat they eat [1]. The choice and eating of meat and meat-based products depend on factors including religious faith, geographical

region, meat type, age group, and consumers' purchasing capacity. Religious faith is the most dominant aspect affecting the selection of meats, especially for Muslim and Jewish communities. Muslims only consume Halal meats and meat-based products, while Jewish communities choose kosher meats [2]. Halal meats can derive from wild animals such as deer, ostrich, rabbits, birds, or domesticated animals such as cattle, poultry, and camels. All these animals are halal following the proper slaughtering processes according to the principles of Syariah law. Non-halal meats (haram meats) are swine (pig), wild boar meat and carnivorous animals. Furthermore, food products containing donkey, frog, dog, and cat meat are considered non-halal and determined not fit for consumption for Muslim consumers [3].

In line with the increased consumption of meats and meat-based food products, the presence of non-halal meats must be anticipated. Meat-based food products such as meatballs, sausages, and nuggets may contain non-halal meats; as a consequence, according to Indonesian Act No. 33 (2014) on Halal Products' assurance, products containing meats must be assessed by laboratory checks to ensure that the products are free from non-halal components. Halal meats are meats that are allowed to be consumed by Muslim societies according to Islamic law (Syariah). Due to the development of food technology, non-halal meats such as pork or canine meat are added to food products to reduce the production costs. Non-halal meats include meats from animals that are not slaughtered according to Syariah law, also known as the non-Zabiha slaughtering technique [4]. Therefore, the availability of a standardized analytical method capable of detecting the presence of non-halal meats and PGs with a low detection limit is crucial [5].

Some reviews have been published on the analytical methods used for halal authentication analyses, such as that of El-Seikha et al., who reviewed DNA-based methods for the analysis of non-halal meats [2]. Some authors looked at different analytical methods (physico-chemical approaches, molecular biology, and DNA- and protein-based methods) in their review, such as Zia et al. [6], Hossain et al. [7], Rohman [8], Valdés et al. [9], and Rohman and Windarsih [10]. The electronic nose (E-nose) method has also been developed as an interesting method for the analysis of non-halal meats. The E-nose method has been successfully used to classify pork and beef in meat mixtures [11]. The flavor compounds of pork were identified using gas chromatography–olfactometry–mass spectrometry (GC-O-MS), and 79 compounds were identified [12]. E-nose based on GC-O has also been used to differentiate between the dry rendered fat of chicken, pork, sheep, and beef [13]. However, the reviews on a specific method (LC-MS in this case) and chemometrics involved in the metabolomics study are very limited. LC-MS/MS is suitable for comprehensive metabolite analysis to identify metabolite compositions in food samples. It is important to detect and differentiate non-halal meats in food products based on their metabolite compositions. In addition, advanced statistical analyses such as chemometrics could be utilized for the investigation of potential biomarkers of non-halal meats. Therefore, the aim of this review is to highlight LC-MS/MS in combination with chemometrics for the analysis of non-halal meats in meat mixtures or in food products.

2. Methods

A total of 500 papers related to the halal authentication analysis of food products were used in this review. The inclusion criteria for the selected papers were: (1) studies related to metabolomics' application using LC-MS for the halal authentication of meat products between 2005 and 2022, (2), studies on the application of chemometrics for the halal authentication of meat products using LC-MS data, and (3) all papers written in English. The keywords of LC-MS/MS, metabolomic, non-halal meats, halal authentication, and chemometrics were used during the article search.

3. Metabolomics for Non-Halal Meats' Analysis

The term metabolomics can be defined as the "comprehensive analysis of the whole metabolome, which refers to the full complement of small molecule metabolites in a cell, tissue or organism, under a given set of conditions". Metabolomic-related studies are a relatively new area of science, which are being used to gain a greater understanding of the chemical constituents and flux within biological systems [14]. Metabolomics has been developed and applied in many research areas and is becoming the most active field of investigation among omics techniques because metabolomics could be used to represent phenotype. In recent years, the utilization of metabolomics in food science, and specifically in food authentication, has gradually increased to address some issues related to food adulterations, food origins, and food contamination [15].

Metabolomics is divided into two main approaches: targeted and untargeted metabolomics approaches. Each approach is used for different purposes and functions [16]. Targeted metabolomics focused on the analysis of one or several metabolites that were previously defined in certain samples. Metabolites that have been identified as markers are often used as target of analysis in targeted approach. Targeted metabolomics has been developed and used since the introduction of metabolomics technology, including in food analysis [17]. Most metabolomics research that has been develop and applied in food analysis and food authentication used a targeted approach. For example, it has been used for the analysis of selected harmful compounds in foods, such as oxidation products, due to processing treatments such as heating [18,19]. Using predefined metabolites as a target of analysis provides an effective and rapid analysis of food authentication. However, targeted metabolomics is limited to the analysis of one or few predefined metabolites. It cannot be used for the analysis of new or unknown metabolites. Moreover, it is not suitable for the analysis of new samples without knowing the markers that will be the target of analysis [20].

The development of untargeted metabolomics has emerged as a potential and promising approach in metabolomics analysis for food authentication, including halal analysis. Untargeted metabolomics is capable of the comprehensive identification of not only predefined metabolites, but also the unknown metabolites in a particular system [17]. Due to its ability to obtain high-coverage metabolites, it offers advantages in the identification of as many metabolites as possible in food samples for differentiation [21]. Moreover, an untargeted approach could be used to identify potential metabolite markers in new samples by using proper data processing. This has proved to be an effective strategy for the identification of species, geographical origin, and genetic markers in food samples [22]. Untargeted metabolomics is also known as the fingerprinting technique, and provides comprehensive information about the metabolite patterns, which is useful for sample differentiation [23]. Untargeted metabolomics seems to be more promising for detecting and analyzing non-halal meats in food products because it can be used to identify the metabolites of non-halal meats. There must be differences in the metabolites of non-halal meats, which is very useful for samples differentiation [24].

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) methods are the two main analytical platforms that have been developed and used for metabolomics analysis [25,26]. NMR offers the simultaneous analysis of both primary and secondary metabolites with minimum sample preparation steps, thus reducing the time needed for analysis. It has been widely used in many types of metabolomics research, such as plant sciences, clinical diseases, drug discoveries, and food analysis [27,28]. However, it has several limitations in terms of metabolite separation, especially in complex samples, which often result in signal overlapping. In addition, it requires more samples to be used because its sensitivity is much lower than the MS technique [29]. The MS technique is known for its high sensitivity and can detect compounds at very low concentrations. It has advantages in high-throughput screening, identifying as many metabolites as possible in the samples and providing a fast, selective, and effective assessment for food authentication [30]. The

MS-based technique has proved to be effective as the metabolite-based technique for identification of non-halal meats. It was successfully used to identify pork in beef meat based on lipids composition [31].

In metabolomics analysis, sample preparation is a crucial initial step. It is affected by several factors and depends on the purpose of the analysis. Different sample preparation techniques are applied for targeted and untargeted approaches. For the targeted approach, an extraction technique capable of the selective extraction of target metabolites is required. It is important to completely extract the target metabolites and purification steps are often performed to remove matrices in complex samples. Conversely, the untargeted approach requires a non-selective extraction technique capable of a comprehensive extraction of as many metabolites in the samples as possible [32]. There is no one solvent with the ability to extract all types of metabolites due to the wide polarity ranges of the metabolites. The selection of the solvent used for metabolite extraction depends on the target of analysis. Polar metabolites could be extracted using polar solvents, while a non-polar solvent is suitable for the extraction of non-polar metabolites. Solvents such as methanol and acetonitrile have been known as general solvents for metabolomics' extraction due to their ability to extract a wide range of metabolites with different polarities, from polar to non-polar metabolites [33]. A combination of methanol or acetonitrile with water using a particular ratio has been used to modify the polarity. For the extraction of non-polar metabolites, specifically lipid metabolomes, a different extraction technique using non-polar solvents has been developed. Conventional lipid extraction methods, such as Bligh and Dyer, Folch, and modifications to these methods, have been widely used for lipid extraction and are still used at present. These methods are two-phase extraction techniques, which are capable of extracting a wide range of lipids and lead to improved lipid characterization [34]. However, two-phase extraction requires many solvents and more extraction steps. A one-phase extraction technique for lipid extraction has been introduced and it is known for advantages such as reducing the volume of solvent used and reducing the extraction time. One-phase lipid extraction using methanol, dichlorometane, chloroform, isopropanol, methyl tert-butyl ether, either in individual form or in combination with certain ratios, has been reported in the lipid extraction of various types of samples, including food samples. This technique is considered green chemistry, due to the limited amount of solvent used [35]. The schematic diagram used to search the biomarkers regarding non-halal meats is depicted in Figure 1.

Some databases are introduced to assist researchers in finding metabolites that can be used as biomarkers during the analysis and identification of non-halal meats and related purposes. As an example, either open-source search engines such as Comet, X! Tandem, and ProteinProspector, or commercial-based search engines such as Proteome Discoverer, ProteinPilot™, are widely used for the identification of protein metabolites. Recently, Amir et al. compared open-source and commercial database search engines using the massive tandem MS of pork-based food products for halal authentication analysis [37].

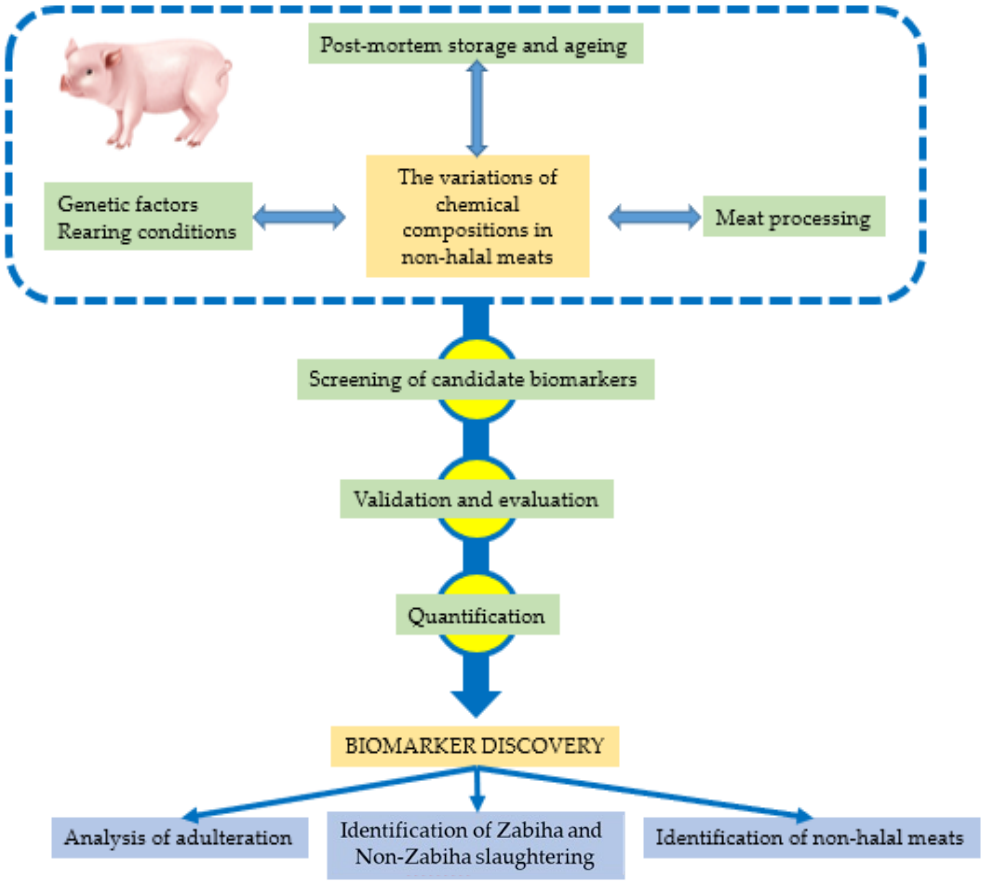

Figure 1. The workflow used to search metabolites used as biomarkers, intended for the identification of halal meats. Adapted from Ref. [36].

4. LC-MS Technique for Metabolomics Analysis

Metabolomics analysis using mass spectrometry can be combined with other separation techniques to optimize metabolite separation, such as gas chromatography, liquid chromatography, and capillary electrophoresis. Gas chromatography–mass spectrometry (GC-MS) is used for the analysis of volatile metabolites. Some non-volatile metabolites can be analyzed using gas chromatography, but this requires a derivatization process. However, not all non-volatile compounds could be subjected to derivatization, reducing the number or compounds that can be analyzed using gas chromatography [38]. Capillary electrophoresis–mass spectrometry (CE-MS) has been used to identify a wide number of polar, highly polar and ionic metabolites. However, it has limitations in the analysis of non-ionic metabolites [39]. Liquid chromatography–mass spectrometry (LC-MS) is known for its high-throughput analysis of the separation of metabolites with different polarities. This technique is capable of detecting a high coverage of metabolites with high robustness and high reproducibility. It has been widely used over a vast area of research, including food authentication and halal food authentication [40].

Liquid chromatography (LC) plays important roles in metabolite separation. Generally, two types of analysis use the LC technique, namely, normal phase chromatography (NPC) and reversed-phase chromatography (RPC). These two techniques have been developed and used for the analysis of many types of sample [32]. NPC is used for the analysis of polar compounds whereas RPC is utilized for the analysis of semi polar and non-polar compounds. The main differences between NPC and RPC are the stationary phase and the mobile phase used. NPC uses a polar stationary phase with a non-polar mobile phase. Meanwhile, the stationary phase in RPC tends to be non-polar, with a polar mobile phase [41]. The development of a liquid chromatography technique resulted in a new

technique known as hydrophilic interaction liquid chromatography (HILIC). This technique is designed for the analysis of polar compounds, similarly to the NPC technique, but it differs in terms of the mobile phase. The mobile phase used in HILIC comprises polar solvents, similar to the RPC technique. Recently, HILIC has been preferred to NPC because the non-polar mobile phase used in NPC can cause problems and is more harmful to the detector such as MS [42]. In addition, the non-polar mobile phase could affect the ionization process of the mass spectrometer. Metabolomics using the RPC technique has become the main technique used in metabolomics analysis. Liquid-chromatography using the reversed-phase technique could be used to separate a wide range of metabolites, from non-polar to semi-polar metabolites such as amino acids, flavonoids, alkaloids, and lipids [41]. The overview generated using two metabolomics data repositories, namely, EBI MetaboLight and NIH Metabolomics Workbench, showed that metabolomics analysis using liquid chromatography employing the RPC technique became the most widely used technique, accounting for 76% and 72%, respectively, of the total [43]. The selection of solvent in LC-MS metabolomics analysis also plays a crucial role in compounds' separation. Acetonitrile, methanol, and water are the most common solvents used as a mobile phase in metabolomics analysis. The mobile phase is often added with a low concentration of acids, such as 0.1% formic acid, for the better separation of analytes, resulting in a good resolution and good peak shape [44]. For the analysis of lipid metabolites, ammonium format was added to optimize the separation of lipids for better lipid characterization [45].

Meats contain a wide range of metabolites, from polar to non-polar metabolites, such as amino acids, organic acids, fatty acids, lipids, and many more. Using the suitable system, liquid chromatography is capable of separating broad metabolites in non-halal meat samples, depending on the target metabolites. A good chromatogram resolution could be obtained using the LC-technique, providing a better identification of metabolite composition. Some studies successfully applied the LC-technique for metabolite separation in meat samples [46].

Mass spectrometer is an important aspect of metabolite detection, coupled with liquid chromatography. The compounds separated by the liquid chromatography system enter the mass spectrometer to be ionized prior to fragmentation. The three main parts of the mass spectrometer are known as the ion source, mass analyzer, and detector [47]. The ion source is the place at which ions are generated from each separated compound. Two ionization techniques, known as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are predominantly used in mass spectrometry. In the majority of metabolomics analyses using LC-MS technique, ESI is preferred to APCI because it is effective at generating ions in a simple way, using either positive or negative ionization modes [40]. The second part of the MS instrument is the mass analyzer, which plays an important role in ion fragmentation. Several types of mass analyzer have been introduced and used in MS analysis. Each mass analyzer differs in its resolving power. Based on the types of mass analyzers, there are three categories of mass analyzer: low-resolution, medium-resolution, and high-resolution mass analyzers. Quadrupole is an example of a low-resolution mass analyzer. It has been widely used in targeted MS analysis. Quadrupole cannot be utilized for untargeted metabolomics analysis due to its low resolving power, which is less than 2000 FWHM (full width at half maximum) [48]. Time of flight (TOF) is an example of a medium-resolution mass analyzer with a resolving power capacity that ranges from 12,000 to 50,000 FWHM. It is often used in combination with Quadrupole known as Q-TOF to obtain an improved resolving power capacity. Q-TOF has been applied in metabolomics research more than Quadrupole due to its untargeted approach [49]. An example of a high-resolution mass analyzer is Orbitrap, which is also often coupled with Quadrupole as Q-Orbitrap. It has a high resolving power capacity of up to 500,000 FWHM. Therefore, it is very suitable for untargeted metabolomics analysis with a high mass accuracy (2–5 ppm). It can be used for high-throughput analysis to identify as many metabolites in particular samples as possible, to obtain a comprehensive metabolites' composition. Moreover, Q-Orbitrap could be used for the analysis of positive and negative

ionization modes in one experiment, because it allows for a fast switching between positive and negative ionization modes [50,51].

Analysis using LC-MS provides a high sensitivity and selectivity for a wide range of metabolites in the presence of complex matrices, such as food products. LC-MS could be applied either to targeted or untargeted metabolomics analysis. A vast number of non-volatile metabolites could be separated and analyzed without any derivatization steps, thus reducing the time needed for sample preparation [52,53]. The utilization of ultra-high performance liquid chromatography (UHPLC) has emerged as a powerful analytical instrument for complex analytes' separation. UHPLC provides some advantages compared to common HPLC, such as a higher theoretical plate, higher resolution, and more reproducible retention time. The high theoretical plate could maximize the separation of metabolites, thus avoiding signal overlapping [54]. In line with the development of mass spectrometry, UHPLC is easily combined with a mass spectrometer. At present, the use of UHPLC-MS in metabolomics analysis for food authentication has significantly increased, as it is an effective and efficient method to ensure the quality, safety and authenticity of food products, including the halal status of food products. UHPLC combined with Q-TOF MS become the most widely used tool in metabolomics analysis. It could be used to identify a wide range of metabolites in untargeted metabolomics analysis due to its acceptable cost. Recently, the utilization of UHPLC-Q Orbitrap mass spectrometry as a sophisticated analytical platform has also been applied for untargeted food metabolomics. Although it has a higher cost than Q-TOF, it has a very high resolving power compared to Q-TOF, which allows for a more comprehensive detection of metabolites [38].

5. Chemometrics

A vast amount of data were obtained from LC-MS metabolomics measurements, especially when using the untargeted metabolomics approach. Thus, advanced statistical tools capable of processing and interpreting LC-MS metabolomics data are required. Chemometrics, an advanced multivariate statistical tool, has been used for the analysis of large amounts of metabolomics data [55]. Chemometrics is a combination of mathematical and statistical techniques, used to process multivariate data obtained from chemical measurements, making it an effective means of big data analysis [25]. Two chemometrics approaches are known, namely, pattern recognition and multivariate calibration or regression. Chemometrics pattern recognition is divided into two categories: unsupervised pattern recognition and supervised pattern recognition [56]. Unsupervised pattern recognition is used for the exploratory analysis of samples, without knowing the sample information. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) are the most unsupervised techniques applied to LC-MS metabolomics data [57]. On the other hand, supervised pattern recognition techniques such as linear discriminant analysis (LDA), partial least square–discriminant analysis (PLS-DA), and orthogonal projection to latent structures–discriminant analysis (OPLS-DA) are powerful for discrimination and classification samples using data obtained from LC-MS. PLS-DA and OPLS-DA could be used for the identification of biomarkers using the variable importance of projections (VIP) value. The specific biomarkers are very important for food authentication, for example, for the detection of non-halal meats in meat products [55].

It is worth noting that the separation pattern between the groups found in PCA may suggest the reliability of the PLS-DA or OPLS-DA model that was trained on the data [58]. Following that, model validation is a vital consideration when undertaking chemometrics; otherwise, this technique may lead the researcher to erroneous results [56]. Several methods can be used to validate the chemometrics models. Eriksson et al. [59] described several validation techniques. The most frequent technique is to observe the value of goodness of prediction (Q^2Y) and goodness-of-fit (R^2Y) by cross-validation, where 0.5 or higher is considered acceptable. External validation, by partitioning the data into a training set and a validation, can also be conducted to further test the predictive power of the models. The permutation test is conducted by developing parallel models where the Y-data are

randomly re-ordered. The R^2Y and Q^2Y values of the original model are then compared with those of the re-ordered model. If the re-ordered model has higher values than the original, then this indicates an overfitted model.

The second type of chemometrics is multivariate calibration, which is used for quantitative analysis to predict the concentration of target analytes using multivariate data. The most-used multivariate calibration techniques for metabolomics analysis are multiple linear regression (MLR), partial least square (PLS), and principal component regression (PCR) [60,61].

Chemometrics has been successfully applied to the identification non-halal meats in food products measured using LC-MS/MS-based metabolomics techniques. The challenge was in properly analyzing enormous amount of data obtained from LC-MS/MS to obtain good interpretation of metabolomics results. The most important advantage of chemometrics is its ability to investigate potential biomarkers of non-halal meat, which is useful for halal authentication. A metabolite of PC(o-18:0/18:2(9Z, 12Z)) was found to be specific for pork and absence in Patin fish meat. Therefore, it can be used as a marker to detect pork adulteration in Patin fish meat [33]. Therefore, chemometrics is promising for use as a powerful tool to process metabolomics data from LC-MS/MS measurements.

6. Application of LC-MS for Identification of Non-Halal Meats

The qualitative analysis (identification) and confirmation of non-halal meats are very challenging, especially in processed meat, due to their composition, inhomogeneity, and complexity, providing a low extractability for the meat components used as analytical targets, such as DNA and protein. The most-reported analytical methods for the analysis of meats in general, including non-halal meats, are DNA-based methods using the polymerase chain reaction. However, there are some concerns related to the thermal stability of the DNA used as markers during the detection of non-halal meats [62]. Fortunately, proteomics techniques have allowed for the detection and identification of proteins present in non-halal meats even after denaturation during food processing and cooking, such as boiling and drying. Some authors have used species-specific peptides as markers in proteomics analysis in foods subject to heating.

Sarah et al. [63] have employed LC–QTOF-MS for the identification of pork by investigating the markers of pork-specific peptide from thermally processed meat, which proved to be capable of differentiating pork from other meats (beef, chicken, and chevon meat). Four peptides were identified using LC–QTOF-MS, namely, FVIEIR, EVTEFAK, LVVITAGAR, and TVLGNFAAFVQK, which were consistently detected in cooked pork meat using MRM mode. Thus, the developed method offers accurate and reliable tools for the detection of pork in food products. Furthermore, peptide mass fingerprinting (PMF) analysis, in combination with targeted tandem LC-MS analysis, complemented the chemometrics of PCA, and OPLS-DA has been proven to identify peptide markers that are specific to pork. As a first step, PCA is used to screen and identify the outliers in classification models, and then OPLS-DA is employed to differentiate pork and other meats (beef and chicken). Using variables of 577 peptide masses from all raw meat samples (pork, chicken, and beef), OPLS-DA offered a variation (R^2) of 96.8%, with a prediction of 93.1% (Q^2). Thus, the OPLS-DA model could differentiate pork from other meats. When applying targeted tandem LC-MS, the specific peptide related to pork myosin-2 marker, (F)DFNSLE(Q), was found. This peptide could be used as a marker to detect pork in food products, and is intended for halal authentication analysis [64].

Mi et al. used the lipidomics approach in combination with the chemometrics of PCA (unsupervised) and PLS-DA (supervised) for the analysis of different pork types (Jilin, Sanmenxia and Tibetan in China) by analyzing lipid classes including sterol, fatty acyls, prenol lipids, polyketides, glycolipids, sphingolipids, and glycerophospholipids. The lipid classes with variable importance in projection (VIP) > 1 were used as variables for the classification of pork types. A clear classification according to type was obtained for three pork samples using PLS-DA, with R2 and Q2 values of 0.861 and 0.752, respectively,

indicating that the developed model provides a good predictive capacity and is robust in the classification of a new dataset. During cross-validation, using the leave-one-out technique, the PLS-DA model exhibited accuracy rates of 91.1% 86.7%, and 86.7% for Jilin, Tibetan and Sanmenxia, respectively. Based on this result, the PLS-DA model offers a more reliable model than PCA for the discrimination of China's domestic pork [65].

Different LC separation techniques were introduced to provide a better separation of proteins used for the identification of non-halal meats. Gel-enhanced LC-MS, assisted by PCA, has been developed to identify potential protein markers for the identification of pork among the halal meats of beef and chicken. Analysis of PCA based on score plot of PC1 and PC2 which are accounted for 62% and 35% of the data variations, respectively; could separate pork, beef, and chicken without any outlier points being observed by ellipse Hotelling'sT2. The variables used for PCA were the separated protein bands from gel-enhanced LC-MS. The proteins that contribute to this separation are troponin T, with a peptide sequence of (R)KPLNIDHLSEDK(L); tropomyosin alpha-1 chain [(K)EAETRAEFAER(S)], [(R)HQGVMVGMGQK(D)], COP9 signalosome complex subunit 4 [(R)VLDYRR(K)] and ribonuclease inhibitor [(R)VLGQGLADSACQLETLR(L)]. Thus, PCA-assisted, gel-enhanced LC-MS could potentially be used as a guideline to separate proteins and the specific peptides of proteins could be potential tools for confirming the presence of pork in the mixture with other meats [66].

Another interesting study involved the employment of a combination of untargeted and pseudo-targeted metabolomic studies to identify different markers, which aimed to distinguish live and dead pork meat using LC-MS and PCA and HCA chemometrics [57]. The untargeted metabolomics of 24 different metabolites were scanned using UHPLC–Triple–TOF–MS, while pseudo-targeted metabolomic studies resulted in 14 different markers that were detected using UHPLC–QTRAP–MS. Assisted by the Metlin database and reference standards, and after being treated with HCA, some of the markers identified as contributing the most to classification are carnosine, acetylcholine, L-histidine, L-carnitine, L-acetylcarnitine, N-acetylhistidine, and two phosphatidylcholines. The PCA score plots used variables of 24 different metabolites obtained from the untargeted metabolomic (method 1), and 14 different markers that resulted from untargeted metabolomic studies (method 2). The pseudo-targeted metabolomic (method 3) could classify dead pork meat, live pork meat and quality control samples with extracted variances of the first three PCs of 80, 78 and 80% of the total variance (R^2), and a predictive ability (Q^2) of 55, 40, 42%, respectively. The authors concluded that the metabolomics studies using LC-MS, combined with pattern recognition, were effective tools for the discrimination of live and dead meats, including the discrimination of live beef meat (halal) and dead beef (non-halal).

A lipidomics study was successfully carried out to discriminate raw pork meat by Mi et al. China's domestic pork, namely, Tibetan, Jilin and Sanmenxia pork, were evaluated by an LC-MS-based lipidomics approach, along with partial least-square discriminant analysis (PLS-DA). It was found that lipidomic analysis, along with the multivariate analysis of PLS-DA, can be employed to differentiate China's domestic pork [65]. A related study by Hu et al. used the LC-MS method coupled with a supervised patter recognition of orthogonal partial least-square discriminant analysis (OPLS-DA) to obtain the lipid metabolism profiling of pig treated with low-dose antibiotics. The lipidome analysis of serum by LC-MS was carried out separately in ESI+ and ESI− modes. OPLS-DA was executed to observe the metabolomic differentiation between the low-dose antibiotics groups and control groups, which presented clear separations in lipid profiles between the two groups [67]. The use of LC-Orbitrap MS and fourier transformation near-infrared spectroscopy (FT-NIRS) with chemometrics were also implemented to determine the geographic origin of Boston butt pork, in the study by Hye et al. Korean and foreign Boston butt samples were distinguished using a biomarker analysis approach. OPLS-DA and canonical discriminant analysis (CDA) played an important role in the selection of major metabolites for discrimination and model prediction [68].

7. Analysis of Non-Halal Meats as Adulterants in Halal Meats

Due to price discrepancies between halal and non-halal meats, unethical producers try to blend or substitute halal meats with non-halal ones to increase profits. LC-MS is an effective tool to detect this adulteration, as it can find specific pork markers in the complex mixtures (meat mixtures or food products containing different types of meats). LC-MS is known for its high throughput analysis, which is capable of an in-depth metabolite analysis that can identify as many metabolites as possible in complex food samples, including detecting the adulteration of meats mixed with non-halal meats. von Bargen et al. [69] applied LC-MS/MS using MRM for an authentication analysis of beef (halal meat) from pork (non-halal meat), applying the multiple reaction monitoring (MRM) method to identify the specific peptide markers. Peptides from myosin-4 (TLAFLFAER), as well as myosin-1 and myosin-4 (SALAHAVQSSR), are specific to pork. The developed method is reported to detect as low as 0.13% pork contamination in beef. Recently, Windarsih et al. [33] also used an untargeted metabolomics and proteomics approach, applying LC-Orbitrap HRMS in combination with PCA and PLS-DA chemometrics to detect pork adulteration in Patin fish (*Pangasius hypopthalmus*) meat. Two peptide markers that are specific to pork, namely, the peptide from myoglobin protein (HPGDFGADAQGAMSK) and β-hemoglobin (FFESFGDLSNADAVMGNPK), could be identified and used to confirm the presence of pork. PLS-DA could classify pork, Patin fish meat and a mixture of pork–patin fish meat with good fitness ($R^2 > 0.95$) and good predictivity ($Q^2 > 0.5$). The presence of pork in amounts as low as 0.5% could be detected using this method.

The metabolomics approaches applying LC-MS combined with PLS-DA were proposed as effective tools for the detection of pork as an adulterant in beef. Based on a PLS-DA score plot, pure beef, pure pork and beef with pork contents of 10%, 25% and 50% could be clearly separated. The variables used during PLS-DA are metabolomics, which increase with increasing levels of pork, namely, 3-oxohexadecanoic acid glycerides, cholesterol esters (22:5), ceramide(d18:1/24:1), decanoylcholine, glycyl-lysine, N-carboxyethyl-aminobutyric acid, oleic acid, phospholipid glyceride (36:4), prostaglandin D2 ethanolamide, and triglyceride (16:0/15:0/18:4) [31].

The presence of pork meat in Bolognese sauce has been successfully detected using the LC-MS/MS technique. The myofibrillar protein was the target of analysis. A specific peptide marker from alfa-collagen chain was found in Bolognese sauce samples containing pork. LC-MS/MS could identify all concentration levels of pork added to Bolognese sauce mixed with beef meat (0%–100% *w*/*w*). The LC-MS/MS method was also applied for the analysis of commercial samples of Bolognese sauce. It was found that several samples contain undeclared pork. The limit of detection (LOD) and limit of quantification (LOQ) measurements demonstrated good accuracy for a 2% concentration of pork in the blind samples. The method has a good ability to detect pork in highly processed food samples such as Bolognese sauce with high accuracy and high precision. This technique was also successfully used for the quantification analysis of pork in Bolognese sauce samples. Validation of this technique was carried out, and it was suggested to be an accurate, rapid, and powerful analytical method of pork detection in Bolognese sauce samples [70].

LC-MS/MS technique has also been successfully used to detect pork content in meats and meat products by determining specific markers of carbonic anhydrase 3. Three peptides were found to be specific to pork, namely, EPITVSSDQMAK, GGPLTAAYR, and HDPSLLPWTASYDPGSAK. These three peptides were capable of pork quantification in food products with high linearity. This method proved to be an excellent analytical technique, that can be applied to the analysis of pork content in various types of food products with complex and different matrices using targets of carbonic anhydrase 3 [71].

8. Application of Metabolomics Studies-Based LC-MS for Analysis of Non-Zabiha Slaughtering

AN interesting study related to the identification of meat metabolites resulting from the Zabiha and non-Zabiha slaughtering of chicken was carried out by Abbas et al. [72].

The Zabiha terms are used to describe the rules of animal slaughtering to ensure that it is Halal; this is typically performed by cutting the neck without detaching the spinal cord, while non-Zabiha refers to completely detaching the neck. Non-zabiha slaughtering methods do not follow Shariah law; therefore, meats obtained from non-Zabiha slaughtering method were categorized as non-halal meats and were not allowed to be consumed by Muslims. The different untargeted metabolites obtained from Zabiha and Non-Zabiha were obtained from LC-ESI-MS/MS measurements. Approximately 150 metabolite features were observed to be significantly different between the two groups (Zabiha and Non-Zabiha), and the most metabolites contributing to this differentiation were 13-keto-9Z,11E-octadecadienoic acid, linolenic acid, lysophosphatidylcholine 16:0, 1-(9Z-octadecenoyl)-sn-glycero- 3-phosphocholine and D-erythro sphingosine. The chemometrics of pattern recognition (PCA and OPLS-DA) could successfully discriminate between Zabiha and Non-Zabiha chicken meat samples by applying selected (25) metabolites as variables. This indicated that metabolomic studies that apply LC-MS/MS and chemometrics to differentiate between animals slaughtered according and not according to Syariah law.

A study on the application of LC-MS/MS and chemometrics to differentiate between broiler chicken meat obtained from halal and non-halal slaughtering methods was conducted by Ali et al. [73] LC-MS/MS could identify metabolite composition in both halal and non-halal meat using a one-phase extraction technique. Non-halal broiler chicken meat samples were successfully differentiated from halal broiler meat based on metabolite composition using PCA and PLS-DA. Some metabolites were found to be potential markers to differentiate non-halal broiler meats. This method could be very useful in the identification of commercial broiler chicken meats on the market.

In the field of food research, the LC-MS method coupled with PCA can be employed for discrimination purposes. Yuswan et al. developed a prediction tool for halal analysis using the improved gel-enhanced LC-MS method combined with PCA to identify potential markers of non-halal pork among halal beef and chicken [66]. Another study from Abbas et al. [72] developed a liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS)-based untargeted metabolomics, accompanied by PCA techniques, to discriminate between poultry samples slaughtered with and without detaching the spinal cord. This research is becoming more important, as many communities have shown concern regarding the procedure used to slaughter animals for meat consumption, for ethical, religious, or cultural reasons.

Sidwick et al. [74] has successfully studied the difference between normal-slaughtered and dead-on arrival poultry meat. Dead-on-arrival poultry meat cannot be consumed by Muslims because it is categorized as a non-halal meat. Liquid chromatography tandem mass spectrometry using Q-TOF mass spectrometry was successfully utilized to differentiate dead-on-arrival poultry meats based on metabolite composition. An investigation of the metabolite markers was performed, using chemometrics to identify dead-on arrival meats. The sphingosine metabolite was identified as a potential marker to detect dead-on-arrival poultry meat. This could be applied to processed chicken meats, which would help to reveal meat adulteration.

In some cases of analytical chemistry, sample clustering using PCA cannot be successfully executed. Hierarchical cluster analysis (HCA) can be applied to overcome this problem. This algorithm evaluates and discovers the highest within-class similarity and highest between-class dissimilarity, followed by a clustering process considering, for example, distance, variable, scale, sample, and linkage method [75]. Nair et al. and Babu et al. developed an LC-MS method combined with both PCA and HCA to analyze biological solution samples. The identification of *Mycoplasma hyopneumoniae* infection in pig serum was achieved by Nair et al., and could be used as a diagnostic tool for infection. An ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC–QTOFMS) system utilizing the BEH C18 column was set using a gradient of mobile phase of water to 95% aqueous can containing 0.1% formic acid over a 10-min run. Metabolite markers were analyzed by ionization mass profiles, followed by PCA and

HCA modeling [76]. Babu et al. developed an unsupervised pattern recognition for PCA and HCA to evaluate sterols, bile acids, and acylcarnitines from humans, mice, and pigs after detection using liquid chromatography with tandem mass spectrometry (LC-S/MS). Surprisingly, the simultaneous detection and annotation of sterols, bile acids, and acylcarnitines from standards and biological samples represents a valuable tool for screening these metabolites with high precision. This method can be applied in routine analysis to evaluate biological samples in future metabolomics studies [77].

9. Conclusions and Future Perspectives

Metabolomics offers great potential for the authentication of food products, including the halal authentication of meat products. The identification of metabolite profiles of meat products has advantages for halal authentication. The presence of non-halal meats could be detected using a metabolomics approach combined with the chemometrics of multivariate analysis. LC-MS/MS has advantages for the high-throughput screening of metabolites with high sensitivity and specificity. This technique is suitable for a metabolomics analysis of food samples with complex matrices. Chemometrics could be used for the identification of metabolite patterns, to differentiate and classify samples. The chemometrics of supervised pattern recognition, such as PLS-DA and OPLS-DA, could be used to investigate potential biomarkers of each specific food. These biomarkers are very important for the differentiation between halal and non-halal meat products. It is suggested that a metabolomics approach using LC-MS/MS and chemometrics could be a powerful, rapid, effective and efficient analytical method for the halal authentication of meat products. This technique could be proposed as a new standard analytical method, and used by institutions responsible for the halal authenticity testing of meat products. Therefore, further research focusing on validating the method of metabolomics analysis is important for future to obtain an accurate, valid, precise, reliable and reproducible analytical technique based on LC-MS metabolomics.

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