The Metabolomics Approaches for Analysis of Non-Halal Meats

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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.

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

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 entry 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. 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].

3. 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 [36]. 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. [37] 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 [38].

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 [39].

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 [40].

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 [41]. 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 [39]. 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 [42]. 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 [43].

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