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Metabolomics-Based Profiling of Porcine and Bovine Gelatin for Halal Authentication

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ABSTRACT

Gelatin is widely used in the food, pharmaceutical, and cosmetic industries, where its source is critical for halal authentication and adulteration detection. This study employs a metabolomics-based approach using liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (LC-QTOF-MS/MS) to comprehensively profile the metabolic differences between porcine and bovine gelatin. Untargeted metabolomics was conducted to identify species-specific metabolic signatures. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied to detect metabolic variations and classify gelatin sources with high accuracy. The results showed that PCA clearly distinguished between bovine and porcine gelatin. Several metabolites with high variable importance in projection (VIP) scores, including sphingomyelin SM(d18:0/18:1(9Z)), cytidine 5′-diphosphocholine, 3-methylcytidine, NAD+, ATP, and Tyr-Tyr, were found in higher abundance in porcine gelatin, whereas cytidine-5′-triphosphate, tryptophan, and phenylalanine were more prevalent in bovine gelatin. These metabolic differences arise due to species-specific variations in collagen breakdown and nucleotide turnover pathways. The LC-QTOF-MS/MS-based metabolomics workflow provides high sensitivity and a non-targeted analytical platform for gelatin authentication, preventing fraudulent substitutions in the food supply chain. This method offers a robust, data-driven solution for distinguishing gelatin sources and could also be used in halal applications.

Keywords: *Metabolomics, Bovine Gelatin, Porcine Gelatin, Halal, LC-QTOF-MS/MS*

Introduction

Gelatin is a biopolymer obtained through the partial hydrolysis of collagen, a structural protein found in animal tissues such as skin, bones, and connective tissue (Yörük et al., 2024). It is widely used in the food, pharmaceutical, and cosmetic industries due to its excellent gelling, stabilizing, emulsifying, and texturizing properties (Rohman et al., 2020). However, the animal origin of gelatin is a major concern for specific consumer groups, particularly those adhering to halal, kosher, or vegetarian/vegan dietary restrictions (Mahamud et al., 2023).

The global gelatin market is projected to reach USD 5.0 billion by 2025, driven by increasing demand across various industries. The majority of

commercially available gelatin is derived from porcine skin (46%), bovine hides (29.4%), porcine and bovine bones (23.1%), and to a lesser extent, fish skin (1.5%) (Rather et al., 2022). Among these, porcine and bovine sources represent the dominant share of the market (Adel & Sheet, 2025). Given their widespread use, accurately identifying the animal origin of gelatin is crucial for ensuring proper labeling, maintaining consumer confidence, and supporting regulatory and religious compliance.

As a result, the authentication of gelatin sources is essential to ensure compliance with religious and ethical dietary laws and to prevent fraudulent substitution or mislabeling in commercial products. The most analytical methods for gelatin source determination include electrophoresis (Abdullah Amqizal et al., 2017), enzyme-linked immunosorbent assays (ELISA) (Tukiran et al., 2015), Fourier-transform infrared spectroscopy (FTIR) (Cebi et al., 2019), DNA-based techniques (Sultana et al., 2018), LC-MS/MS (Kwon et al., 2025) . While these approaches have been widely applied, many suffer from limitations such as low sensitivity, insufficient specificity, and lacking comprehensiveness.

In recent years, comprehensive metabolomics analysis of small-molecule metabolites within biological systems has emerged as a promising strategy for food authentication (Selamat et al., 2021). Among the analytical platforms available, LC-QTOF-MS/MS enables untargeted metabolomic profiling with high sensitivity, resolution, and reproducibility (Zhong et al., 2022). This technique allows for the detection of a broad range of metabolites, making it particularly suitable for differentiating gelatin sources based on their distinct biochemical signatures.

This study aims to develop a metabolomics-based approach using LC-QTOF-MS/MS to discriminate between porcine and bovine gelatin sources. The application of PCA and PLS-DA enables the identification of metabolic markers, offering a robust solution for halal authentication.

Materials and Methods

Materials

Porcine gelatin standard was obtained from Sigma-Aldrich (St. Louis, MO, USA), and bovine gelatin was sourced from Cartino Gelatin Co., Ltd. (Thailand). Trypsin from bovine pancreas and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany), and formic acid was sourced from Fisher Scientific (Buchs, Switzerland).

Sample Preparation

10 mg of bovine gelatin and porcine gelatin standard were each weighed into separate 2 mL microcentrifuge tubes. Next, 500 µL of a working solution (pH 8.0) was added to each sample. The mixtures were incubated in a shaking incubator at 600 rpm and 37 °C for 15 minutes. Subsequently, 10 µL of trypsin enzyme solution (1 mg/mL) was added to each tube, followed by incubation in the shaking incubator at 600 rpm and 37 °C for 16 hours.

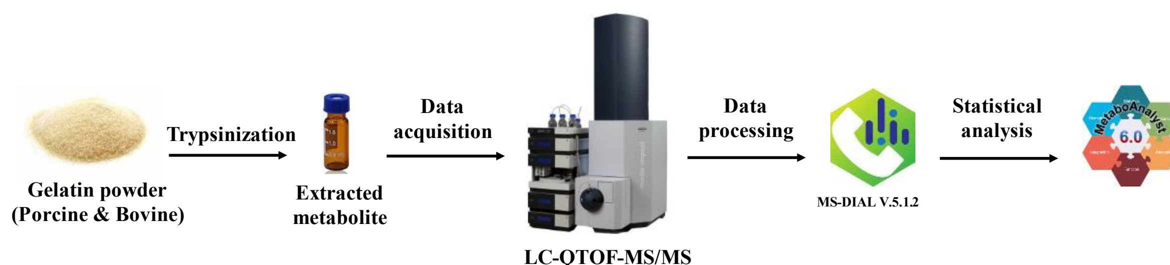

Figure 1. Schematic overview of the metabolomics analysis process for gelatin.

After enzymatic digestion, the samples were centrifuged at 13,000 rpm for 5 minutes. A volume of 400 µL of the supernatant was discarded, and 400 µL of the working solution was added to each tube. Finally, the resulting solutions were filtered through a 0.45 µm syringe

filter and transferred into 1.5 mL LC vials. The samples were stored at -80 °C until LC-MS/MS analysis. The workflow of metabolomics analysis in gelatin is shown in Figure 1.

LC-QTOF-MS/MS Analysis

Metabolomic profiling was performed using a Dionex Ultimate 300 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) connected to a QTOF impact II mass spectrometer (Bruker Daltonics, Bremen, Germany). Metabolite separation was carried out on a C18 reversed-phase column (2.1 × 100 mm, 1.9 µm particle size; Thermo Scientific, Sunnyvale, CA, USA). The column oven was maintained at 40 °C, while the autosampler was set to 7 °C to preserve sample integrity. The gradient elution method was employed using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile, v/v). The gradient program was as follows: The mobile phase B gradually increased from 0% to 10% over the first 10 minutes, then to 20% by 30 minutes, followed by 35% at 40 minutes and 45% at 45 minutes. It was then rapidly decreased to 1% at 50 minutes, with a total run time of 50 minutes. The flow rate was maintained at 0.3 mL/min throughout the run.

Mass spectrometric data were acquired using electrospray ionization (ESI) in positive ionization modes, with a mass scan range of m/z 50–1000. Nitrogen served as both the nebulizing and collision gas. Collision energy was set at 20.0 eV and the dry gas temperature was maintained at 250 °C with a flow rate of 8.0 L/min. Capillary voltages were adjusted to 3800 V for positive mode.

Data Processing and Metabolite Identification

Raw LC-QTOF-MS/MS data were converted into ABF (Analysis Base File) format and imported into MS-DIAL version 5.1.2 for data processing, including deconvolution, peak detection, alignment, and compound identification. Compound annotation was performed using an authentic standards library in MSP format (last updated in August 2024) for positive ionization modes. The resulting peak area data were then uploaded to MetaboAnalyst 6.0 for statistical analysis. Prior to analysis, the data were log-transformed and Pareto-scaled. Multivariate analysis was conducted using PLS-DA, and potential biomarkers were identified based on VIP scores and statistically significant differences in metabolite levels between groups ($p < 0.05$).

Results and Discussion

Metabolic Profiling and Sample Discrimination

A total of 49 metabolites were detected through untargeted metabolomics analysis using LC–QTOF–MS/MS in both porcine and bovine gelatin samples. Subsequent multivariate analysis using PCA revealed a clear and distinct separation between the porcine and bovine gelatin groups, as shown in Figure 2. This distinct clustering indicates significant differences in the metabolic profiles of the two gelatin sources. The separation observed in the PCA two-dimensional (2D) score plot suggests that the detected metabolites can effectively discriminate between the origins of the gelatin samples.

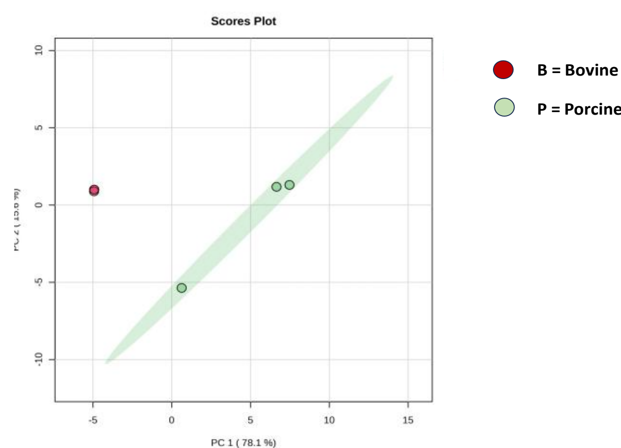

Figure 2. PCA 2D score plot of metabolites in porcine and bovine gelatin.

PCA facilitates the visualization and interpretation of underlying patterns, such as sample groupings or outliers, by transforming the original, potentially correlated variables (metabolites) into a smaller set of uncorrelated variables known as principal components (PCs). In this study, the group separation along the first two principal components PC1 and PC2

highlights the substantial variation in metabolite composition between the two species. PC1 and PC2 accounted for 78.1% and 15.6% of the total variance, respectively, further confirming the discriminatory power of the metabolite profiles.

To identify the most discriminative metabolites contributing to the differentiation between porcine and bovine gelatin, VIP scores were calculated using PLS-DA. Among the identified metabolites, 25 exhibited VIP scores greater than 1.0, indicating their significant contribution to group separation. These metabolites are summarized in Table 1.

Table 1. Metabolites with VIP scores > 1.0 identified in porcine and bovine gelatin.

No.	VIP Scores	Metabolite name	Formula	RT(min)	Detected m/z
1	1.5880	Cytidine-5'-triphosphate	$C_9H_{16}N_3O_{14}P_3$	9.270	484.2588
2	1.4870	Tryptophan	$C_{11}H_{12}N_2O_2$	11.686	205.0927
3	1.3654	Sphingomyelin SM(d18:0/18:1(9Z))	$C_{41}H_{83}N_2O_6P$	14.219	731.5930
4	1.3393	Cytidine 5'-diphosphocholine	$C_{14}H_{26}N_4O_{11}P_2$	1.088	489.4421
5	1.3168	Phenylalanine	$C_9H_{11}NO_2$	1.005	166.1049
6	1.2970	3-Methylcytidine	$C_{10}H_{15}N_3O_5$	0.988	258.1050
7	1.2890	NAD+	$C_{21}H_{28}N_7O_{14}P_2$	15.613	664.1122
8	1.2468	Adenosine triphosphate	$C_{10}H_{16}N_5O_{13}P_3$	11.634	505.9886
9	1.2432	Tyr-Tyr	$C_{18}H_{20}N_2O_5$	1.325	345.1384
10	1.2332	Coenzyme Q10	$C_{59}H_{90}O_4$	21.447	869.6860
11	1.2310	3-Methyl-N-phenylaniline	$C_{13}H_{13}N$	19.623	184.1109
12	1.2299	FAD	$C_{27}H_{33}N_9O_{15}P_2$	15.709	786.1673
13	1.2242	Coenzyme A	$C_{21}H_{36}N_7O_{16}P_3S$	18.861	768.5505
14	1.1842	Vitamin B12	$C_{63}H_{88}N_{14}O_{14}P$	1.111	678.3049
15	1.1614	5-Hydroxylysine	$C_6H_{14}N_2O_3$	1.055	163.1048
16	1.1331	Nicotinamide hypoxanthine dinucleotide	$C_{21}H_{27}N_6O_{15}P_2$	16.221	665.5268
17	1.1243	N-2-Hydroxycyclopentyladenosine	$C_{15}H_{21}N_5O_5$	0.915	352.1648
18	1.0990	Acetyl-CoA	$C_{23}H_{38}N_7O_{17}P_3S$	19.563	810.1216
19	1.0961	PC (16:0/18:1(9Z))	$C_{42}H_{82}NO_8P$	21.447	760.5999
20	1.0760	D-Ala-D-ala	$C_6H_{12}N_2O_3$	19.612	161.0959
21	1.0733	Leucine_Enkephalin	$C_{28}H_{37}N_5O_7$	1.056	556.2841
22	1.0493	putative heterocyst glycolipid	$C_{32}H_{64}O_8$	24.234	576.4599
23	1.0470	N-L-Leucyl-L-leucine	$C_{12}H_{24}N_2O_3$	7.973	245.1809
24	1.0449	Betaine	$C_5H_{11}NO_2$	0.946	257.1433
25	1.0311	S-Adenosyl-L-homocysteine	$C_{14}H_{20}N_6O_5S$	1.056	385.3889

The PLS-DA model showed excellent performance, with a goodness of fit (R^2) of 0.99 and a predictive ability (Q^2) of 0.79. These values demonstrate that the model provides a highly accurate representation of the data, explaining a large proportion of variance ($R^2 = 0.99$) and exhibiting strong predictive power ($Q^2 = 0.79$). Such metrics indicate that the model is both robust and reliable in distinguishing the metabolic profiles of the sample groups. The model's ability to accurately classify samples underscores its suitability for metabolomic discrimination and biomarker identification in this study.

Identification of Metabolites

The identified metabolites highlight underlying biochemical differences in the source origins used for gelatin production. These variations may arise from species-specific metabolic pathway and differences in protein composition during the gelatin extraction process. Several metabolites with high VIP scores including sphingomyelin SM(d18:0/18:1(9Z)), cytidine 5′-diphosphocholine, 3-methylcytidine, NAD^+, ATP, and Tyr-Tyr were found in greater abundance in porcine gelatin. In contrast, cytidine 5′-triphosphate, tryptophan, and phenylalanine

were more prevalent in bovine gelatin, as shown in Figure 3.

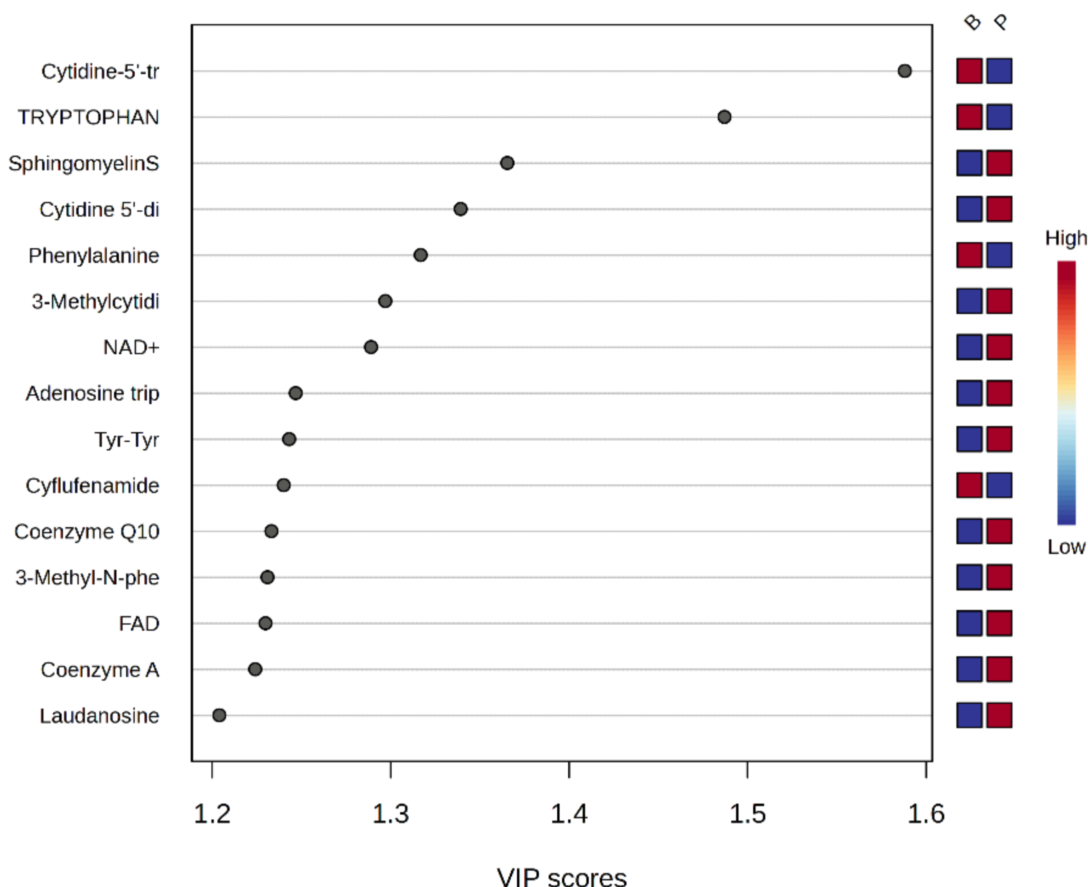

Figure 3. VIP scores plot of metabolites in porcine and bovine gelatin.

Porcine gelatin was characterized by elevated levels of sphingomyelin SM(d18:0/18:1(9Z)), a structural lipid commonly found in animal cell membranes. The presence of this lipid in porcine gelatin may reflect the origin of the raw materials that are rich in sphingolipids. According to a study by (Enomoto et al., 2019), various sphingomyelin species, including SM(d18:0/18:1), were identified in pork meat using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), supporting the finding of SM enrichment in porcine-derived products. In Addition, several studies have reported that tyrosine is found in higher abundance in porcine tissues compared to bovine sources (Hassan et al., 2025; Kumar et al., 2015). This difference may be attributed to variations in the protein composition and amino acid profiles of the two species, particularly in collagen-rich tissues commonly used for gelatin production.

Higher levels of cytidine-5′-triphosphate were observed in bovine gelatin samples. The presence of tryptophan is consistent with the study conducted by (Jamaluddin et al., 2024), as indicated by peaks at 1335.45 and 1336.42 cm^{-1} in bovine gelatin, attributed to the C–N vibrations of tryptophan. In addition, the elevated level of phenylalanine in bovine gelatin aligns with findings from studies by (Harlina et al., 2024; Hassan et al., 2025; Maritha et al., 2023), which reported that bovine gelatin contains a higher concentration of phenylalanine compared to porcine gelatin.

The hierarchical clustering heatmap generated using MetaboAnalyst 6.0 provided a visual overview of the metabolic differences between porcine and bovine gelatin samples, is shown in

figure 4. This analysis simultaneously clustered both samples and metabolites based on similarities in metabolite abundance patterns, allowing for intuitive interpretation of species-specific metabolic profiles.

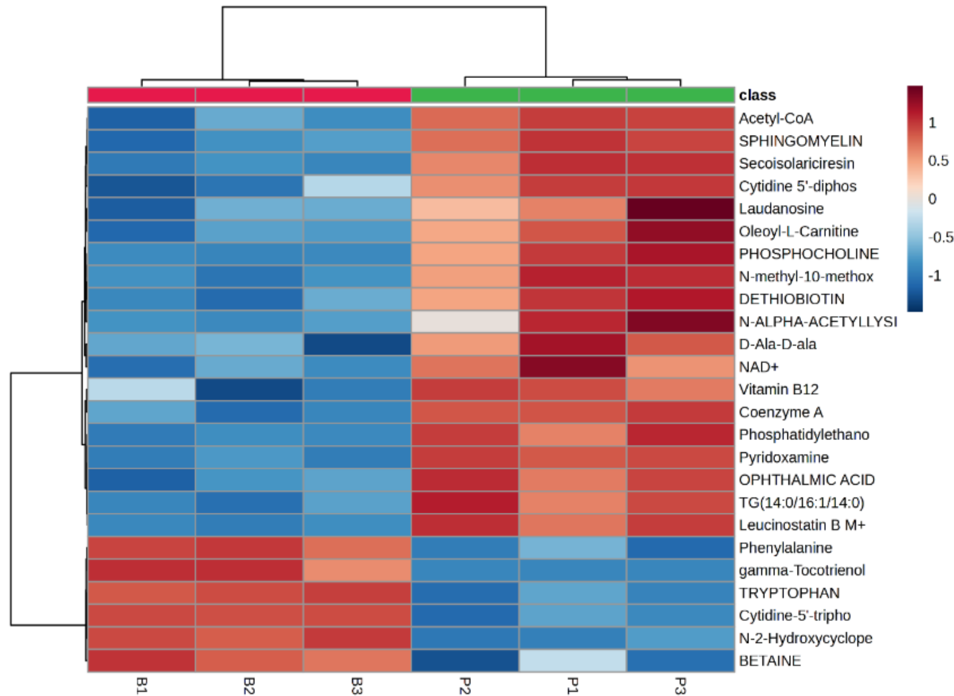

Figure 4. Heatmap analysis in porcine and bovine gelatin.

In the heatmap, color intensity represents the relative abundance of each metabolite across the samples: red indicates higher relative abundance, blue indicates lower relative abundance, and intermediate shades (white or light red/blue) represent moderate metabolite levels. This color gradient facilitates the rapid identification of biochemical patterns. For example, a cluster of red-colored cells corresponding to porcine samples suggests that certain metabolites are consistently more abundant in porcine gelatin, while a blue pattern for the same metabolites in bovine samples indicates lower abundance.

The results revealed a clear separation between porcine and bovine gelatin samples, as evidenced by the distinct clustering observed in the sample dendrogram. Samples derived from the same species clustered closely together, indicating consistent and species-specific metabolic signatures. These findings align with the PCA and PLS-DA results, further confirming that the metabolomic profiles of bovine and porcine gelatin are significantly different.

Conclusion

This study presents a comprehensive metabolomics-based approach for gelatin authentication using LC-QTOF-MS/MS. The results demonstrate that clear metabolic signatures can effectively differentiate between porcine and bovine gelatin, offering a powerful tool for halal adulteration prevention. The identification of species-specific metabolites, particularly those with high VIP scores, provides a robust biochemical foundation for gelatin source differentiation. This is especially significant for halal authentication, as the consumption of porcine-derived products is prohibited in Islam. By enabling accurate discrimination between gelatin sources, this metabolomics approach offers a valuable analytical tool for regulatory agencies, food manufacturers, and certification bodies to ensure compliance with halal standards.

In a broader context, the application of metabolomics contributes to enhanced

transparency, traceability, and consumer confidence in the global food supply chain. This is particularly relevant in multicultural markets where religious or ethical dietary restrictions such as halal, kosher, or vegetarian play a critical role in consumer decision-making. As such, metabolomics-based techniques have strong potential to become standard practice for the authentication of animal-derived ingredients in processed foods and nutraceuticals. Future research may extend this method to complex food matrices containing gelatin and further validate the identified metabolic markers across a wider range of samples and processing conditions.

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Profiliranje svinjskog i goveđeg želatina zasnovano na metabolomici za halal autentifikaciju

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Sažetak

Želatin se široko koristi u prehrambenoj, farmaceutskoj i kozmetičkoj industriji, gdje je njegovo porijeklo ključno za halal autentifikaciju i otkrivanje falsifikata. Ova studija koristi pristup zasnovan na metabolomici, koristeći tečnu hromatografiju u kombinaciji s kvadrupolnim vremenski-of-flajt tandem masenim spektrometrom (LC-QTOF-MS/MS), kako bi se detaljno profilisale metaboličke razlike između svinjskog i goveđeg želatina. Neselektivna (untargeted) metabolomika je provedena s ciljem identifikacije metaboličkih potpisa specifičnih za vrstu. Primijenjene su analiza glavnih komponenti (PCA) i parcijalna najmanje kvadrata diskriminantna analiza (PLS-DA) za otkrivanje metaboličkih varijacija i klasifikaciju izvora želatina s visokom tačnošću. Rezultati su pokazali da PCA jasno razlikuje goveđi od svinjskog želatina. Nekoliko metabolita s visokim VIP vrijednostima, uključujući sfingomijelin SM(d18:0/18:1(9Z)), citidin 5′-difosfokolin, 3-metilcitidin, NAD+, ATP i Tyr-Tyr, pronađeni su u većoj količini u svinjskom želatinu, dok su citidin-5′-trifosfat, triptofan i fenilalanin bili prisutniji u goveđem želatinu. Ove metaboličke razlike proizlaze iz specifičnih varijacija po vrstama u razgradnji kolagena i putevima razmjene nukleotida. Metabolomički pristup zasnovan na LC-QTOF-MS/MS nudi visoku osjetljivost i neselektivnu analitičku platformu za autentifikaciju želatina, sprječavajući prevarne zamjene u lancu prehrambene opskrbe. Ova metoda predstavlja robusno, na podacima zasnovano rješenje za razlikovanje izvora želatina i može se koristiti i u halal kontekstu.

Ključne riječi: *Metabolomika, goveđi želatin, svinjski želatin, halal, LC-QTOF-MS/MS*