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Abstract

Species substitution, most notably substituting buffalo for beef, makes it difficult to ensure that meat quality is authentic and halal. In this study, 600 MHz ^{1}H NMR metabolomics and chemometrics were employed for the detection of buffalo meat adulteration in a meatball containing beef. The composition of beef and buffalo meat was mixed with emulsion to prepare meatballs at a 99:1, 95:5, 90:10, and 80:20 (w/w) ratio. Metabolomic profiling of the untargeted metabolites was carried out, which revealed 45 metabolites including significant changes in amino acid, purine catabolism, and glycolysis metabolic pathway, with an increase in buffalo content leading to a significant decrease in most of free amino acid and energy related metabolites; taurine and ATP levels increased as a function of buffalo content. Supervised orthogonal partial least square-discriminant analysis models were created to achieve high discrimination rates at substitution of beef with buffalo content of more than 10% with $Q^2 > 0.876$, while ATP, inosine, and one unidentified but high discriminatory factor were found as the most discriminating markers. The results revealed that the NMR-based metabolomics approach is a non-destructive approach for verifying the meat authenticity in the heat-treated food and a potential tool for the halal monitoring industry in the world.

Keywords: metabolomics, food authenticity, meatballs, chemometrics, food safety; livestock

Introduction

In the meat sector, food adulteration is one of the major issues, leading to food quality issues, consumer confidence and compliance issues [1]. Species substitution in processed meat products is more concerning because this can be done economically to lower the price of the product by using cheaper meat to replace the most expensive meat [2,3] In the halal market, this practice, as species fraud not declared to the consumer, not only constitutes the act of economic fraud but it is also a religious violation [4]. Therefore, this is critical in processed meat, where meatballs are commonly sold with ground, mixed and heated characteristics [5]. Meatballs are a type of food that is frequently adulterated due to its ground structure, mixing, and heating characteristics [6].

Buffalo meat, due to its abundance and low value is widely employed as a substitution product of beef in several Asian regions [7,8]. The problem of detecting beef-buffalo mixture is challenging because both meat come from two similar ruminant species [9]. In addition to these factors, heat treatment leads to denaturation, oxidation and Maillard's reactions during meat products processing, which significantly change the composition of meat [10], affecting meat authenticity, quality attributes, flavor stability, and sensory acceptance.

Current detection methods of meat products, including PCR, protein and spectroscopy techniques can be highly sensitive in detecting species [11], but they may be less useful in heat-treated meat products. The DNA and protein analysis can be affected by DNA and protein degradation, while spectroscopy can be interfered with by food additive and meat product components [12,13]. In this regard, non-destructive and metabolomic-based methods have emerged recently in food authentication to analyze specific metabolites that reflect species difference.

NMR-based metabolomics is one of the emerging techniques in food authentication studies for identifying specific small molecular metabolites that can distinguish various food samples, such as meat, in a short and reproducible manner [14–17] which was combined with PCA, PLS-DA, and OPLS-DA analysis [4,18–20]. Several applications of NMR-based metabolomics, as it has been combined with chemometrics, were demonstrated to be effective in species authentication and adulteration detection in meat [19,21].

Although there are studies that have already conducted on the authenticity verification of meat products such as beef adulterated with other meat [21,22], studies specifically investigating buffalo adulteration in beef meatballs remain scarce. Therefore, we investigated the potential of using NMR-based metabolomics combined with PCA and OPLS-DA to authenticate and detect buffalo adulteration in beef meatballs. Based on the existing literature, it is hypothesized that NMR-based metabolomics combined with chemometrics can be successfully applied to identify meat species and authenticate the origin of heat-treated meatballs containing buffalo and beef meat. The proposed method is expected to be rapid, non-destructive and reliable to support the verification of meat authenticity and halal meat production, as well as quality assurance of meat products.

Materials and Methods

Sample preparation

Fresh beef (*Bos indicus*) and buffalo (*Bubalus bubalis*) skeletal muscle tissues (males, 2-3 years) were obtained from a certified local market and transported on ice to the laboratory within 24 h postmortem. Visible connective tissue and fat were manually trimmed prior to processing. Four meatball formulations were prepared using beef and buffalo meat at ratios of 99:1 (MB1), 95:5 (MB5), 90:10 (MB10), and 80:20 (MB20) by weight (Table 1). All non-meat ingredients were held constant across treatments: mixed phosphate (6.22 g), salt (21.77 g), pepper (9.33 g), mixed flour (62.21 g), sugar (24.88 g), garlic (9.33 g), and ice (311.04 g), giving a standardized batch weight of 2,000 g per formulation. Each formulation was homogenised using an Electric Meat Grinder (HR1503/00, Phillips, China) until a uniform consistency was achieved. Meatballs (approximately 15 g each) were formed manually and cooked by cooking at 80°C for 5 min until the internal temperature reached 75°C. After cooling to ambient temperature, meatball samples were immediately measured quality characteristics, and other samples stored at −80 °C until analysis. Fifteen independent biological replicates were prepared for each formulation (n = 15 per group), yielding 60 experimental samples in total.

Meat quality measurements

Meat quality attributes associated with meat were assessed comprehensively on the longissimus lumborum muscle within 24 h postmortem through standardized non-overlapping analysis procedures. Measurement of ultimate pH was done at 24 h using a calibrated portable pH meter (HI981036, Hanna Instruments, Woonsocket, RI, USA) and instrumental color (CIE L*, a*, b*) was measured after 30 min bloom using a CR-400 colorimeter (Konica Minolta Sensing Inc., Osaka, Japan) under D 65 illumination in accordance with AMSA guidelines [23]. The determination of water activity was done at 25°C using a chilled-mirror dew-point meter (Novasina AG, Lachen, Switzerland) calibrated against certified salt standards. Drip and cooking losses were gravimetrically determined using the Honikel method [24]. A Warner–Bratzler Shear Force (WBSF) was measured on cooked steaks in accordance with AMSA guidelines [25] using TA.XT Plus texture analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK). Texture profile analysis (TPA) was also measured according to standard protocols [26]. The electronic nose (Electronic Nose Co., Ltd., Bangkok, Thailand) with eight MOS sensors (TGS series) was used to analyze meat volatile profiles following standard procedures [27]. Finally, chemical composition was followed AOAC International guidelines [28].

NMR-based metabolomic analysis

^{1}H NMR-based metabolic profiling was performed at the National Phenome Institute (KKUNPhI), Khon Kaen University in December 2025. Briefly, 100 mg of meatball sample was extracted using a mixture of 400 µL methanol and 85 µL water (v/v). Samples were homogenized using a bead beater (3,500 × g for 45 s, two cycles with a 5 s pause between cycles), followed by centrifugation at 14,000 × g for 14 min at 4°C. The supernatant was transferred to a new tube, and chloroform (400 µL) and HPLC-grade water (200 µL) were added. The mixture was vortexed, sonicated for 15 min (twice), and incubated at 4 °C for 15 min. Subsequently, samples were centrifuged at 1,000 × g at 4 °C for 15 min to achieve phase separation. The aqueous phase was collected and dried.

Dried extracts were reconstituted in 650 µL of NMR buffer containing 1.5 mM KH_2PO_4, 2 mM NaN_3, and 0.05% (w/v) trimethylsilylpropionic acid (TSP) in D_2O (pH 7.4). The solution was sonicated for 15 min and centrifuged at 15,000 × g at 4 °C for 10 min. An aliquot of 580 µL was transferred into 5 mm NMR tubes. Quality control (QC) samples were prepared by pooling 20–30 µL aliquots from each sample. ^{1}H NMR spectra were acquired using a 600 MHz spectrometer (Ascend Evo, Bruker Biospin, Switzerland) at 298 K. A one-dimensional NOESY pulse sequence (noesypr1d) was employed for water suppression. Spectra were processed at the Chenomx Inc. using Chenomx NMR Suite (version 10, Chenomx Inc., Canada), including phase and baseline correction, and chemical shifts were referenced to TSP-d_4. Metabolites were identified and quantified using the Chenomx library based on chemical shift and signal pattern matching, with concentrations referenced to the internal standard TSP (δ 0.00 ppm). The spectral region corresponding to residual water (4.70–5.00 ppm) was excluded from analysis.

Statistical and chemometric analysis

All analyses were conducted in R (v4.6.0) [29]. Univariate analysis was performed by one-way ANOVA with Tukey's post-hoc test. Chemometric analysis was also conducted in R using the ropls (v1.34.0), pROC (v1.19.0.1), and ggplot2 (v4.0.2) packages. The concentration data were Pareto-scaled (mean-centered and divided by the square root of the standard deviation) to reduce the influence of high-abundance metabolites while preserving data structure.

Unsupervised principal component analysis (PCA) was performed on the Pareto-scaled matrix using ropls [30]. Two models were constructed: (i) an all-sample model including QC injections to verify analytical stability, and (ii) an experimental-sample model (QC excluded) to visualize group separation. All six pairwise PCA models were additionally fitted to inspect local separation patterns. Score plots were annotated with 95% confidence ellipses.

Supervised orthogonal partial least squares-discriminant analysis (OPLS-DA) [31] was applied to each of the six pairwise group combinations using ropls [30] with one predictive component, automatic orthogonal components, and Pareto-scaled data (scaleC = "none"). Model quality was assessed by R^2X, R^2Y, and Q^2. Model validity was confirmed by 1,000-cycle permutation testing; intercepts of the regression lines for permuted R^2Y and Q^2 were inspected to exclude overfitting [32,33]. All R^2X values were computed from model score and loading slots to include orthogonal components.

For biomarker selection, a possible candidate metabolite was designated a potential adulteration biomarker if it simultaneously satisfied: (i) VIP > 1.0 [34], (ii) Wilcoxon rank-sum test $p < 0.05$ [35], (iii) Benjamini–Hochberg false discovery rate (FDR) < 0.05 [36], and (iv) fold-change (FC) threshold ≥ 1.50 or ≤ 0.67. Diagnostic performance was assessed per biomarker using receiver operating characteristic (ROC) analysis and area under the curve (AUC) with 95% bootstrap confidence intervals (pROC, [37]).

Results

Meat quality characteristics

Descriptive statistics (mean ± SD) for raw beef and buffalo meat used in this study showed that buffalo meat had a higher ultimate pH than beef (5.63 ± 0.02 vs. 5.45 ± 0.03). For color, beef was lighter and more yellow (CIE L* = 42.47 ± 0.44; CIE b* = 18.80 ± 0.10) than buffalo (CIE L* = 36.00 ± 0.45; CIE b* = 16.00 ± 0.62), and marginally redder (CIE a* = 29.90 ± 0.20 vs. 29.10 ± 0.98). Buffalo meat had higher water activity (Aw = 0.98 ± 0.01 vs. 0.96 ± 0.00) but lower drip loss (4.14 ± 1.62% vs. 4.55 ± 0.21%) and cooking loss (35.26 ± 5.84% vs. 39.51 ± 3.49%) than beef. Texture profile analysis revealed that beef was substantially harder (15,748.72 ± 3,749.35 g vs. 10,932.78 ± 4,332.45 g) and had higher gumminess, springiness, and cohesiveness than buffalo meat. Beef also showed greater adhesiveness (more negative value). Electronic nose responses were higher for buffalo meat on Sensors 1, 2, 4, 5, 6, 7, and 8, while beef produced a stronger response only on Sensor 3. Regarding proximate composition, beef had lower moisture (75.14 ± 0.04% vs. 77.48 ± 0.18%) but higher protein (22.52 ± 0.04% vs. 20.96 ± 0.05%), fat (1.92 ± 0.04% vs. 1.32 ± 0.02%), and ash (0.41 ± 0.03% vs. 0.23 ± 0.14%) contents than buffalo meat.

The mean values of the quality attributes for the four formulations of meatballs are presented in **Table 2**. Treatment had a significant effect on surface color (CIE L*, a*, b*; $p \leq 0.025$), water activity (Aw; $p = 0.008$), and most TPA parameters — hardness ($p = 0.004$), cohesiveness ($p = 0.012$), gumminess ($p < 0.001$), chewiness ($p = 0.021$), and resilience ($p = 0.002$). The treatment did not significantly affect adhesiveness and springiness ($p > 0.05$). Responses of all eight sensors using the electronic nose were also not significant ($p > 0.05$). The buffalo meat proportion had a non-linear relationship with the product redness as the highest CIE a* value were recorded by MB10 (90:10) meatballs and the lowest by MB1 (99:1). The difference in Aw between MB1 and MB5 was statistically significant from MB10 and MB20. Texture measurements of hardness, gumminess, chewiness and resilience generally followed this same pattern, with MB1 and MB5 being hardest and MB20 (80:20) the softest. There was the highest cohesiveness for MB1 and MB10, and the lowest cohesiveness for MB5 and MB20.

Treatment significantly influenced the proximate composition ($p < 0.0001$ for moisture content, protein content and fat content; $p = 0.003$ for ash content). MB5 had the highest moisture (76.48%) and MB20 the lowest (75.43%). The proportion of buffalo increased the protein content of the meat, with the highest being MB20 (18.52%) and the lowest in MB5 (18.09%). However, content of buffalo increased and fat decreased: MB1 was the highest (1.72%) and MB5 had the lowest (1.35%). MB1, MB10 and MB20 were similar (highest) and MB5 was the lowest (4.07%) in terms of ash content.

NMR-based metabolomic profiling

The detected metabolites from the beef–buffalo meatballs tested by ^{1}H NMR metabolomics were quantified in a matrix of 45 metabolites from various biochemical classes (**Table 3**). The results showed that the concentrations of metabolites were affected by increasing amounts of buffalo meat for most of the metabolites detected. The trends were generally of a negative association between most of the metabolites and buffalo meat and a positive association for a specific group.

Thirteen free amino acids were significantly altered by adulteration level ($p < 0.05$). Alanine, glycine, isoleucine, leucine, valine, arginine, lysine, phenylalanine, tyrosine, methionine, proline, glutamate, and glutamine all decreased monotonically with increasing buffalo meat content. The branched-chain amino acids (BCAAs) leucine and valine exhibited particularly stated reductions: leucine declined from 0.023 ± 0.003 mM in MB1 to 0.013 ± 0.002 mM in MB20, and valine from 0.021 ± 0.003 mM to 0.011 ± 0.004 mM. Glutamate decreased from 0.046 ± 0.008 mM to 0.035 ± 0.004 mM. For leucine, valine, methionine, glutamate, and tyrosine, Tukey's HSD resolved MB20 as a distinct grouping from lower-adulteration formulations, indicating that these metabolites are particularly sensitive discriminators of high-level buffalo substitution. In aggregate, the reduction in the free amino acid pool encompassed aliphatic, branched-chain, basic, aromatic, and sulfur-containing subclasses, indicating a broad and systemic shift in nitrogen metabolism associated with increasing buffalo meat proportion.

In contrast to the general declining trend, taurine exhibited a consistent and significant increase with rising buffalo meat content, rising from 2.351 ± 0.185 mM in MB1 to 2.730 ± 0.201 mM in MB20 ($p <$ 0.0001). Tukey's HSD distinguished low-adulteration groups (MB1, MB5) from high-adulteration groups

(MB10, MB20), establishing taurine as a robust positive marker of buffalo meat content. Betaine and pyroglutamate remained unchanged across formulations, whereas cystathionine decreased significantly with increasing buffalo proportion.

Anserine-like3, carnosine, and glutathione all declined significantly with increasing adulteration. Carnosine showed the most pronounced response, decreasing sequentially from 0.016 ± 0.005 mM in MB1 to 0.005 ± 0.003 mM in MB20 ($p < 0.0001$). Anserine-like3 decreased from 0.435 ± 0.046 mM to 0.361 ± 0.024 mM, and glutathione declined toward the quantification limit in the higher-adulteration formulations.

The purine metabolite pool exhibited a divergent pattern: ATP increased significantly from 0.028 ± 0.005 mM in MB1 to 0.035 ± 0.008 mM in MB20 ($p = 0.005$), whereas IMP and inosine decreased. IMP declined from 0.272 ± 0.031 to 0.221 ± 0.016 mM ($p < 0.0001$), and inosine from 0.032 ± 0.005 to 0.019 ± 0.006 mM ($p < 0.0001$). This reciprocal shift—elevated ATP concurrent with diminished IMP and inosine—is consistent with interspecies differences in the rate and extent of post-mortem purine catabolism.

Lactate decreased markedly from 6.432 ± 0.602 mM in MB1 to 5.608 ± 0.402 mM in MB20 ($p = 0.0001$), together with significant reductions in 2-hydroxyvalerate, acetate, citrate, and fumarate. Formate and succinate did not differ significantly among groups. Creatine declined from 2.584 ± 0.212 mM to 2.062 ± 0.160 mM ($p < 0.0001$), with Tukey's HSD resolving three distinct groups (MB1 > MB5/MB10 > MB20); creatinine similarly decreased from 0.108 ± 0.008 to 0.090 ± 0.013 mM ($p < 0.0001$).

Sucrose and myo-inositol decreased significantly in MB20 relative to lower-adulteration groups, whereas glycerol showed a non-monotonic pattern, reaching its nadir in MB10 before a modest recovery in MB20 yet remaining significantly lower than MB1. Glucose was not significantly different across formulations. Carnitine decreased substantially from 0.414 ± 0.031 mM in MB1 to 0.332 ± 0.038 mM in MB20 ($p < 0.0001$), while *O*-acetylcarnitine was unchanged.

Ethanol decreased modestly but significantly across adulteration levels ($p = 0.008$), whereas isopropanol and methanol were not significantly altered. Niacinamide showed a decline from MB1 to MB20 ($p < 0.0001$), resolved into three distinct Tukey groups (MB1 > MB5/MB10 > MB20). Betaine, glucose, *O*-acetylcarnitine, methanol, isopropanol, and Unknown1 were not significantly affected by adulteration level, underscoring the selectivity of the metabolic response to buffalo meat substitution. Collectively, univariate identified systematic adulteration-dependent shifts across amino acid metabolism, purine catabolism, glycolysis, creatine metabolism, and fatty acid transport, demonstrating that beef–buffalo meatballs at different adulteration levels possess molecularly distinguishable metabolomic fingerprints.

Chemometric Analysis

Principal component analysis

PCA of all samples including pooled QC (**Figure 1**) demonstrated that QC injections clustered tightly near the score-plot centroid, indicating acceptable instrument stability throughout the analytical sequence and confirming the suitability of the data for metabolomic fingerprinting. Experimental groups (MB1, MB5, MB10, MB20) exhibited partial cluster overlaps in the global PCA (**Figure 2**), consistent with the inherently exploratory nature of PCA, which maximizes explained variance rather than group discrimination. A progressive increase in cluster separation with increasing compositional difference was nonetheless evident: the MB1-vs.-MB20 and MB10-vs.-MB20 pairwise PCAs achieved better group resolution than the MB1-vs.-MB5 and MB5-vs.-MB10 comparisons, which retained substantial overlap. This pattern confirms that low-level adulteration (1–5%) produces metabolic fingerprints insufficiently distinct for reliable unsupervised discrimination.

OPLS-DA model performance

Application of supervised OPLS-DA (**Figure 3**) markedly improved group discrimination relative to PCA in most pairwise comparisons. The MB1-vs.-MB5 model, however, produced poor model quality ($R^2X = 0.595$, $R^2Y = 0.228$, $Q^2 = 0.033$) and yielded no biomarkers satisfying the selection criteria (VIP > 1, FDR < 0.05, FC > 1.5 or < 0.67), indicating that the metabolic differences between these two closely spaced adulteration levels are insufficient to support a reliable predictive model under the conditions of this study. By contrast, the MB1-vs.-MB10 comparison produced a robust model ($R^2X = 0.628$, $R^2Y =$

0.941, Q^2 = 0.876; permutation $pR^2Y = pQ^2$ = 0.001), identifying ATP and inosine as discriminatory variables. The MB1-vs.-MB20 comparison achieved the strongest overall performance (R^2X = 0.845, R^2Y = 0.978, Q^2 = 0.940; permutation p = 0.001), with four discriminatory metabolites: Unknown1, myo-inositol, ATP, and inosine. The MB10-vs.-MB20 model also exhibited high discriminatory power (R^2X = 0.747, R^2Y = 0.975, Q^2 = 0.919; permutation p = 0.001), with Unknown1 as the sole variable passing all selection criteria. These findings collectively indicate that metabolomic discrimination scales with adulteration magnitude, and that statistically valid, non-overfitted OPLS-DA models are attainable for adulteration differences ≥10%. Notably, the MB5-vs.-MB10 and MB5-vs.-MB20 models yielded high R^2Y and Q^2 values (0.982/0.950 and 0.986/0.938, respectively) with significant permutation tests, yet no individual metabolite satisfied the biomarker selection criteria.

Biomarker identification

Biomarker selection from pairwise OPLS-DA analyses (**Table 4**) corroborated model-level findings. For MB1 vs. MB10, both ATP (VIP = 1.199, FC = 0.544, $\log_2$FC = −0.877, AUC = 1.000) and inosine (VIP = 1.131, FC = 0.520, $\log_2$FC = −0.944, AUC = 1.000) were significantly lower in MB1 than in MB10. For MB1 vs. MB20, Unknown1 was the highest-ranked variable (VIP = 2.384, FC = 2.123, $\log_2$FC = +1.086, AUC = 0.871, 95% CI: 0.699–1.000), present at higher concentrations in MB1 than in MB20. myo-Inositol (AUC = 0.933), ATP (AUC = 0.969), and inosine (AUC = 0.973) were also significantly reduced in MB1 relative to MB20. For MB10 vs. MB20, Unknown1 remained the dominant discriminatory variable (VIP = 3.141, FC = 1.693, $\log_2$FC = +0.760, AUC = 0.871), suggesting a role as a fingerprint metabolite across mid-to-high adulteration transitions. Structural characterization of Unknown1 is required before it can be advanced from candidate to confirmed biomarker status.

Discussion

Physicochemical Properties of Raw Meat and Meatball

The physical, chemical, and textural characteristics of beef and buffalo meat are different, and they are due to fundamental structural and biochemical differences between beef and buffalo meat [38]. Meat acidity differs between buffalo and beef, with the buffalo meat having a higher ultimate pH (5.63 vs 5.45) than beef which indicates less extensive postmortem glycolysis, low glycogen depletion and low lactate accumulation [39]. The meat color was also darker and less yellow, which is known as having a higher myoglobin content and proportion of oxidative muscle fibres [23,40,41]. Even though buffalo meat had a higher water activity than beef, they found that the buffalo meat had lower drip and cooking losses, showing that it may have better water-holding ability due to its higher pH and improved myofibrillar water retention [42]. Conversely, beef exhibited higher levels of hardness, cohesiveness, gumminess, adhesiveness and springiness, which could be attributed to the higher fat content and differences in the properties of the proteins in muscles and the connective tissue of beef [43]. Protein, fat and ash content was also higher in beef compared to buffalo while moisture content was lower in the same.

An increase in the percentage of buffalo meat gradually lowered meatball hardness, gumminess, chewiness and resilience, which may be attributed to the low fat content and different gelation characteristics of buffalo myofibrillar proteins [43] to give a softer and less elastic protein matrix. Formulations with higher beef levels, on the other hand, exhibited firmer textures, as a result of the physicochemical properties of raw beef. Non-linear responses were observed for the variables redness with peak at MB10, and protein structural change during cooking, indicating interaction between these variables that is not additive in determining the final product color [23]. The higher proportion of buffalo also led to a higher water activity and water retention, which is in line with the higher water holding capacity of buffalo meat, and protein content was slightly higher, while fat content was lower, due to buffalo meat being leaner. Most notably, there were no significant differences between the treatments in the E-nose analysis even though there was clear difference in the physicochemical properties of the buffalo, suggesting that the generation of VOCs was less sensitive to the low level buffalo substitution.

Metabolomic Profile of Beef–Buffalo Meatballs

In the present study, it is found that using the metabolomics approach based on ^{1}H NMR spectra and combined with the univariate and chemometric analysis can successfully detect the changes in composition of beef meatball emulsions adulterated with buffalo meat at concentrations ranging from 1%

up to 20% (w/w). Consistently, buffalo proportion resulted in changes in amino acid-related metabolomic pathways, purine metabolites, glycolytic pathways, creatine metabolism, and carnitine metabolism, which demonstrated the untargeted metabolomics ability to highlight the global compositional shifts in meat products [1,38,44]. The reduction of most free amino acids, dipeptides and energy related metabolites with increasing buffalo content, is probably due to species-related differences in muscle composition, post-mortem metabolism and proteolysis, and not because there was a single marker compound. The results highlight the benefit of metabolomics over DNA-based approaches, which reveal not only whether the species has been substituted but also may provide information on the wider biochemical changes that occur when adulteration is occurring [1,22].

The decrease in BCAAs (leucine, isoleucine and valine) in addition to lysine, arginine, methionine, glutamate and phenylalanine, indicates considerable changes in nitrogen metabolism and protein turnover with increasing buffalo meat proportion. These amino acids are significant in the flavor development and overall product quality of meat, and their reduction is probably due to differences in species muscle protein composition, postmortem proteolysis and extraction of metabolites during emulsion processing [38]. The reduction of glutamate is especially important in terms of umami taste and metabolomic discrimination due to its role in amino acid metabolism and flavor formation in food [45]. Taurine content, on the other hand, increased gradually with the addition of buffalo meat. However, the taurine generally vary with different animal species or muscle types [46].

The content of carnosine and anserine-like compounds in skeletal muscle as intracellular pH buffers and antioxidants, decreased with the increase of buffalo meat content, which may be due to the muscle fiber composition differences between buffalo and cattle [47]. Likewise, creatine and creatinine exhibited dose-dependent decreases, which reflected less amounts of energy-reserve metabolites in buffalo-rich formulations and made a significant contribution to treatment discrimination. In purine metabolism, there was an increase in ATP and a decrease in IMP and inosine in the higher buffalo formulation, indicating that there may be species differences in post-mortem nucleotide degradation and energy metabolism. The differences in meat metabolism also lead to purine metabolites being closely linked to meat-flavor development and post-mortem aging, further highlighting the value of purine metabolites as species authentication markers in meat authentication [22,38,48].

As buffalo meat increased, there was a corresponding decrease in lactate, and this contrasted with species differences in post-mortem glycolysis and energy metabolism (Lawrie & Ledward, 2006). Other metabolites, such as citrate, fumarate, and 2-hydroxyvalerate, are also reduced, suggesting changes in pathways related to the TCA cycle, which is crucial for meat authentication and quality assessment [1,38]. Other lipids, such as carnitine and myo-inositol, also decreased, indicating a difference in the lipid metabolic and osmotic regulation between the beef and buffalo muscle. While these metabolites were not significantly different, betaine, glucose, O-acetylcarnitine, methanol, and isopropanol were not significantly altered, suggesting that buffalo contamination does not occur across the metabolome but rather occurs in specific metabolic pathways.

Chemometric Analysis and Candidate Biomarkers

NMR-based metabolomics uses the ability to produce comprehensive metabolic 'fingerprints' to differentiate between authentic beef and adulterated products, detecting differences between the two, for example, beef and pork, and between non-halal and halal beef. This is done by optimized sample preparation, advanced NMR acquisition, and chemometric/machine learning-based models that identify and quantify specific metabolites of interest, such as specific amino acids and lipid profiles. The techniques provide powerful non-destructive analysis of subtle compositional differences that have potential for fraud detection—particularly when combined with other analytical systems and AI analytical tools for increased sensitivity and automation [4,48,49]. Untargeted ^{1}H NMR metabolomics with OPLS-DA is a scientifically sound alternative to authentication based on DNA and protein that is often unsuccessful for thermally processed matrices [22,50,51]. The results presented here show that a detection threshold for buffalo substitution higher than 5% is reached. Adulteration level is 10%, and the models show good predictive power ($Q^2 \geq 0.876$) and statistical significance.

Biochemically, ATP and inosine are identified as key variables with species-specific post-mortem energy metabolism and enzyme kinetics [38]. These nucleotides are identified as markers in species monitoring of meat [22,48]. Moreover, the finding of myo-inositol in the MB1-vs.-MB20 model implies species-specific differences in membrane homeostasis and physiological state, which could also be confirmed by further MS/MS structural analysis [1,38]. The high potency unknown1 (VIP score up to 3.141) arose with fingerprint of mid to high adulteration. As for the contemporary standards, this feature should be structurally characterized using 2D NMR and MS/MS prior to its elevation to a confirmed biomarker [1,38]. Interestingly, some models (MB5-vs.-MB10/20) obtained high R^2Y and Q^2 without using individual biomarkers. This is a diffuse distributed variance and confirms that multivariate separation cannot be understood without considering the biological context and selection criteria, but not only with statistical criteria [52]. The results are consistent with the supervised chemometrics approach for the classification of red meat based on metabolites such as lactate, creatine, and acetate [1,22,48]. Existing studies typically rely on LC-MS or FTIR to detect non-Halal adulterants [53,54], which could be applied to the detection of (Halal) species adulteration as well as quantitative estimation for Halal regulatory frameworks [54].

Limitations and Future Perspectives

Although great progress has been made, there are still several obstacles in the way of the widespread use of metabolomics as a routine tool for authentication of processed meat products. The complexity and heterogeneity of meat matrices can mask subtle differences in the metabolic process, especially when different industrial processing conditions are implemented and when several adulterants are present in the samples. Furthermore, the usefulness and transferability of predictive models rely on large, diverse and well-characterized datasets. Future work should thus be conducted to achieve methodological standardization, interlaboratory validation and to develop Certified Reference Materials for ensuring analytical reproducibility. The addition of more species, geographic locations, nutrition, and processing methods to metabolomic databases will further increase the specificity of biomarkers and their applicability to other parts of the world.

Conclusion

Using ^{1}H NMR-based metabolomics combined with chemometrics, we established a comprehensive analytical strategy for detecting buffalo meat adulteration in the beef meatball emulsion range at 1–20% (w/w) levels. Increasing buffalo content caused significant changes in a number of metabolic processes, such as amino acid metabolism, purine degradation, glycolysis, creatine metabolism and fatty acid transport. Only the increase of taurine levels was observed consistently with the increasing buffalo content, suggesting its utility as a species-specific positive marker. Good models ($Q^2 \geq 0.876$, perm $p = 0.001$) were found in all the OPLS-DA models when the adulteration level was more than 10% (w/w), identifying ATP, inosine and Unknown1 as the most important variables for differentiation between the meat. The proposed ^{1}H NMR-based approach for detecting buffalo meat adulteration in beef meatballs may be useful for evaluating the authenticity and monitoring meat quality for regulatory purposes.

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Tables

Table 1. Formulation of beef meatballs containing varying proportions of buffalo meat.

Ingredient (g)	**MB1 (99:1)**	**MB5 (95:5)**	**MB10 (90:10)**	**MB20 (80:20)**
Beef	1,539.66	1,477.45	1,399.69	1,244.17
Buffalo meat	15.55	77.76	155.52	311.04
Mixed phosphate	6.22	6.22	6.22	6.22
Salt	21.77	21.77	21.77	21.77
Pepper	9.33	9.33	9.33	9.33
Mixed flour	62.21	62.21	62.21	62.21
Sugar	24.88	24.88	24.88	24.88
Garlic	9.33	9.33	9.33	9.33
Ice	311.04	311.04	311.04	311.04
Total	**2,000**	**2,000**	**2,000**	**2,000**

Table 2. Quality attributes of beef meatballs formulated with varying proportions of buffalo meat

Item	MB1 (99:1)	MB5 (95:5)	MB10 (90:10)	MB20 (80:20)	SEM
Surface color					
Lightness (CIE L*)	65.12^{a}	62.43^{b}	62.41^{b}	61.58^{c}	0.202
Redness (CIE a*)	3.78^{c}	4.35^{b}	4.82^{a}	3.90^{c}	0.076
Yellowness (CIE b*)	11.51^{ab}	11.21^{b}	11.44^{ab}	11.71^{a}	0.059
Water activity (Aw)	0.962^{b}	0.964^{b}	0.973^{a}	0.974^{a}	0.001
Texture profile analysis (TPA)					
Hardness (g)	$6{,}065.17^{a}$	$5{,}776.18^{a}$	$5{,}103.25^{ab}$	$3{,}952.67^{b}$	230.54
Adhesiveness	−1.51	−1.77	−1.47	−2.04	0.165
Springiness (mm)	0.74	0.77	0.78	0.77	0.017
Cohesiveness	0.75^{a}	0.70^{b}	0.75^{a}	0.69^{b}	0.008
Gumminess	$4{,}448.33^{a}$	$3{,}966.49^{a}$	$3{,}811.01^{a}$	$2{,}722.38^{b}$	155.37
Chewiness	$3{,}484.15^{a}$	$3{,}139.53^{a}$	$3{,}097.15^{a}$	$2{,}146.51^{b}$	162.38
Resilience	0.42^{a}	0.39^{ab}	0.42^{a}	0.36^{b}	0.007
Odor profile-based E- nose response (%)					
Sensor 1	0.388	0.743	0.685	0.809	0.064
Sensor 2	0.488	0.763	0.677	0.833	0.065
Sensor 3	1.816	1.962	2.201	2.038	0.094
Sensor 4	0.211	0.198	0.311	0.292	0.023
Sensor 5	0.337	0.345	0.230	0.316	0.023
Sensor 6	0.417	0.747	0.568	0.840	0.065
Sensor 7	0.471	0.827	0.708	0.895	0.069
Sensor 8	2.391	2.494	2.445	2.537	0.075
Proximate composition (%)					
Moisture	75.58^{c}	76.48^{a}	75.81^{b}	75.43^{c}	0.062
Protein	18.38^{c}	18.09^{d}	18.45^{b}	18.52^{a}	0.023
Fat	1.72^{a}	1.35^{d}	1.43^{c}	1.68^{b}	0.022
Ash	4.30^{a}	4.07^{b}	4.31^{a}	4.37^{a}	0.032

$^{a–c}$ Means within the same row bearing different superscript letters differ significantly ($p < 0.05$).

MB1 = beef: buffalo 99:1; MB5 = 95:5; MB10 = 90:10; MB20 = 80:20; SEM = standard error of means.

Sensor 1: butane, methane, propane; Sensor 2: smoke, alcohols; Sensor 3: organic solvent vapors; Sensor 4: methyl mercaptan, trimethylamine; Sensor 5: isobutane, ethanol, ammonia; Sensor 6: propane, isobutane, methane; Sensor 7: alcohols, organic solvents; Sensor 8: ammonia.

Table 3. NMR-identified metabolites (mM) in beef meatballs with varying proportions of buffalo meat

Metabolite	**MB1 (99:1)**	**MB5 (95:5)**	**MB10 (90:10)**	**MB20 (80:20)**	***p* Value**
Amino Acids (n = 13)					
1) Aliphatic Amino Acids					
Alanine	0.228 ± 0.023^{a}	0.216 ± 0.020^{a}	0.213 ± 0.012^{a}	0.205 ± 0.015^{b}	0.012
Glycine	0.180 ± 0.016^{a}	0.182 ± 0.014^{a}	0.169 ± 0.014^{a}	0.162 ± 0.009^{b}	0.0004
2) Branched-Chain Amino Acids (BCAAs)					
Isoleucine	0.014 ± 0.005^{a}	0.014 ± 0.003^{a}	0.013 ± 0.003^{a}	0.011 ± 0.002^{b}	0.010
Leucine	0.023 ± 0.003^{a}	0.018 ± 0.006^{b}	0.016 ± 0.004^{b}	0.013 ± 0.002^{c}	< 0.0001
Valine	0.021 ± 0.003^{a}	0.018 ± 0.004^{a}	0.015 ± 0.003^{b}	0.011 ± 0.004^{c}	< 0.0001
3) Basic Amino Acids					
Arginine	0.015 ± 0.007^{a}	0.012 ± 0.005^{a}	0.008 ± 0.004^{b}	0.007 ± 0.004^{b}	0.0005
Lysine	0.043 ± 0.010^{a}	0.040 ± 0.010^{a}	0.033 ± 0.008^{b}	0.032 ± 0.006^{b}	0.002
4) Aromatic Amino Acids					
Phenylalanine	0.090 ± 0.021^{a}	0.080 ± 0.013^{a}	0.075 ± 0.012^{b}	0.069 ± 0.013^{b}	0.004
Tyrosine	0.007 ± 0.003^{a}	0.006 ± 0.002^{a}	0.004 ± 0.002^{b}	0.004 ± 0.001^{b}	0.0001
5) Sulfur-Containing Amino Acids					
Methionine	0.021 ± 0.003^{a}	0.018 ± 0.004^{a}	0.014 ± 0.003^{b}	0.013 ± 0.003^{b}	< 0.0001
6) Cyclic Amino Acids					
Proline	0.010 ± 0.003^{a}	0.010 ± 0.003^{a}	0.008 ± 0.003^{a}	0.006 ± 0.002^{b}	0.006
7) Acidic Amino Acids					
Glutamate	0.046 ± 0.008^{a}	0.038 ± 0.007^{b}	0.036 ± 0.007^{b}	0.035 ± 0.004^{b}	0.0001
Glutamine	0.011 ± 0.006^{a}	0.011 ± 0.002^{a}	0.010 ± 0.003^{a}	0.006 ± 0.003^{b}	0.0007
Amino Acid Derivatives (n = 4)					
Betaine	0.121 ± 0.017	0.118 ± 0.009	0.113 ± 0.010	0.114 ± 0.009	0.200
Cystathionine	0.049 ± 0.008^{a}	0.041 ± 0.007^{b}	0.038 ± 0.005^{b}	0.037 ± 0.003^{b}	< 0.0001
Pyroglutamate	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.999
Taurine	2.351 ± 0.185^{b}	2.415 ± 0.160^{b}	2.582 ± 0.175^{a}	2.730 ± 0.201^{a}	< 0.0001
Dipeptides & Peptides (n = 3)					
Anserine-like3	0.435 ± 0.046^{a}	0.422 ± 0.043^{a}	0.390 ± 0.030^{b}	0.361 ± 0.024^{b}	< 0.0001
Carnosine	0.016 ± 0.005^{a}	0.014 ± 0.003^{a}	0.009 ± 0.002^{b}	0.005 ± 0.003^{c}	< 0.0001
Glutathione	0.001 ± 0.001^{a}	0.001 ± 0.001^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}	< 0.0001
Nucleotides & Nucleosides (n = 3)					
ATP	0.028 ± 0.005^{b}	0.032 ± 0.005^{a}	0.033 ± 0.004^{a}	0.035 ± 0.008^{a}	0.005
IMP	0.272 ± 0.031^{a}	0.264 ± 0.016^{a}	0.238 ± 0.029^{b}	0.221 ± 0.016^{b}	< 0.0001
Inosine	0.032 ± 0.005^{a}	0.024 ± 0.007^{b}	0.023 ± 0.005^{b}	0.019 ± 0.006^{c}	< 0.0001
Organic Acids & TCA Cycle Intermediates (n = 9)					
1) Hydroxy Acids					
2-Hydroxyvalerate	0.010 ± 0.004^{a}	0.010 ± 0.002^{a}	0.008 ± 0.002^{a}	0.006 ± 0.002^{b}	0.002
2) Short-Chain Organic Acids					
Acetate	0.019 ± 0.005^{a}	0.021 ± 0.004^{a}	0.019 ± 0.004^{a}	0.016 ± 0.003^{b}	0.020
Formate	0.384 ± 0.030^{a}	0.378 ± 0.051^{a}	0.388 ± 0.028^{a}	0.410 ± 0.030^{a}	0.087

Metabolite	**MB1 (99:1)**	**MB5 (95:5)**	**MB10 (90:10)**	**MB20 (80:20)**	***p* Value**
3) TCA Cycle Intermediates					
Citrate	0.004 ± 0.002^{a}	0.003 ± 0.002^{a}	0.003 ± 0.001^{a}	0.002 ± 0.001^{b}	0.037
Fumarate	0.005 ± 0.002^{a}	0.006 ± 0.002^{a}	0.004 ± 0.001^{b}	0.004 ± 0.001^{b}	0.004
Malate	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	0.999
Succinate	0.055 ± 0.011^{a}	0.059 ± 0.012^{a}	0.055 ± 0.010^{a}	0.048 ± 0.007^{b}	0.055
4) Glycolysis Byproduct					
Lactate	6.432 ± 0.602^{a}	6.018 ± 0.506^{a}	5.833 ± 0.327^{b}	5.608 ± 0.402^{b}	0.0001
Malonate	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}	0.999
Creatine Metabolism (n = 2)					
Creatine	2.584 ± 0.212^{a}	2.373 ± 0.195^{b}	2.224 ± 0.151^{b}	2.062 ± 0.160^{c}	< 0.0001
Creatinine	0.108 ± 0.008^{a}	0.107 ± 0.012^{a}	0.105 ± 0.010^{a}	0.090 ± 0.013^{b}	< 0.0001
Carbohydrates & Polyols (n = 4)					
Glucose	0.234 ± 0.034^{a}	0.208 ± 0.033^{a}	0.213 ± 0.035^{a}	0.205 ± 0.027^{a}	0.072
Sucrose	0.041 ± 0.010^{a}	0.034 ± 0.010^{a}	0.035 ± 0.008^{a}	0.030 ± 0.007^{b}	0.021
Glycerol	0.241 ± 0.021^{a}	0.219 ± 0.027^{a}	0.209 ± 0.023^{b}	0.224 ± 0.021^{a}	0.003
myo-Inositol	0.089 ± 0.010^{a}	0.094 ± 0.015^{a}	0.091 ± 0.015^{a}	0.079 ± 0.009^{b}	0.011
Lipid Metabolism & Carnitines (n = 2)					
Carnitine	0.414 ± 0.031^{a}	0.405 ± 0.046^{a}	0.390 ± 0.046^{a}	0.332 ± 0.038^{b}	< 0.0001
O-Acetylcarnitine	0.023 ± 0.007	0.024 ± 0.005	0.023 ± 0.005	0.022 ± 0.004	0.689
Alcohol & Volatile Compounds (n = 3)					
Ethanol	0.033 ± 0.006^{a}	0.029 ± 0.005^{a}	0.028 ± 0.006^{a}	0.025 ± 0.007^{b}	0.008
Isopropanol	0.061 ± 0.010^{a}	0.056 ± 0.013^{a}	0.054 ± 0.009^{a}	0.052 ± 0.009^{a}	0.090
Methanol	0.207 ± 0.024^{a}	0.204 ± 0.020^{a}	0.201 ± 0.021^{a}	0.198 ± 0.012^{a}	0.631
Vitamins & Cofactors (n = 1)					
Niacinamide	0.006 ± 0.002^{a}	0.004 ± 0.001^{b}	0.004 ± 0.001^{b}	0.003 ± 0.001^{c}	< 0.0001
Unclassified (n = 1)					
Unknown1	0.203 ± 0.042^{a}	0.194 ± 0.022^{a}	0.194 ± 0.024^{a}	0.175 ± 0.028^{a}	0.088

Values shown as Mean ± SD.

$^{a–c}$ Means within the same row bearing different superscript letters differ significantly ($p < 0.05$).

Table 4. Candidate biomarkers for discrimination of beef–buffalo meatball adulteration groups

Comparison	**Metabolite**	**VIP**	***P* Value**	**FDR**	**FC**	**$\log_2$FC**	**AUC**	**95% CI**
MB1 *vs.* MB10	*ATP*	1.199	< 0.001	< 0.001	0.544	−0.877	1.000	1.000–1.000
	Inosine	1.131	< 0.001	< 0.001	0.520	−0.944	1.000	1.000–1.000
MB1 *vs.* MB20	*Unknown 1*	2.384	0.001	0.002	2.123	+1.086	0.871	0.699–1.000
	myo-Inositol	1.656	< 0.001	< 0.001	0.593	−0.754	0.933	0.835–1.00

								0
	ATP	1.180	<0.001	<0.001	0.513	−0.962	0.969	0.905–1.000
	Inosine	1.177	<0.001	<0.001	0.475	−1.073	0.973	0.918–1.000
MB10 *vs.* MB20	*Unknown 1*	3.141	<0.001	0.005	1.693	+0.760	0.871	0.699–1.000

FC = mean concentration (group 1) / mean concentration (group 2) from unscaled data. AUC = area under the receiver operating characteristic curve; 95% CI computed by DeLong’s method. All models fitted as OPLS-DA (Pareto scaling, 1 predictive component); permutation *p* values based on 1,000 cycles.

Figures

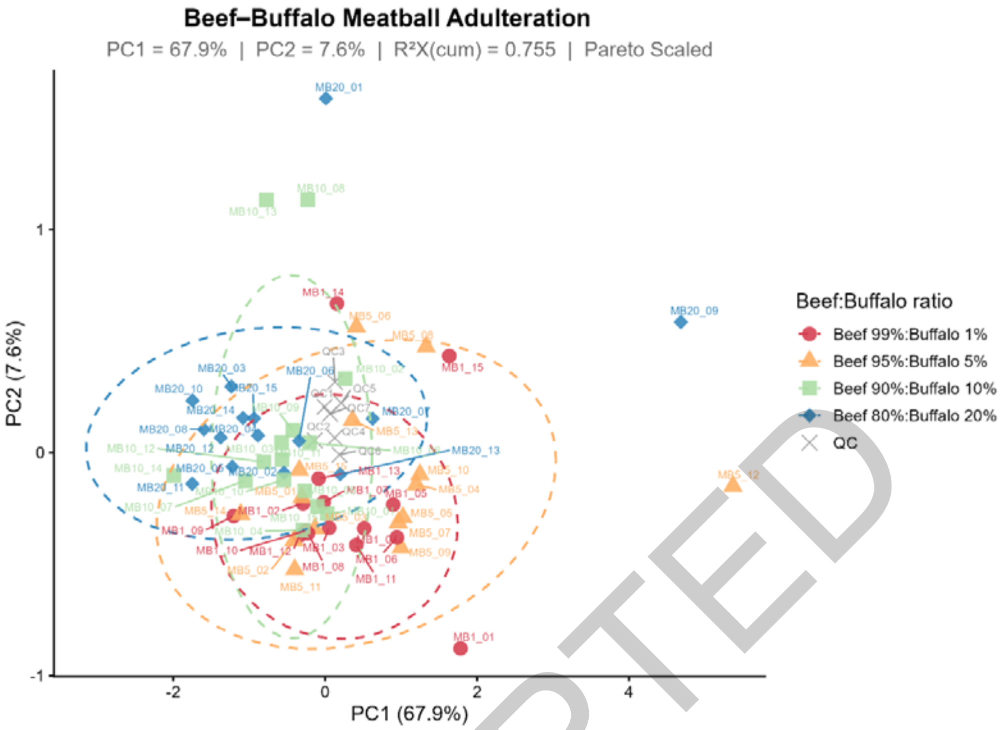

Figure 1. PCA score plot of beef meatballs with varying proportions of buffalo meat

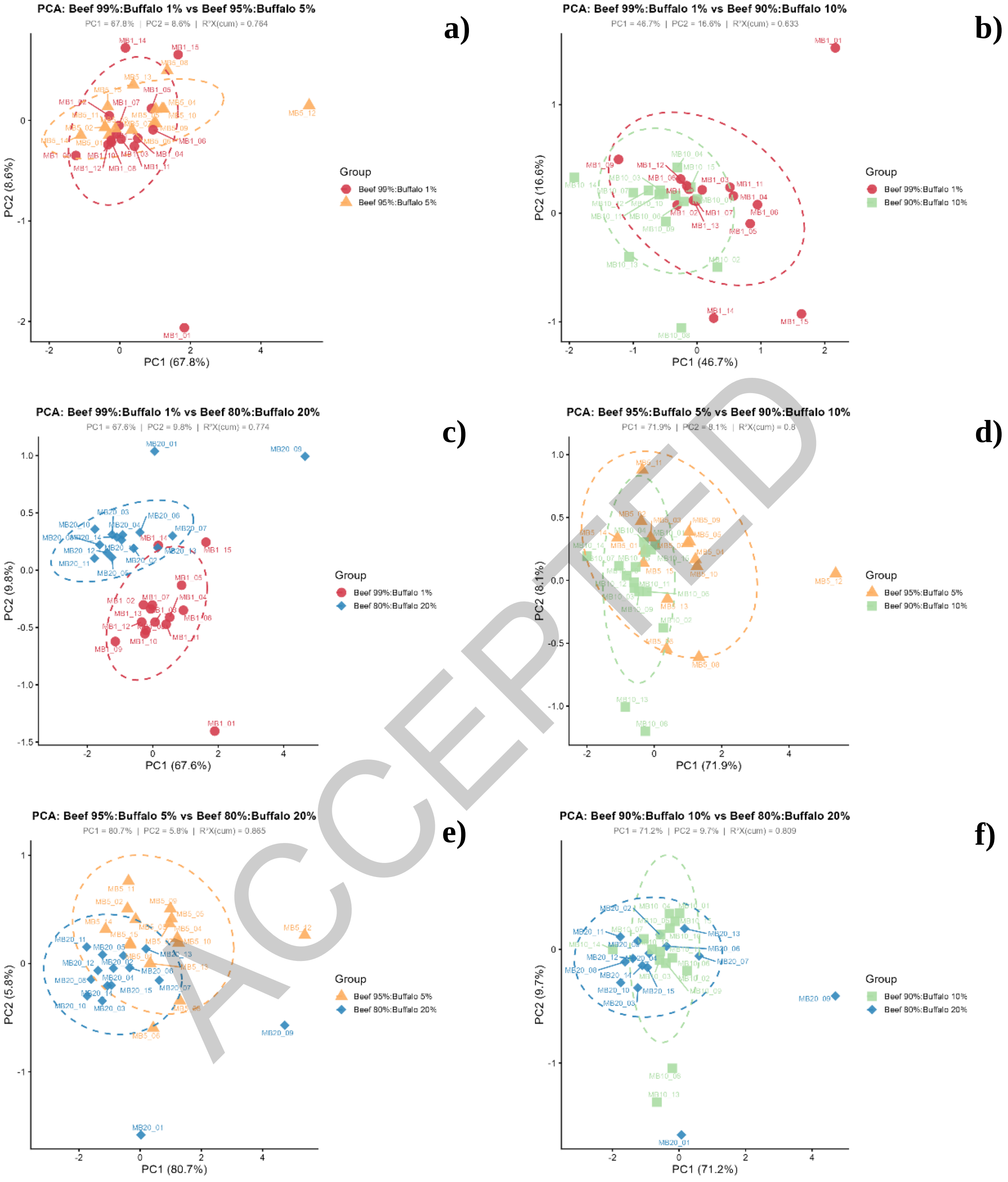

Figure 2. Pairs of PCA score plots (a–f) for beef meatballs with varying proportions of buffalo meat

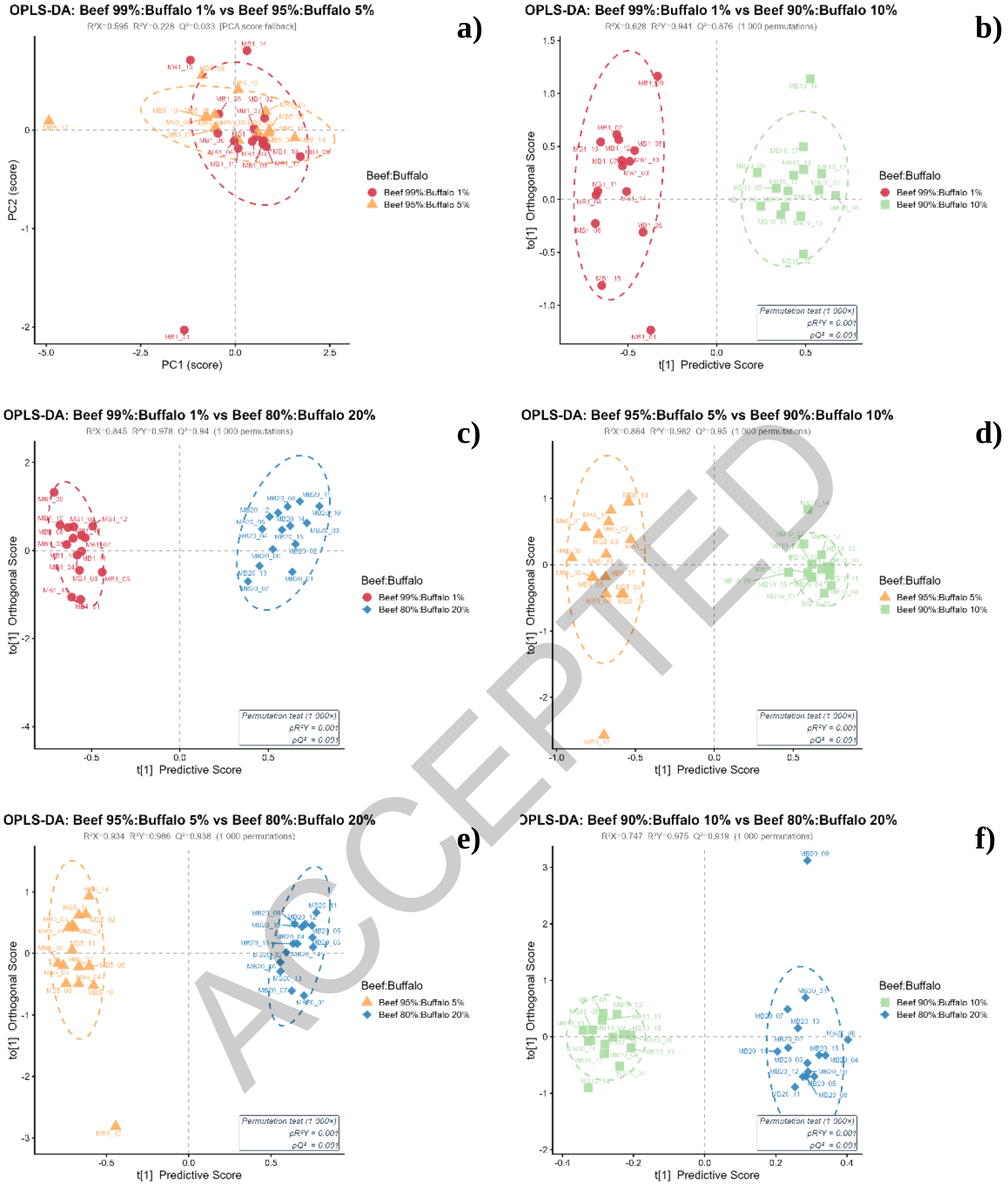

Figure 3. Pairs of OPLS-DA score plots (a–f) for beef meatballs with varying proportions of buffalo meat

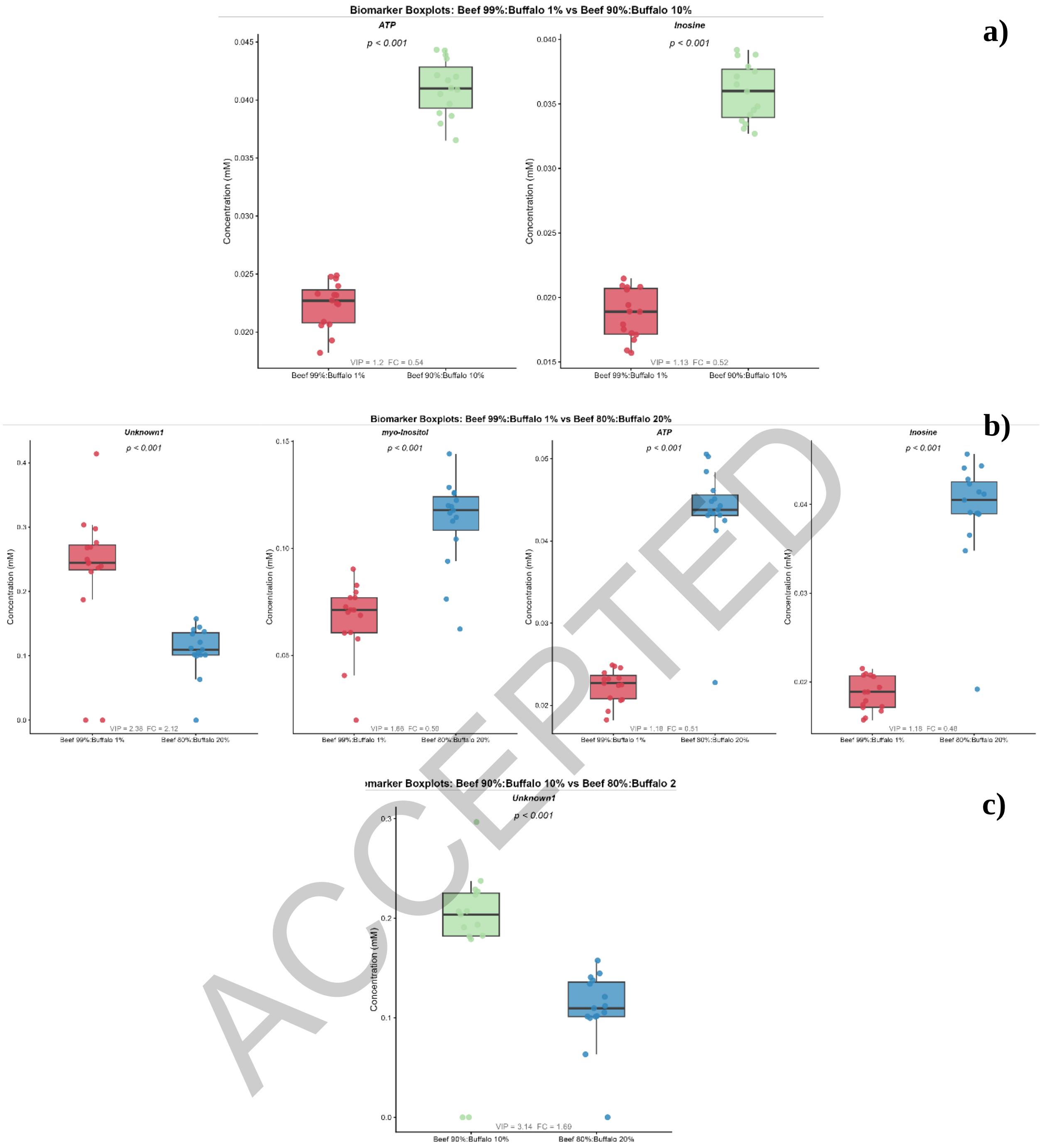

Figure 4. Biomarkers boxplots: (a) MB1 *vs*. MB10, (b) MB1 *vs*. MB20, and (c) MB10 *vs*. MB20