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Novel Methodologies for Food Quality and Provenance Fingerprints Assessment

Lead Guest Editor: Xavier Cetó
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Editorial Board

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Editorial

Novel Methodologies for Food Quality and Provenance Fingerprints Assessment

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The development of novel reliable methodologies that allow the control, assessment, and prediction of the characteristics of food products is a field under expansion nowadays, especially those that allow their characterization, classification, and authentication.

On the one side, the highly competitive global environment in food industry requires continuous innovation and a better sustainable usage of our natural resources in order to improve the high standards of food producers, leading to high value-added products. The linkage of new research ideas with food production provides a competitive advantage to food makers to fulfil the competitive market challenges.

On the other side, industry is increasingly interested on fast-response and low-cost methods to be used for the detection of adulterations or contamination of the products (either during or after its production) or to assess whether they guarantee quality control standards.

Food authentication is a multidisciplinary field that has input from instrumentation, biology, informatics, mathematics and statistics, agriculture, economics, and food technology. Current approaches for quality control are shifting from compound-oriented to pattern-oriented strategies, the former focusing on the identification of few specific compounds, while the latter targeting at the extraction of a global fingerprint of all the compounds present in the sample. Thus, fingerprinting techniques require the use of an analytical method that can simultaneously detect a large spectrum of compounds and provide comprehensive information of the sample, in combination with chemometric tools that allow the interpretation and extraction of meaningful data from the complex readings.

In this regards, this special issue has risen with the idea to cover advances in fast, reliable, and affordable characterization, classification and authentication of food products, and methodologies devoted to improve their quality.

When assessing food quality, perception of such foods by consumers is as critical as can be the identification of bioactive compounds or the detection of contaminations and/or adulterations. Characterization of food by analytical means can help on the comprehension of the chemical process that affects foods' physical characteristics and, consequently, consumers' quality perception. In this line, L. Yang evaluated the synergistic effect of trisodium citrate and microbial transglutaminase treatment on the textural properties of acidified yak skim milk gels. Results of the study allowed identification of the conditions that led to gels with higher stiffness, water holding capacity, and storage modulus.

In another subject, proteomic analysis has also gained an increasing role in the authentication of food products over the last years. Usage of proteins as markers complements traditional methodologies in the verification of the claims made about certain food products. To this aim, sample preparation and protein extraction is a key step in proteomic analysis so as to maximize protein recovery and minimize proteolysis and modification. In this regards, the contribution of A. della Malva et al. provide a comparison between

two methods for the extraction and separation of myofibrillar proteins including solutions with different ionic strength in meat and fish muscles. The authors show how both extraction methods provided good solubilisation of myofibrillar proteins and derived fragments, with the denaturing solution leading to a more complex profile in terms of number of bands and fragments extracted (with lower molecular weight), while the use of the nondenaturing solution revealed a major intensity for most of the myofibrillar protein (with higher molecular weight) analysed.

In another work, M. Negera and A. P. Washe evaluate the potential of certain natural dietary spices, which are commonly used in food flavouring and preservation, to detoxify or degrade chemical carcinogens such as aflatoxin B1 (AFB1). The usage of natural plant extracts represents an interesting alternative to biological agents for aflatoxin detoxification as those would not alter significantly the nutritional properties of the product, plus its usage fits with a sustainable production of traditional food products. The reported findings revealed nonobvious benefits of the use of natural dietary spices as effective solution for AFB1 degradation and decontamination of food, providing scientific credit to the nonobvious benefits of indigenous knowledge of using natural spices for food flavouring and preservation to control aflatoxin.

Lastly, authentication of food products and detection of adulterations is particularly critical, given the difficulties to relate those with the concentration of specific compounds that can be assessed using classical analytical techniques. The application of pattern recognition or multivariate calibration methods allows one to infer underlying relationships between the measured analytical signals and the properties of the samples, approaches that are fundamental in food authentication.

In this regards, L. Du et al. propose the use of Fourier-transform infrared spectroscopy (FT-IR), in combination with chemometric methods such as principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), for the authentication of raw from reconstituted milk. To this purpose, the authors adulterated different raw milk samples with commercial milk powders at different levels, succeeding in the correct discrimination of raw and reconstituted milk samples.

In the same line, Tian et al. propose the combined use of an electronic tongue and an electronic nose for the detection of the adulteration of minced mutton with pork meat. To this aim, the authors made use of two commercial systems to measure the samples, and combined data were analysed by means of canonical discriminant analysis (CDA) and artificial neural networks (ANNs). The results showed that the authors were not only able to correctly discriminate both type of meats but also able to numerically predict the degree of adulteration (pork content).

Conflicts of Interest

The guest editors declare that they have no conflicts of interest regarding the publication of this special issue.

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Research Article

Combination of an E-Nose and an E-Tongue for Adulteration Detection of Minced Mutton Mixed with Pork

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An E-panel, comprising an electronic nose (E-nose) and an electronic tongue (E-tongue), was used to distinguish the organoleptic characteristics of minced mutton adulterated with different proportions of pork. Meanwhile, the normalization, stepwise linear discriminant analysis (step-LDA), and principle component analysis were employed to merge the data matrix of E-nose and E-tongue. The discrimination results were evaluated and compared by canonical discriminant analysis (CDA) and Bayesian discriminant analysis (BAD). It was shown that the capability of discrimination of the combined system (classification error 0%~1.67%) was superior or equable to that obtained with the two instruments separately, and E-tongue system (classification error for E-tongue 0~2.5%) obtained higher accuracy than E-nose (classification error 0.83%~10.83% for E-nose). For the combined system, the combination of extracted data of 6 PCs of E-nose and 5 PCs of E-tongue was proved to be the most effective method. In order to predict the pork proportion in adulterated mutton, multiple linear regression (MLR), partial least square analysis (PLS), and backpropagation neural network (BPNN) regression models were used, and the results were compared, aiming at building effective predictive models. Good correlations were found between the signals obtained from E-tongue, E-nose, and fusion data of E-nose and E-tongue and proportions of pork in minced mutton with correlation coefficients higher than 0.90 in the calibration and validation data sets. And BPNN was proved to be the most effective method for the prediction of pork proportions with R^2 higher than 0.97 both for the calibration and validation data set. These results indicated that integration of E-nose and E-tongue could be a useful tool for the detection of mutton adulteration.

1. Introduction

There is a consistent growth in the demand for meat with high nutritional value, enriched with several vitamins, minerals, and essential polyunsaturated fatty acids and low in fat and cholesterol [1]. However, it is noted that processed mutton products have been adulterated with other types of cheaper meat, including vegetarian meat, to increase its profit [2, 3]. The worse is that pork back fat has been added to these processed mutton products in some cases [1]. In addition, the horse-meat scandal in 2013 and the frequently reported mutton adulteration have seized the attention of consumers all over the world. Reliable methods to detect mutton adulteration based on E-tongue were established [4], and it is necessary to establish fast and reliable methods to verify meat adulteration in different viewpoints.

For meat species and meat adulteration detection, many studies were reported. The methods used included molecular biology-based methods [5, 6], enzymatic immunological methods [7], chromatographic methods [8, 9], spectroscopy methods [10–12], and electronic sensory evaluation [9, 13].

With the advantages of small amount of the sample required, speed, simplicity, high sensitivity, and good correlation with data from sensory analyses, the use of the electronic sensory evaluation of E-nose and E-tongue to evaluate the quality of meat has become more popular. E-tongue was shown to be able to discriminate different

species of chicken [14, 15] and fishes [16]. E-nose was shown to be useful in meat products differentiation and authenticity assessment [9] and the identification and differentiation of pork for halal authentication [17] and pork adulteration in mutton [13].

However, the use of E-nose or E-tongue could only reflect one aspect for the sample, which may cause inaccuracy of the classification. By fusion of E-nose or E-tongue responses, the discrimination of meat adulteration could be improved by giving the overall sensory evaluation of meat, closer to human judgment. The combination of E-nose and E-tongue had been reported in the researches in geographical origin identification of potato creams [18] and virgin olive oil [19–21]; varieties and grade level discrimination of Chinese green tea [22], black tea [23], red wine [24], and fruit juice [25, 26]; cultivars discrimination and characterisation of *Perilla frutescens* [27] and species differentiation of coffee [28]; freshness evaluation of wine [29] and milk [30]; quality differentiation [31]; and authenticity assessment [32]. These studies provided references for the in-depth study of the food quality inspection of E-nose and E-tongue.

For the fusion of E-nose or E-tongue responses, three ways [33] of abstraction were reported. For the original sensor fusion method, known as the low-level fusion, the data from E-nose and E-tongue were simply concatenated as the input data set for model construction, with the number of rows equal to the number of samples and the number of columns equal to the total number of signals from E-nose and E-tongue. Sometimes, the combined data set faced with normalization or extraction of the eigenvalue. However, for fusion of extracted features, named midlevel fusion, feature extraction methods were applied to each data source before the extracted features are combined. The commonly used selection feature methods are analysis of variance, principle component analysis, stepwise discriminant analysis, Bayes discriminant analysis, and so on. Fusion of extracted features is more popular, as the redundant information and multiple collinearity problem of data set were eliminated, and the data dimensions were reduced. For the high-level fusion, separate models were built for each data set of E-nose and E-tongue, respectively, and then the models were combined to give fused responses. It is the fusion of results.

Of the three levels of data fusion, low and midlevel fusion are the most used. Low-level fusion is the first attempt approach, but when data are very different in size or scale, the more tuneable midlevel fusion can yield better results [34]. High-level fusion, instead, is the least used approach, as the best classification or prediction model had to be determined to ensure that the fused results are better than individual models.

In this study, low-level fusion and midlevel fusion methods were studied for the fusion of E-nose or E-tongue data in the discrimination of mutton authentication. The aim of this study was to evaluate the effectiveness of data fusion methods for E-nose and E-tongue in the authentication of mutton according to its proportion of pork and to correlate the fusion data set with pork proportion in minced mutton.

2. Materials and Methods

2.1. Meat Samples. All the hind leg mutton samples detected by E-nose and E-tongue and used for determination of physical properties were obtained from the local logistics center for agricultural products, Hangzhou, China. The hind leg pork samples were purchased from Wal-Mart Stores, Hangzhou, China. The meat samples were brought to laboratory and stored at −18°C.

2.2. Sample Preparation. The fat and connective tissue were removed before samples were processed. The mutton and pork samples were cut into 1 cm^3 cubic and minced for 2 min by a mincer, respectively. The adulterated mutton samples were made by mixing minced pork at levels of 0%, 20%, 40%, 60%, 80%, and 100% by weight with minced mutton and followed with mincing for 1 min. After each sample was minced, the mincer was cleaned with detergent water and rinsing with distilled water, to prevent from cross interference of odour and flavor information. The adulterated meat samples were brought to room temperature before being detected by E-nose and E-tongue.

For detection of E-nose, the optimized detection parameters were as follows: 10 g of the minced meat was placed in a beaker of 250 mL at the temperature of 25°C ± 3°C, and the beaker was sealed by plastic for a headspace generation time of 30 min. The headspace gas was detected by E-nose.

For detection by E-tongue, the optimized detection parameters were as follows: the taste substances of minced meat samples were extracted using potassium chloride solution (0.1 mol/L) for 30 minutes at 4°C with a shaking (SKY-2112B, SUKUN, China) rate of 1500 rpm. The mixture was passed through the filter paper, and the supernatant was measured by E-tongue.

2.3. Detection Procedures of Electronic Nose (E-Nose) and Electronic Tongue (E-Tongue). To collect the odour fingerprint of the adulterated mutton, an E-nose of PEN 2 (Airsense Corporation, Germany) was used. The E-nose system consisted of three parts: the first is the sampling apparatus, the second is the detector unit containing of a sensor array of 10 different metal oxide sensors, and the third is pattern recognition software of Win Muster v.1.6. The nomenclature and characteristics of the 10 metal oxide sensors are listed in Table 1. It shows that each sensor has a certain degree of affinity towards specific chemical or volatile compounds.

For detection of adulterated mutton by E-nose, the experimental condition described in our former research [13] was used. The headspace generated by 10 g of samples in a beaker of 250 mL was detected for 80 s, at the flow rate of 200 $mL \cdot min^{-1}$.

The α-Astree taste system was employed to detect the gustatory information of the adulterated mutton samples. The E-tongue system comprised three parts of the sensor array, the reference electrode of Ag/AgC, and the autosampler with a mechanical stirrer. In the sensor array, the sensors were made from silicon transistors with an organic

Table 1: Sensors used and their main applications in PEN 2 electronic nose.

Number in array	Sensor name	General description	Reference
S1	W1C-aromatic	Aromatic compounds	Toluene, 10 ppm
S2	W5S-broad range	Very sensitive, broad range sensitivity, react on nitrogen oxides, very sensitive with negative signal	NO_2, 1 ppm
S3	W3C-aromatic	Ammonia, used as a sensor for aromatic compounds	Benzene, 1 ppm
S4	W6S-hydrogen	Mainly hydrogen, selectively (breath gases)	H_2, 100 ppb
S5	W5C-arom-aliph	Alkanes, aromatic compounds, less polar compounds	Propane, 1 ppm
S6	W1S-broad-methane	Sensitive to methane (environment) ca, 10 ppm, broad range, similar to no. 8	CH_4, 100 ppm
S7	W1W-sulfur-organic	Reacts on sulfur compounds, H_2S 0.1 ppm. Otherwise sensitive to many terpenes and sulfur organic compounds which are important for smell, limonene, pyrazine	H_2S, 1 ppm
S8	W2S-broad-alcohol	Detects alcohols, partially aromatic compounds, broad range	CO, 100 ppm
S9	W2W-sulph-chlor	Aromatic compounds, sulfur organic compounds	H_2S, 1 ppm
S10	W3S methane-aliph	Reacts on high concentration >100 ppm, sometimes very selective (methane)	CH_4, 100 ppm

Table 2: Sensors used in α-Astree E-tongue and their thresholds ($mol \cdot L^{-1}$).

Basic taste	Taste substance	ZZ	BA	BB	CA	GA	HA	JB
Sour	Citric acid	10^{-7}	10^{-6}	10^{-7}	10^{-7}	10^{-7}	10^{-6}	10^{-6}
Salty	KCL	10^{-7}	10^{-4}	10^{-4}	10^{-5}	10^{-4}	10^{-4}	10^{-4}
Sweet	Glucose	10^{-7}	10^{-4}	10^{-7}	10^{-7}	10^{-4}	10^{-4}	10^{-4}
Bitter	Caffeine	10^{-5}	10^{-4}	10^{-4}	10^{-5}	10^{-4}	10^{-4}	10^{-4}
Savoury	L-Arginine	10^{-6}	10^{-4}	10^{-6}	10^{-5}	10^{-5}	10^{-4}	10^{-5}

Table 3: Data fusion methods for E-nose and E-tongue.

Fusion level	Methods	No. of parameters
Low	Normalization after combination	17
	Feature extracted by step-LDA	16
	Extraction of features by principle components	9
Midlevel	Combination of extracted principle components	11

coating that governs sensitivity and selectivity of each individual sensor [35, 36]. The thresholds [35] of 7 sensors to 5 basic tastes [36] are shown in Table 2.

The E-tongue system was conditioned with the conditioning sequence with the solution of 0.01 mol/L HCl to determine if the measured values are stable and repeatable. The calibration sequence was followed to adjust the measured values in the measuring range of the instrument. In the end, the diagnostic sequence was conducted with solution of 0.01 mol/L HCl, NaCl, and MSG to determine whether the measured values conform to the discrimination performance characteristics predefined by Alpha MOS for each sensor type.

The detection procedure of E-tongue is as follows: for each sample, the measurement time was set to 120 s, and the sensors were rinsed for 10 s in potassium chloride solution (0.1 mol/L) before the detection of the next sample. All the adulterated samples were detected at room temperature with 20 duplications.

2.4. Data Fusion Method. Either in the view of odour or flavor, E-nose and E-tongue have showed high ability in discrimination of adulterated mutton. Aiming at the combination of odour or flavor and giving an overall sensory result, the data fusion methods of low-level and midlevel were employed for the fusion of E-nose and E-tongue data. The information of data fusion methods for E-nose and E-tongue is described in Table 3.

For the low-level fusion method, (1) the E-nose data (120 repetitions of meat samples × 10 sensors of E-nose) and E-tongue data (120 repetitions of meat samples × 7 sensors of E-tongue) were gathered together to form a new data matrix with 120 rows and 17 columns for each sample. For all the adulterated mutton samples, the combined data matrix had 120 rows and 17 columns. (2) The [0, 1] maximum-minimum normalized processing was conducted after data combination. Data selected by stepwise LDA were conducted by SAS version 8 (SAS Institute Inc., Gary, USA) on combined data of E-tongue and E-nose. The selection of the variable starts with the largest value of the F statistic (F value) and the probability of F lower than 0.15. The procedure was repeated with the unselected variables and ends when the probability of F is higher than 0.15. As a result, 16 variables were selected for further analysis. Sensor responses with small influence on the identification process were switched off in further analysis. (3) Data extracted by principle component analysis was applied on the directly combined data set of E-nose and E-tongue (120 × 17), 99.0% cumulative calibrated variance was acquired, and as a result, 9 PCs (99.20%) were extracted.

For midlevel level fusion methods, principle component analysis was applied to extracted features of E-nose and E-tongue signals before combining. For reducing the dimension of data set by linear combination of primary variables into the unrelated comprehensive index, principal component analysis is often used as the data extraction method. In order to retain most information of the original data, 99.0% cumulative calibrated variance was acquired. As a result, 6 PCs (99.52%) based on E-nose and 5 PCs (99.43%) based on E-tongue were used to form the combination matrix.

2.5. Data Analysis. To evaluate the discrimination effects of different fusion methods for E-nose and E-tongue,

discriminant analysis methods of canonical discriminant analysis (CDA) with linear discriminant function and quadratic discriminant function and Bayes discriminant analysis (BDA) were used. To build the predictive models for the proportions of pork in adulterated mutton with the best fusion data set, methods of multiple linear regression (MLR), partial least square analysis (PLS), and backpropagation neural network (BPNN) were used. Both the qualitative and quantitative methods were compared to find the better one. The SAS version 8 (SAS Institute Inc., Gary, USA) was used for data processing, and all the figures were plotted by OriginPro 8 (OriginLab, USA).

3. Results and Discussion

3.1. Sensor Array Response to Meat Odour and Taste. In order to study the changing trend of E-nose and E-tongue sensors to adulterated mutton samples, the sensor responses of E-nose and E-tongue to minced mutton adulterated with 40% of pork are given in Figures 1 and 2. For 10 sensors of E-nose, at the initial response phase of 0–40 s, the responses of S2, S8, and S6 increased with the collection time, and it is in accordance with the results of Zhan [37] that mutton was rich in volatile compounds and large content of alcohol, aldehydes, and ketones. The responses of S3, S1, and S5 dropped, for the content of aromatic compounds. However, the responses of S7, S9, S4, and S10 stayed stable during 0–50 s. The responses of S7 and S9 increased slightly after 50 s, while the responses of S4 and S10 stayed unchanged, as the content of hydrogen and methane-aliph was low. The responses of S2 kept rising during the whole collection time as it is very sensitive and with broad range sensitivity. The other 9 sensors' responses stabilized after collection time of 60 s. For further data analysis, 10 sensors responses at the 75^{th} s were used, forming a data set of 10×20 for 6 adulterated mutton samples.

The adulterated mutton sample was detected by E-tongue, and the typical responses of E-tongue taste sensors to minced mutton adulterated with 40% of pork are shown in Figure 2. Taste sensors need time to adapt to the soluble flavor substances in the sample extraction. It was observed that most sensors adapt to the taste within 10 s, while BB and BA need longer time of 20 s and 100 s. For data analysis, the stable response at the 120^{th} s of 7 sensors of E-tongue responses was extracted, forming a data set of 7×20 for 6 adulterated mutton samples.

3.2. Comparison of E-Nose and E-Tongue Results on Discrimination of Mutton Adulteration. For adulterated mutton samples, the odour and flavor information were obtained by E-nose and E-tongue, respectively. The stable values at the 75^{th} s of E-nose responses and the stable values of the 120^{th} s responses of E-tongue were extracted and used. For discrimination of mutton samples adulterated with different proportions of pork, three discriminant analysis methods were used and the results were compared, including canonical discriminant analysis (CDA) with linear discriminant function, canonical discriminant analysis with quadratic discriminant function, and Bayes discriminant analysis. The discrimination results of E-nose and E-tongue are shown in Table 4. For E-nose, the percentage of misclassified was in the range of 5.83%~10.85%, and the best discrimination results were obtained by Bayes discriminant

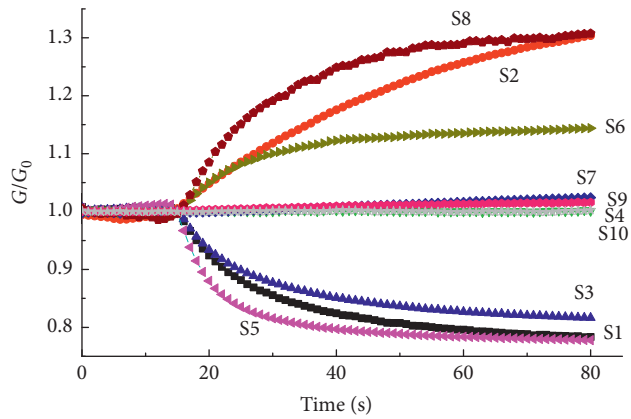

Figure 1: E-nose sensors' response curve to minced mutton adulterated with 40% of pork (S1: ■; S2: ●; S3: ▲; S4: ▼; S5: ◀; S6: ▶; S7: ◆; S8: ●; S9: ●; S10: ★).

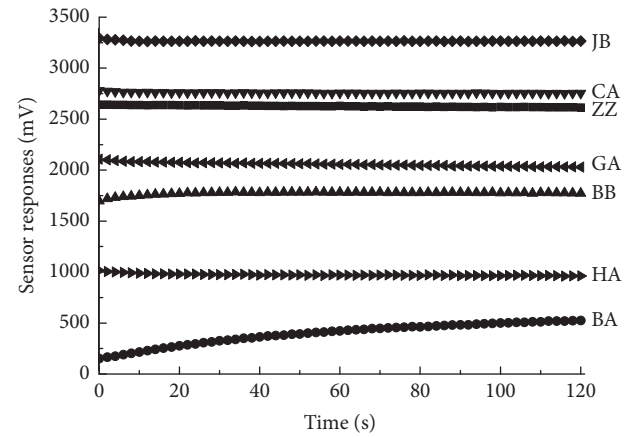

Figure 2: Typical response of E-tongue sensors to taste substance of minced mutton adulterated with 40% of pork (ZZ: ■; BA: ●; BB: ▲; CA: ▼; GA: ◀; HA: ▶; JB: ◆).

Table 4: Comparison results of discriminant analysis for responses of E-nose and E-tongue to minced mutton adulterated with pork.

Discrimination method	Evaluation indices	E-nose 75^{th} s	E-tongue 120^{th} s
Bayesian discriminant analysis	Number of misclassified	7	0
	Percentage of misclassified (%)	5.83	0
CDA-linear discriminant function	Number of misclassified	13	3
	Percentage of misclassified (%)	10.83	2.5
CDA-quadratic discriminant function	Number of misclassified	13	2
	Percentage of misclassified (%)	10.83	1.67
	Averaged percentage of misclassified (%)	**9.16**	**1.39**

analysis. For E-tongue, the percentage of misclassified was in the range of 0~2.5%, and the best discrimination results were obtained by Bayes discriminant analysis.

As a result, both E-nose and E-tongue could discriminate the adulteration of mutton with percentage of misclassified mutton samples adulterated with different proportions of pork lower than 11%, and E-tongue showed higher ability in the adulteration detection of mutton. However, both E-nose and E-tongue detection showed only one aspect of sensory evaluation. Aiming at combination of odour or flavor and giving an overall sensory result, the data fusion methods of low-level and midlevel were employed for the fusion of E-nose and E-tongue data.

3.3. Comparison of Fusion Methods for E-Nose and E-Tongue Data on Adulteration Classification of Mutton Samples. As shown in Section 3.2, both E-nose and E-tongue could discriminate minced mutton adulterated with different proportions of pork with high accuracy. However, there were still few samples misclassified. The combination of E-nose and E-tongue was conducted using low-level and midlevel fusion methods. Discrimination methods of canonical discriminant analysis (CDA) with linear discriminant function, canonical discriminant analysis with quadratic discriminant function, and Bayes discriminant analysis were used, and the results were compared to get better identification results. The results obtained for each of the tasks are summarized in Table 5.

For low-level fusion, the following tasks were performed:

(1) E-nose and E-tongue data were combined directly, containing both useful and redundant information. The combined data set was analyzed by BDA and CDA with linear discriminant function and quadratic discriminant function after the normalization process. The results showed that only one sample was misclassified by CDA, with 99.45% correctly classified.

(2) After the combination of E-nose and E-tongue data, step-LDA was employed to eliminate redundant information, and the responses of sensor W1S of E-nose were removed. Compared with the directly combined data set, the discrimination results were same with the directly combined data set.

(3) Thus, PCA analysis was employed to analyze the combined data set of E-nose and E-tongue, and the first 9 principle components, containing 99.20% cumulative variance, were selected for discriminant analysis. The elimination of S6 leads to worse discrimination results, with 2 samples misclassified, with the average 1.11% percentage of misclassified mutton samples.

For the midlevel fusion, principal component analysis was used to extract features, reducing the dimension of data set by linear combination of primary variables into the unrelated comprehensive index, which is often used as the data extraction method. In order to retain most information of the original data, 99.0% cumulative calibrated variance was acquired. Thus, for E-nose, the first 6 principle components, containing 99.52% cumulative variance, and the first 5 principle components of E-tongue, containing 99.43% cumulative variance, were selected to form a new data set comprising 11 components. The discrimination results were highly improved, with 100% correctly classified.

It can be seen that the data fusion processes showed good results as regards to the classification of minced mutton adulterated with different proportions of pork. The misclassified samples were lower than 1.67% in most cases. Compared with one detection method of E-nose or E-tongue, the discrimination results were improved greatly with the fusion data of E-nose and E-tongue, especially for the fusion data set containing 6 PCs of E-nose and 5 PCs of E-tongue. The comparison showed that the results were always improved through combining taste and smell information in our work.

3.4. Adulteration Classification of Mutton Samples. To visualize the discrimination results, the results of CDA with linear discriminant function using data set of E-nose and E-tongue and fusion of E-nose and E-tongue were shown in Figure 3. For the CAN1 and CAN2, they explained 93.41%, 93.09%, 90.24%, 89.99%, 88.68%, and 89.52% of the total variance for different data sets with the value of 100%, respectively. So, it can be inferred that the first two CANs can give most information of the data set. After applying the CDA method, the samples could be grouped into 6 clusters according to their pork proportions although there were few samples misclassified into other groups. Two discriminant functions showed good separation in the direction of CAN1 among 5 groups of minced meat containing mutton, and CAN1 increased with decreasing pork proportion. Furthermore, the pork samples scattered in the left side of the figure (Figure 3), which was far from the other groups. With different proportions of mutton, all minced mixed meat had the smell of mutton with diverse intensity, which could be used as an index in discrimination of adulteration.

For discrimination, samples in groups of 40%, 60%, and 80% overlapped with each other when analyzed by E-nose (Figure 3(b)), and samples in groups of 40% and 20% overlapped with each other when analyzed by E-tongue with CDA (Figure 3(a)). When data fusion methods were used, the discrimination results of CDA were improved in the scatter plot of Figure 3 and Table 5, except that one or two samples were close to the adjacent groups. The best method was found to be the combination of principle components of each data set for fusion of E-nose and E-tongue data (6 PCs of E-nose and 5 PCs of E-tongue), with no sample misclassified by CDA.

For BDA, the discrimination results were same with the results obtained with E-tongue data only, better than with E-nose data. The discrimination results were not always improved using fusion data, which were similar with the results of Cosio at 2007 [38].

3.5. Rapid Characterisation of Pork Proportions in Minced Mutton. In order to establish the relationship between E-panel and pork proportions in minced mutton and to

Table 5: Comparison results of discriminant analysis for different data fusion methods for responses of E-nose and E-tongue to minced mutton adulterated with pork.

Fusion methods	Original sensor fusion method				Combination of extracted data
	Normalization		Feature extraction		
			Stepwise LDA	Extraction of principle components	Extraction of principle components
Bayesian discriminant analysis	Number of misclassified	0	0	0	0
	Percentage of misclassified	0	0	0	0
CDA-linear discriminant function	Number of misclassified	1 (20% to 40%)	1 (20% to 40%)	2 (40% to 20%)	0
	Percentage of misclassified	0.83%	0.83%	1.67%	0
CDA-quadratic discriminant function	Number of misclassified	1 (40% to 20%)	1 (40% to 20%)	2 (1 was 40% to 20%; 1 was 20% to 40%)	0
	Percentage of misclassified	0.83%	0.83%	1.67%	0
Averaged		0.55%	0.55%	1.11%	0

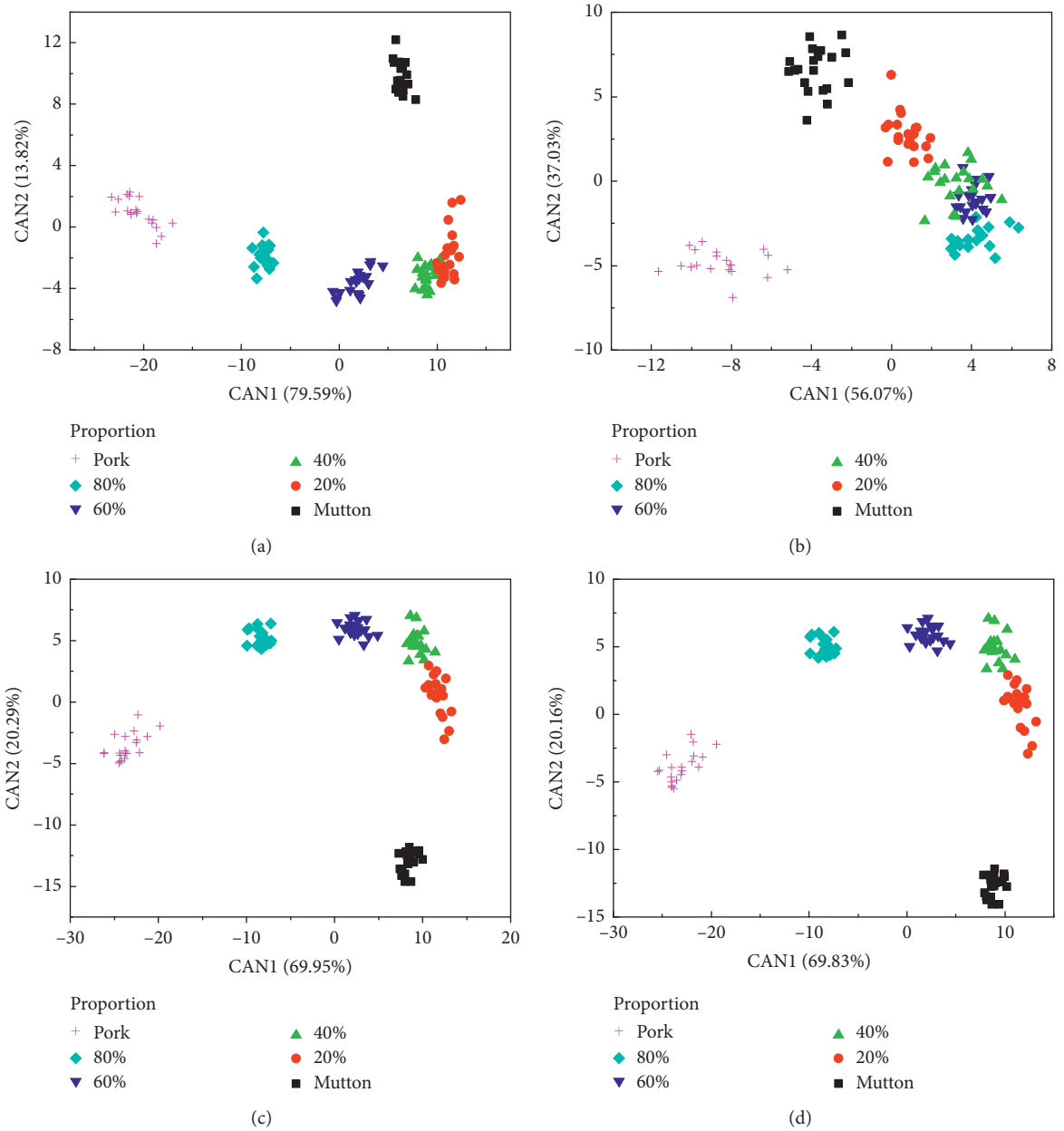

Figure 3: Continued.

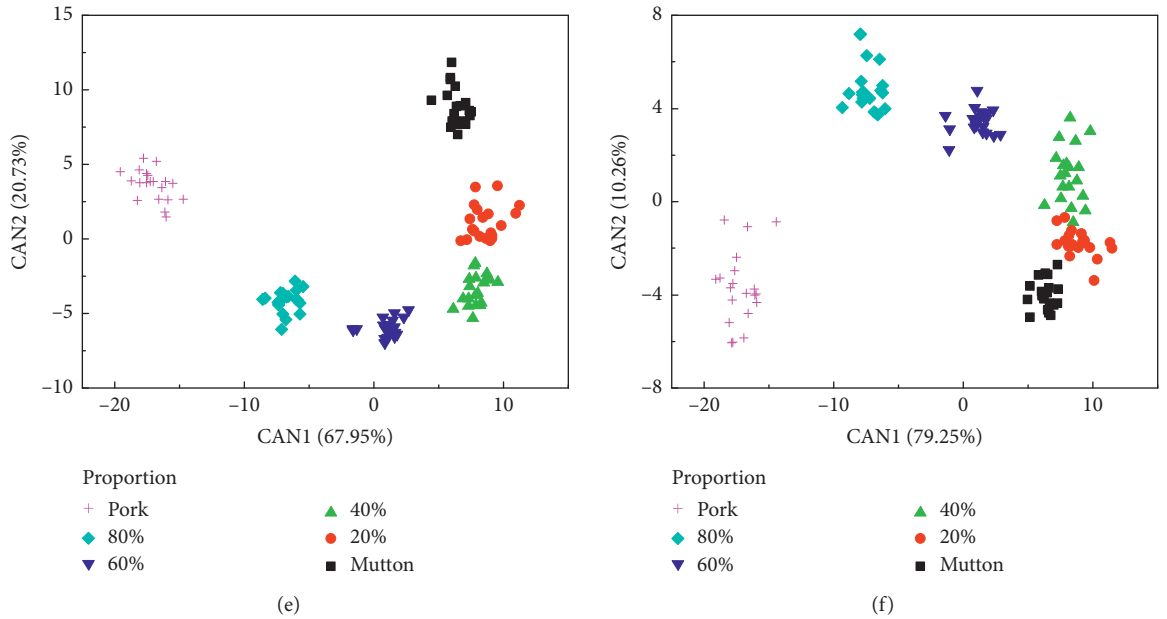

Figure 3: Scatter plot of CDA with linear discriminant function: (a) E-tongue; (b) E-nose; (c) original sensor fusion of E-tongue and E-nose; (d) features extracted by stepwise LDA on original sensor fusion data; (e) combination of features of E-nose and E-tongue extracted by PCA; (f) features extracted by PCA on original sensor fusion data.

classify meat samples according to their proportions of pork, analytical methods of partial least square (PLS), multiple linear regressions (MLR), and backpropagation neural network (BPNN) were used, and the results were compared to the best prediction model.

Here, the fusion data set, which contains 6 PCs of E-nose and 5 PCs of E-tongue, was used as input data to build the predictive models. The samples used for the training data set and testing data set were randomly selected in the experiment. 96 meat samples were used to build the predictive model for the proportions of pork adulterated into minced mutton, and 24 meat samples were used to validate the model. The performance of the model was evaluated by correlation coefficient (R^2) and root mean square error (RMSE) between experimental values and predicted values.

For BPNN, the experimental design was completely randomized with each sample as an experimental unit. The architecture of the artificial neural network chosen was $N \times (2N + 1) \times M$ three-layered backpropagation, where N is the number of inputs and M is the number of outputs. The three layers are as follows: the input layer was designed as 11 neurons according to the sensor array fused by combination of 6 PCs of E-nose and 5 PCs of E-tongue; 1 hidden layer; and the output layer had 6 neurons for different proportions of pork in minced mutton.

The results obtained by the training model of BPNN showed that all the training and testing samples were correctly classified according to their proportions of pork. And the correlations between the observed and predicted proportions of pork in minced mutton were higher than 0.99 (Table 6 and Figure 4) both for the training and testing sets. The low errors of prediction and the high correlation of the BPNN model suggested that the fusion of E-nose and E-tongue can be successfully applied in the determination of the adulteration detection of pork in minced mutton.

The multivariate projection method of PLS, with the leave-one-out technique, was applied to model the relationship between dependent variables and independent variables. The accuracy was estimated using the parameters (R^2 and RMSE) obtained from the fitted equation. As shown in Table 6 and Figure 4, good correlations of calibration were found between E-nose data and content of pork with a determination of coefficient $R^2 = 0.9791$. When the model was applied to predict the other 24 samples, similar results could be found, and good prediction results for content of pork with the coefficient R^2 of 0.9758 was obtained. The PLS-E-tongue methods have been proved to be suitable for prediction of chemical parameters [39, 40]. So, pork proportion in minced mutton could be determined simultaneously by PLS using the E-tongue data in this work.

The relationship between fusion signals of E-nose and E-tongue and the pork content in adulterated mutton was studied by the MLR algorithm, and the results are shown in Table 6 and Figure 4. A linear correlation between fusion signals and pork content were found, with $R^2 = 0.9746$ and RMSE = 5.04% for the training set. And when it was used in the testing data set, high predictive ability was found with $R^2 = 0.9755$ and RMSE = 5.52%.

In conclusion, excellent prediction ability was found using PLS, MLR, and BPNN for the prediction of pork proportion in minced mutton with R^2 higher than 0.9609 and RMSE lower than 10.94%. Compared with predictive results obtained by E-nose, BPNN, MLR, and PLS improved the predictive results of R^2 or RMSE value, indicating that the combination of more information could lead to better results. However, compared with E-tongue, the predictive

Table 6: Prediction of pork proportion in minced mutton by signals of E-nose, E-tongue, and the combined data set of E-nose and E-tongue.

Data used	Methods	Calibration		Validation	
		R^2	RMSEC (%)	R^2	RMSEP (%)
E-nose (variables selected by stepwise LDA)	PLS	0.9609	6.72	0.9092	10.94
	MLR	0.9609	6.72	0.91	10.94
	BPNN	0.9886	3.78	0.9762	5.26
E-tongue	PLS	0.987	3.90	0.9823	7.96
	MLR	0.9869	3.90	0.9827	7.52
	BPNN	0.9950	2.44	0.9956	2.23
Fusion of E-nose and E-tongue	PLS	0.9791	4.90	0.9758	5.37
	MLR	0.9746	5.04	0.9755	5.52
	BPNN	0.9966	2.23	0.9928	3.01

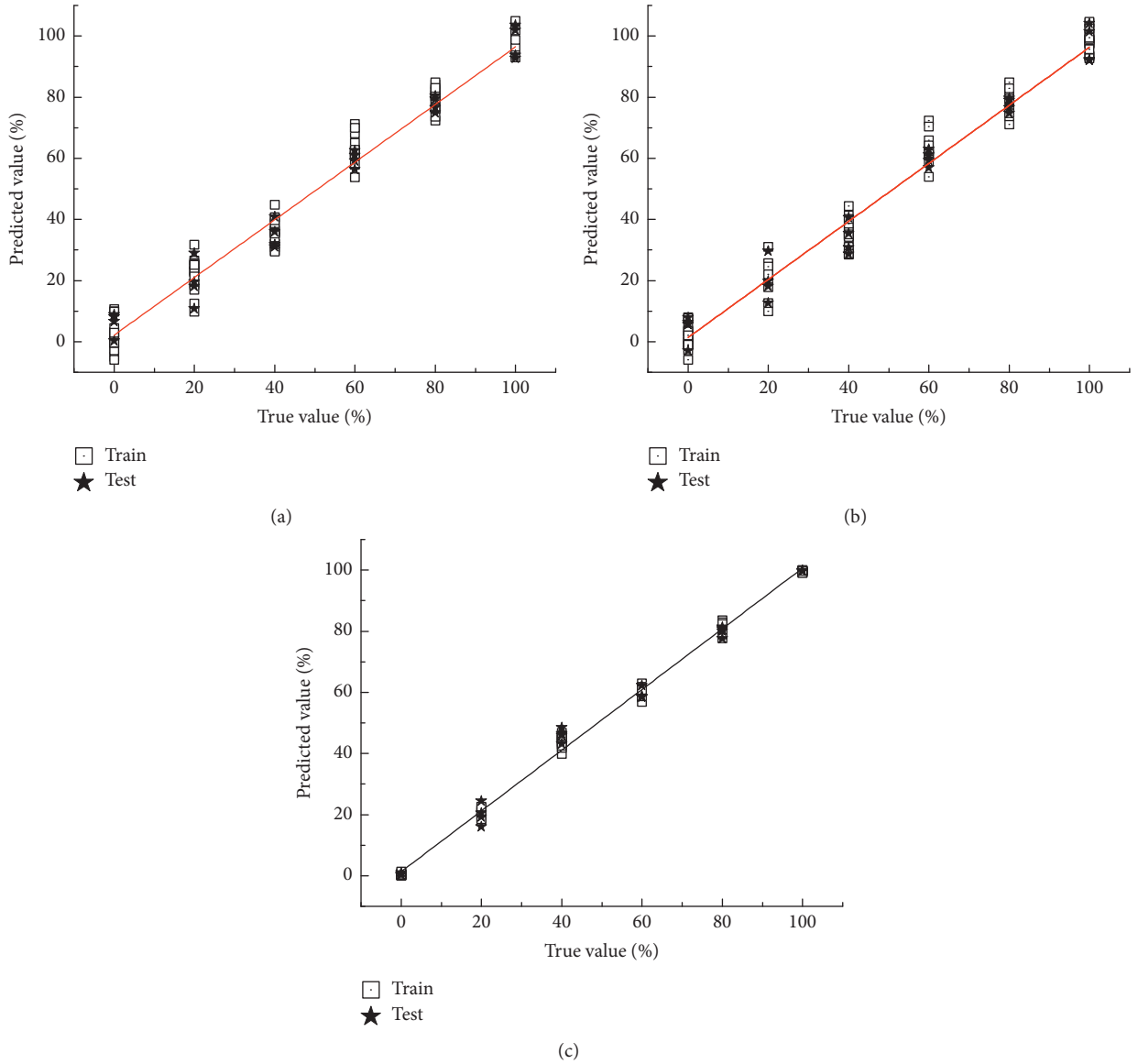

Figure 4: Regression between the expected and true proportion of pork in minced mutton using combined data set of E-nose and E-tongue: (a) PLS; (b) MLR; (c) BPNN.

results of PLS and MLR were similar or even lower with data combined. The best predictive results were found by the BPNN model with the combined data set, with high correlation (higher than 0.99) for the training and testing subsets.

4. Conclusions

The data fusion methods (low-level and midlevel) proved the effective combination of E-nose and E-tongue for the authentication of mutton accordingly to the proportion of pork. Of the two levels of fusion methods, midlevel level fusion, using principle component extraction, exhibited good discrimination results with discrimination methods of CDA, with all samples correctly classified, same with BDA using E-tongue data for the fusion data with any methods. Strong correlation was observed between the E-panel system and proportion of pork in minced mutton, and BPNN was the most suitable one for the prediction of pork proportion. The results obtained by this study are promising in terms of further development of rapid detection method based on E-panel systems for meat authentication in the meat industry.

Data Availability

The statistical data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

The statistical data used to support the findings of this study are included within the supplementary information files. (*Supplementary Materials*)

References

[1] K. Paulos, S. Rodrigues, A. F. Oliveira, A. Leite, E. Pereira, and A. Teixeira, "Sensory characterization and consumer preference mapping of fresh sausages manufactured with goat and sheep meat," *Journal of Food Science*, vol. 80, no. 7, pp. S1568–S1573, 2015.

[2] D. Cozzolino and I. Murray, "Identification of animal meat muscles by visible and near infrared reflectance spectroscopy," *LWT—Food Science and Technology*, vol. 37, no. 4, pp. 447–452, 2004.

[3] M. Kamruzzaman, D.-W. Sun, G. Elmasry, and P. Allen, "Fast detection and visualization of minced lamb meat adulteration using NIR hyperspectral imaging and multivariate image analysis," *Talanta*, vol. 103, no. 2, pp. 130–136, 2013.

[4] X. Tian, J. Wang, R. Shen, Z. Ma, and M. Li, "Discrimination of pork/chicken adulteration in minced mutton by electronic taste system," *International Journal of Food Science & Technology*, vol. 54, no. 3, pp. 670–678, 2019.

[5] B. Druml, S. Grandits, W. Mayer, R. Hochegger, and M. Cichna-Markl, "Authenticity control of game meat products–A single method to detect and quantify adulteration of fallow deer (Dama dama), red deer (Cervus elaphus) and sika deer (Cervus nippon) by real-time PCR," *Food Chemistry*, vol. 170, pp. 508–517, 2015.

[6] M. M. Rahman, M. E. Ali, S. B. A. Hamid, S. Mustafa, U. Hashim, and U. K. Hanapi, "Polymerase chain reaction assay targeting cytochrome b gene for the detection of dog meat adulteration in meatball formulation," *Meat Science*, vol. 97, no. 4, pp. 404–409, 2014.

[7] Q. Rao and Y.-H. P. Hsieh, "Evaluation of a commercial lateral flow feed test for rapid detection of beef and sheep content in raw and cooked meats," *Meat Science*, vol. 76, no. 3, pp. 489–494, 2007.

[8] A. M. A. Di Giuseppe, N. Giarretta, M. Lippert, V. Severino, and A. Di Maro, "An improved UPLC method for the detection of undeclared horse meat addition by using myoglobin as molecular marker," *Food Chemistry*, vol. 169, pp. 241–245, 2015.

[9] M. Nurjuliana, Y. B. Che Man, D. Mat Hashim, and A. K. S. Mohamed, "Rapid identification of pork for halal authentication using the electronic nose and gas chromatography mass spectrometer with headspace analyzer," *Meat Science*, vol. 88, no. 4, pp. 638–644, 2011.

[10] N. Morsy and D.-W. Sun, "Robust linear and non-linear models of NIR spectroscopy for detection and quantification of adulterants in fresh and frozen-thawed minced beef," *Meat Science*, vol. 93, no. 2, pp. 292–302, 2013.

[11] M. Zhao, G. Downey, and C. P. O'Donnell, "Detection of adulteration in fresh and frozen beefburger products by beef offal using mid-infrared ATR spectroscopy and multivariate data analysis," *Meat Science*, vol. 96, no. 2, pp. 1003–1011, 2014.

[12] A. I. Ropodi, D. E. Pavlidis, F. Mohareb, E. Z. Panagou, and G.-J. E. Nychas, "Multispectral image analysis approach to detect adulteration of beef and pork in raw meats," *Food Research International*, vol. 67, pp. 12–18, 2015.

[13] X. Tian, J. Wang, and S. Cui, "Analysis of pork adulteration in minced mutton using electronic nose of metal oxide sensors," *Journal of Food Engineering*, vol. 119, no. 4, pp. 744–749, 2013.

[14] J. Han, L. Huang, Z. Gu, S. Tian, and S. Deng, "Evaluation of meat quality and freshness based on the electronic tongue," *Journal of Chinese Institute of Food Science and Technology*, vol. 8, no. 3, pp. 125–132, 2008, in Chinese.

[15] P. Wang, Y. Zhang, X. Xu, and G. Zhou, "Discrimination on chicken meat products with electronic tongue," in *Proceedings of the Chinese Society of Agricultural Engineering*, 2011, in Chinese.

[16] A. L. A. Rudnitskaya, B. Seleznev, and Y. Vlasov, "Recognition of liquid and flesh food using an 'electronic tongue'," *International Journal of Food Science and Technology*, vol. 37, no. 4, pp. 375–385, 2002.

[17] M. García, M. Aleixandre, J. Gutiérrez, and M. C. Horrillo, "Electronic nose for wine discrimination," *Sensors and Actuators B: Chemical*, vol. 113, no. 2, pp. 911–916, 2006.

[18] T. Horrillo, S. Marco, A. Perera et al., "Potato creams recognition from electronic nose and tongue signals: feature

extraction/selection and RBF neural networks classifiers," in *Proceedings of the 5th Seminar on Neural Network Applications in Electrical Engineering, 2000 (NEUREL 2000)*, pp. 69–74, IEEE, Belgrade, Serbia, September 2000.

[19] Z. Haddi, M. Boughrini, S. Ihlou et al., "Geographical classification of Virgin Olive Oils by combining the electronic nose and tongue," in *Proceedings of the Sensors, 2012 IEEE*, pp. 1–4, IEEE, Taipei, Taiwan, October 2012.

[20] Z. Haddi, H. Alami, N. El Bari et al., "Electronic nose and tongue combination for improved classification of Moroccan virgin olive oil profiles," in *Food Research International*, M. Luz Méndez Méndez, Ed., pp. 277–289, Academic Press, Cambridge, MA, USA, 2016.

[21] C. Apetrei, M. Ghasemi-Varnamkhasti, and I. Mirela Apetrei, *Olive Oil and Combined Electronic Nose and Tongue, Electronic Noses and Tongues in Food Science*, M. Luz Rodríguez Méndez, Ed., pp. 277–289, Academic Press, Cambridge, MA, USA, 2016.

[22] D. Huo, Y. Wu, M. Yang, H. Fa, X. Luo, and C. Hou, "Discrimination of Chinese green tea according to varieties and grade levels using artificial nose and tongue based on colorimetric sensor arrays," *Food Chemistry*, vol. 145, pp. 639–645, 2014.

[23] R. Banerjee, B. Tudu, L. Shaw, A. Jana, N. Bhattacharyya, and R. Bandyopadhyay, "Instrumental testing of tea by combining the responses of electronic nose and tongue," *Journal of Food Engineering*, vol. 110, no. 3, pp. 356–363, 2012.

[24] M. L. Rodriguez-Mendez, A. A. Arrieta, V. Parra et al., "Fusion of three sensory modalities for the multimodal characterization of red wines," *IEEE Sensors Journal*, vol. 4, no. 3, pp. 348–354, 2004.

[25] Z. Haddi, S. Mabrouk, M. Bougrini et al., "E-nose and e-tongue combination for improved recognition of fruit juice samples," *Food Chemistry*, vol. 150, pp. 246–253, 2014.

[26] S. Qiu, J. Wang, and L. Gao, "Qualification and quantisation of processed strawberry juice based on electronic nose and tongue," *LWT—Food Science and Technology*, vol. 60, no. 1, pp. 115–123, 2015.

[27] M. Laureati, S. Buratti, A. Bassoli, G. Borgonovo, and E. Pagliarini, "Discrimination and characterisation of three cultivars of *Perilla frutescens* by means of sensory descriptors and electronic nose and tongue analysis," *Food Research International*, vol. 43, no. 4, pp. 959–964, 2010.

[28] W. Dong, J. Zhao, R. Hu, Y. Dong, and L. Tan, "Differentiation of Chinese robusta coffees according to species, using a combined electronic nose and tongue, with the aid of chemometrics," *Food Chemistry*, vol. 229, pp. 743–751, 2017.

[29] L. Gil-Sánchez, J. Soto, R. Martínez-Máñez, E. Garcia-Breijo, J. Ibáñez, and E. Llobet, "A novel humid electronic nose combined with an electronic tongue for assessing deterioration of wine," *Sensors and Actuators A: Physical*, vol. 171, no. 2, pp. 152–158, 2011.

[30] C. Di Natale, R. Paolesse, A. Macagnano et al., "Electronic nose and electronic tongue integration for improved classification of clinical and food samples," *Sensors and Actuators B: Chemical*, vol. 64, no. 1–3, pp. 15–21, 2000.

[31] C. Apetrei, I. M. Apetrei, S. Villanueva, J. A. de Saja, F. Gutierrez-Rosales, and M. L. Rodriguez-Mendez, "Combination of an e-nose, an e-tongue and an e-eye for the characterisation of olive oils with different degree of bitterness," *Analytica Chimica Acta*, vol. 663, no. 1, pp. 91–97, 2010.

[32] X. Hong and J. Wang, "Detection of adulteration in cherry tomato juices based on electronic nose and tongue: comparison of different data fusion approaches," *Journal of Food Engineering*, vol. 126, pp. 89–97, 2014.

[33] A. R. D. Rosa, F. Leone, F. Cheli, and V. Chiofalo, "Fusion of electronic nose, electronic tongue and computer vision for animal source food authentication and quality assessment—a review," *Journal of Food Engineering*, vol. 210, pp. 62–75, 2017.

[34] E. Borràs, J. Ferré, R. Boqué, M. Mestres, L. Aceña, and O. Busto, "Data fusion methodologies for food and beverage authentication and quality assessment–A review," *Analytica Chimica Acta*, vol. 891, pp. 1–14, 2015.

[35] Alpha MOS, *Technical Note T-P-02, Similarities between Electronic Tongue and Human Tongue*, Alpha MOS, Toulouse, France, 2005.

[36] Alpha MOS, *Technical Note T-SAS-01, Electrochemical Sensors*, Alpha MOS, Toulouse, France, 2002.

[37] P. Zhan, H. Tian, W. Li, Y. Ma, and X. Zhang, "Establishment of mutton characteristics flavor fingerprint," *Modern Food Science and Technology*, vol. 29, no. 10, pp. 2522–2527, 2013.

[38] M. S. Cosio, D. Ballabio, and C. S. Gigliotti, "Evaluation of different storage conditions of extra virgin olive oils with an innovative recognition tool built by means of electronic nose and electronic tongue," *Food Chemistry*, vol. 101, no. 2, pp. 485–491, 2007.

[39] C. Apetrei, I. M. Apetrei, I. Nevares et al., "Using an e-tongue based on voltammetric electrodes to discriminate among red wines aged in oak barrels or aged using alternative methods," *Electrochimica Acta*, vol. 52, no. 7, pp. 2588–2594, 2007.

[40] A. De Saja, E. Polshin, D. Kirsanov et al., "Instrumental measurement of beer taste attributes using an electronic tongue," *Analytica Chimica Acta*, vol. 646, no. 1-2, pp. 111–118, 2009.

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Research Article

Authenticating Raw from Reconstituted Milk Using Fourier Transform Infrared Spectroscopy and Chemometrics

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Fourier transform infrared (FTIR) spectroscopy combined with chemometrics was used to authenticate raw milk from their reconstituted counterparts. First, the explanatory principal component analysis (PCA) was employed to visualize the relationship between raw and reconstituted milk samples. However, the degree of separation between two sample classes was not significant according to direct observation of the scores plot, indicating FTIR spectra may contain complicated chemical information. Second, partial least-squares-discriminant analysis (PLS-DA) that incorporate additional class membership information as modelling input was further calculated. The PLS-DA scores yielded clear separation between two classes of samples. Additionally, possible components from the model loading were studied, and the PLS-DA model was validated internally under the model population analysis framework, as well as externally using an independent test set. This study gave insights into the authentication of milk using FTIR spectroscopy with chemometrics techniques.

1. Introduction

Milk is one of the most consumed food items, which has significant nutritional and economical importance. The reconstitution of milk is an act that adulterates skimmed or whole milk powder in part into raw milk or completely substitutes raw milk [1, 2]. Such fraud can achieve marginal economic gain since the shelf life of milk powder is longer than their raw counterparts. Adulteration of powdered milk in their raw counterparts may alter the original nutritional and functional value of raw milk, and thus, it may provoke a crisis of confidence to consumers for milk industry. Therefore, a rapid, simple, and automated method for milk adulteration detection is required.

Adulteration of commercial milk powder is even more challenging to detect than many other common milk adulterants such as melamine or plant protein, due to the extremely similar chemical composition. Therefore, measurements with both high sensitivity and resolution are preferred. For instance, two-dimensional gel electrophoresis combined with matrix-assisted laser desorption/ionization-mass spectrometry was reported to detect powdered milk in raw cow's milk based on the modified peptide including oxidation, lactosylation, and deamination protein products [1]. The detection of furosine [3] and lysinoalanine [4] by liquid and gas chromatography was introduced. Rather than seeking specific marker components, other kinds of methods applied empirical models or fingerprints to detect adulteration. Differentiation of raw from reconstituted milk by the stable isotope ratios of oxygen and hydrogen was also reported [5]. However, the above methods often require either time and cost-consuming mass spectrometric detection or labor-intensive sample pretreatment or analysis procedures, which render these methods inapplicable to large-scale assay.

Rapid analytical techniques such as spectroscopy or electronic noses, with the combination of empirical modelling, provide a convenient approach to characterize complex food matrices. For example, the adulteration of

whole milk with milk powder was detected by spectrophotometry. The ultraviolet and visible spectroscopy has been applied to the detection and quantification of raw milk with reconstituted full-fat milk powder [2]. The transmittance of raw milk adulterated with full-fat dry milk powder reconstituted milk was observed and possibly explained the phenomenon by turbidity variation induced from low degree of homogenization [6]. In addition, the fluorescence of advanced Maillard products and soluble tryptophan (FAST) index had been devised for distinguishing milk heat treatments [7]. However, these researches were based on empirical observations without clear metrics or limits, and thus limited information is extracted from the spectra. Fingerprints combined with chemometrics methods were suitable for processing complex analytical data in an automated and objective decision-making manner. For instance, the adulteration of reconstituted milk or water with electronic noses constructed with ten different metal oxide sensors was monitored with chemometrics modelling [8].

Fourier transform infrared spectroscopy (FTIR) has been widely used for food quality monitoring including authenticity and traceability, due to its fast speed and nondestructive capabilities [9, 10]. FTIR spectroscopy has been successfully demonstrated in milk authentication such as to detect soymilk adulterated in cow or buffalo milk [11]. It is therefore interesting to test whether FTIR spectroscopy could further identify any reconstitution in raw milk.

Adulteration in food ingredients such as milk or olive oil suggested that chemometrics modelling is becoming an essential part in the fingerprinting analyses [12–14]. Specifically, infrared and Raman spectroscopy studies on detection of food adulterations had resulted in a wide range of successful applications. Raman spectroscopy could detect melamine adulterant in milk powder at the detection limit of 0.13% (w/w) by two vibration modes at 673 and 982 cm^{-1} [12]. Additionally, machine-learning methods provide possibilities to a wide range of application of infrared spectroscopy in food authentication and quality control. For instance, near-infrared reflectance spectral were used to examine the authentication of skim and nonfat dry milk powder using analysis of variance- (ANOVA-) principal component analysis (PCA), pooled-ANOVA, and partial least-squares-regression (PLSR) [13]. The potential of near-infrared (NIR) spectroscopy combined with chemometrics for nontargeted detection of adulterants in skim and nonfat dry milk powder was also studied [14]. Therefore, it is interesting to test whether infrared spectroscopy combined with chemometric modelling techniques can be applied in detecting milk powder in raw milk.

In this study, FTIR combined with chemometrics was developed for the detection of milk adulteration. Specifically, infrared spectral fingerprints combined with chemometrics were tested in detecting reconstituted milk powder in raw milk. The workflow is demonstrated in Figure 1. This study aimed at detecting milk powder adulterated in raw milk using FTIR spectroscopy combined with chemometrics. This work may serve as a reference for quality assurance of raw milk and its related dairy products.

2. Materials and Methods

2.1. Sample Collection. Twenty raw milk samples were provided by local milk farms located in Qingdao, Shandong province, China. These farms were certified suppliers of the Nestle Corporation (Vevey, Switzerland). Each raw milk sample was stored in a separate 100 mL polythene bottle. All samples were immediately frozen after collection. The bottles were placed in a portable Styrofoam box with ice packs and dry ice to maintain optimum low temperature and stored at −20°C once transferred to the laboratory. Four anonymously branded commercial milk powders with unrevealed processing techniques were purchased from local groceries in Shanghai, China.

2.2. Sample Pretreatment. Raw milk samples were directly lyophilized using a Labconco freeze dryer (Kansas City, MO, USA). The freeze-drying process removes any moisture that may interfere the FTIR measurement. It was served as a pretreatment step that maintains the original chemical compositions of raw milk as much as possible and made storage and testing of a large batch of samples possible.

For the preparation of adulterated milk with reconstituted milk powder, first, raw milk was randomly selected as the standard sample. Then, each commercial milk powder was added to the authentic liquid milk in 0.5, 1, 3, 5, and 10% (w/v), resulting in five partially reconstituted samples, respectively. After that, the mixtures were sonicated for 20 min. Finally, the mixtures were lyophilized. The lyophilizates were subjected to FTIR analysis.

2.3. FTIR Analysis. All fingerprints were collected using a Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Smart iTR single bounce germanium crystal attenuated total reflectance (ATR) sampling accessory (Thermo Fisher Scientific). The spectra were collected in the transmittance mode by an average of 60 scans ranging between 650 and 4000 cm^{-1} with a 0.48 cm^{-1} interval. Before each measurement, an independent background scan was performed and subtracted immediately to minimize atmospheric interference and instrument fluctuation.

All samples were prepared and tested in triplicates, including 60 raw milk samples (20 raw milk samples × 3 replicates) and 60 reconstituted milk samples (4 milk powders × 5 adulteration levels × 3 replicates), resulting in totally 120 spectra.

2.4. Chemometrics Modelling. All raw data were imported to MATLAB (version R2018a, The MathWorks, Natick, MA, USA). Different preprocessing strategies such as wavenumber region selection, autoscaling, standard normal variate (SNV), and derivative were applied. All chemometric analyses including preprocessing, PCA, and partial least-squares-discriminant analysis (PLS-DA) were performed using in-house MATLAB routines running on a personal

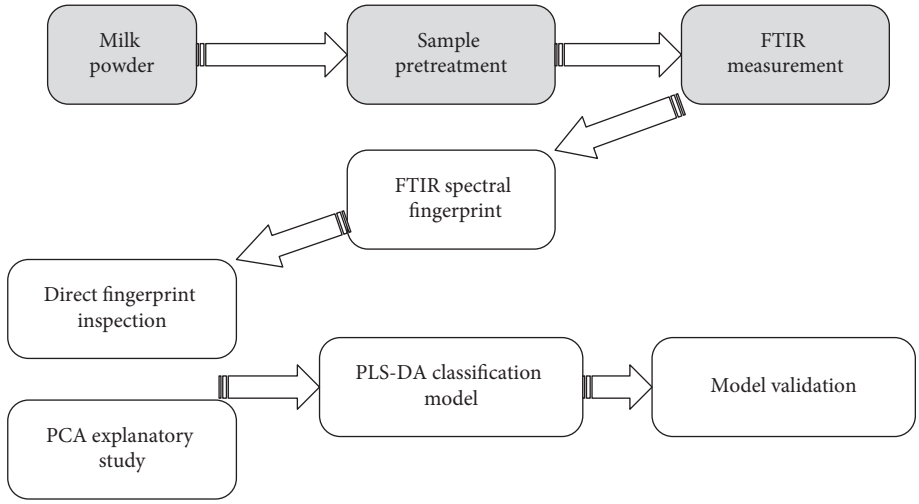

Figure 1: Schematic workflow of this study.

computer under Windows 7 operating system (Microsoft Corporation, Redmond, WA, USA).

For internal validation, statistically relevant comparisons were achieved by the model population analysis (MPA) framework [15]. The MPA is essentially based on cross validation of a series of submodels obtained from the original data set through random sampling. In this work, MPA extract statistical information from models to achieve a statistically unbiased estimation of performance. The internal validation process was evaluated repeatedly for 100 bootstraps. For external validation, the Latin partition approach was employed to split the whole data set into training and test sets prior to classification. To evaluate the result, prediction accuracy of the data set is used. Prediction accuracy is an estimated percentage of correct identifications when the model is applied for unknown samples, which is widely applied to assess the overall performance of a specific classification model.

3. Results and Discussion

3.1. FTIR Spectral Characteristics. The FTIR spectral fingerprints contained representative information for different components in milks. The mean spectra of raw and reconstituted milks are shown in Figure 2. The absorption bands observed at 1630 to 1680 cm^{-1} and 1510 to 1570 cm^{-1} may be induced by C=O stretching vibrations of absorption of amide I and N-H and C-H bending vibration absorption of amide II from milk protein, respectively [16, 17]. The bands around 2920, 2850, and 1743 cm^{-1} may be antisymmetric and symmetric CH_2 stretching and carbonyl group C=O double bond stretching from milk fat, respectively [18]. The absorption bands located at 3200 to 3800 cm^{-1}, 1030 to 1200 cm^{-1}, 900 to 930 cm^{-1}, and 755 to 785 cm^{-1} may be associated with carbohydrate [19, 20]. These peaks also resemble the largest differed variable ranges in fingerprints. However, noting that, the mean spectra occurred in high overlap, suggesting a strong compositional similarity. Additionally, no evident peaks were determined as marker peaks since any single component is unlikely to be a critical differentiation factor. Consequently, it is hard to detect milk adulteration with mere visual inspection. Therefore, applying multivariate methods to address the overall spatial distribution of the data is necessary.

3.2. PCA Explanatory Study. PCA was performed to preliminary visualize the multivariate distribution of all fingerprints. Figure 3 shows the PCA scores plot, with autoscaling preprocessing applied. The PCA scores plot suggested that there were no obvious discriminations between raw and reconstituted samples using raw fingerprints. Specifically, no separation tendencies between raw and reconstituted samples were observed along the axes of both principal components PC 1 and PC 2, the two largest principal components. The combined variances explained by PC 1 and PC 2 were 88% of the total variances, indicating that the most dominant variances of the fingerprints do not closely relate to the reconstituted milk. The PCA result was also consistent with the result from visual inspection. Different preprocessing methods, including SNV alone and SNV combined with first- and second-order derivatives, were also studied by observing the PCA scores plot (data not shown). Regardless of preprocessing methods or the combinations used, there were no obvious discriminations between raw and reconstituted samples. By selecting the wavenumber region of 800–1800 cm^{-1}, the degree of separation between raw and reconstituted milk cannot be improved either (data not shown). Consequently, it is not confirmed by PCA that there can be characteristic bands in the fingerprint region, nor there exhibit characteristics between two kinds of spectra. However, supervised multivariate classification may be capable of extracting information from complex data because the class memberships of samples were also included as the model input. Therefore, PLS-DA was applied to analyze the fingerprints further.

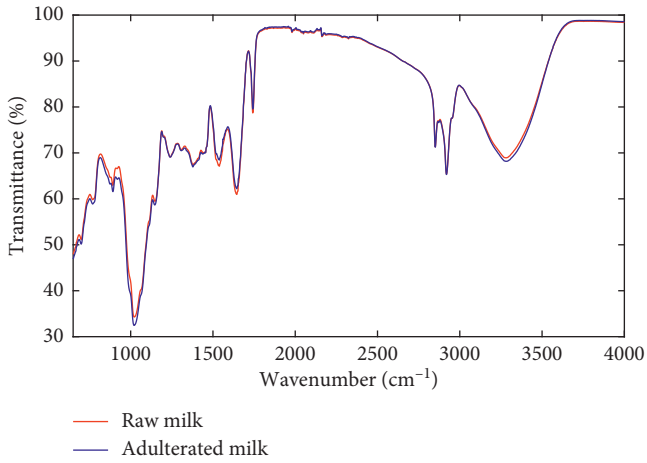

Figure 2: Average FTIR spectral fingerprints of raw and reconstituted milks.

3.3. PLS-DA Model Evaluation. PLS-DA is perhaps one of the most well-known supervised classification methods in chemometrics. This method is based on partial least-squares-regression of continuous predictor variables, which seek for optimal latent variables with maximum covariance. Similar to PCA, PLS-DA was firstly applied as an explanatory approach to study the overall distribution. Different preprocessing methods were applied to the data, including wavenumber selection, autoscaling, first derivatives, and different combinations. It was indicated that PLS-DA achieved generally good separation of classes by PLS-DA scores. The best separation is shown in Figure 4, which is the *X*-scores (scores of the spectral data block) plot of the PLS-DA model by first selecting the wavenumber at 800–1800 cm^{-1}, where the spectral differences were larger than other regions, with autoscaling and first derivative preprocessing. The two largest latent variables are displayed in Figure 4. Although with small portion of overlap, the distribution of tested samples clearly showed two clusters, indicating an intrinsically different fingerprint patterns among two classes of samples. A trend related to the adulteration level was also observed. Specifically, samples adulterated at 0.5%, the lowest adulteration level in this study, is located at the partial overlap with the raw milk sample cluster. Contrarily, samples adulterated at 10% are more significantly apart from raw milk, compared with those at lower adulteration levels.

In explanatory studies, both PCA and PLS-DA scores plots limit their indicative abilities in only two dimensions, namely, the first and second principal components or latent variables. Such analysis approach relies heavily on the final judgement of the researcher for the analysis of visual patterns instead of objective performance metrics. In comparison, the PLS-DA model is able to overcome this shortcoming by the automatic model-building process with a reasonable number of variables. By selecting 90% of original data as the training set, with 11 latent variables though internal validation procedure described in the next section, a final PLS-DA model was built and validated. Figure 5 shows the regression coefficients of the PLS-DA model. Positive and negative coefficients represent the relationships of the peaks to pure and reconstituted samples, respectively. The absolute magnitude of coefficients indicated the relative importance of peaks. Some interesting peaks arise in the PLS-DA coefficients. Peaks at 904 to 1288 cm^{-1} were generally associated with C-H bending, C-O-H in-plane bending, and C-O stretching vibrations of lipids, organic acids, and carbohydrate derivatives. Compared with the raw spectra shown in Figure 2, some peaks (904–1288 cm^{-1}) may be associated with carbohydrates. This might be attributed to a series of the Maillard reaction occurred in milk powder, which result in the reduction of lysine-rich proteins and lactose [21]. Peaks at 1583 cm^{-1} corresponded to unspecified compounds. The result is relevant with the PCA study that characteristic peaks may arise in the fingerprint region when authenticating raw milk. However, it did not agree with our previous findings that PCA and PLS-DA performed consistently in classifying pure milk and their counterparts adulterated with other powdered proteins [22], probably due to the complexity of spectra. It was indicated that, for the complex FTIR spectral fingerprints, the application of supervised classification methods is important because exploratory methods such as PCA did not yield a complete clear characterization.

3.4. PLS-DA Model Validation. Although PLS-DA model finds the possible characteristics between raw and reconstituted milk samples, evaluating the validity of the PLS-DA model is necessary, since PLS-DA may be prone to overfitting. Specifically, the quantitative metrics of PLS-DA prediction power were tested by both internal and external approaches to indicate the suitability and generalizability of the model. Firstly, the complete data set was split into training and external test sets. Secondly, the internal validation was performed solely on the training set by splitting the training set into internal training and calibration set. In internal validation, statistically relevant validation of PLS-DA modelling was achieved by MPA. To achieve a statistically unbiased estimation of performance, a series of PLS-DA models were built and evaluated repeatedly for 100 bootstraps. The average classification accuracy was 98% when 11 latent variables were applied, suggesting a reliable performance.

In external validation, the Latin partition approach was employed to split the whole data set into training (90%) and test (10%) sets prior to the PLS-DA classification. Unlike the previous PCA scores plot that used only two principal components to find possible separation between pure and reconstituted samples, 11 latent variables were applied for the final building of the PLS-DA model after bootstrapped Latin partition evaluation, indicating that there were many independent components presented in the sample to establish an effective model. Figure 6 shows the final prediction output of the PLS-DA model for external validation. All samples in the test set were correctly classified by PLS-DA.

It is also interesting to study the differences between different adulteration levels since Figure 4 presented differences as previously discussed. Therefore, PLSR was

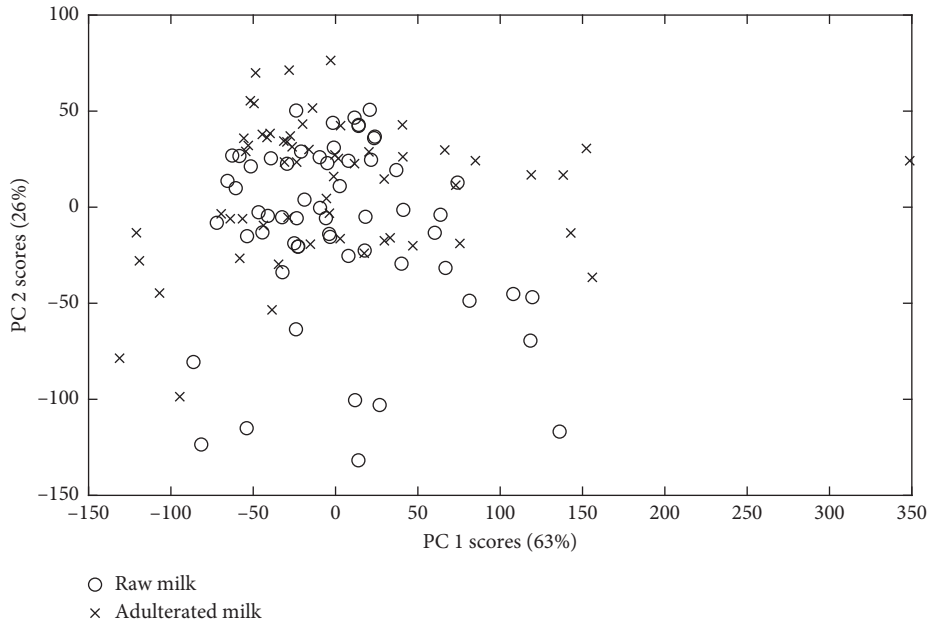

Figure 3: Principal component analysis scores plots of FTIR fingerprints.

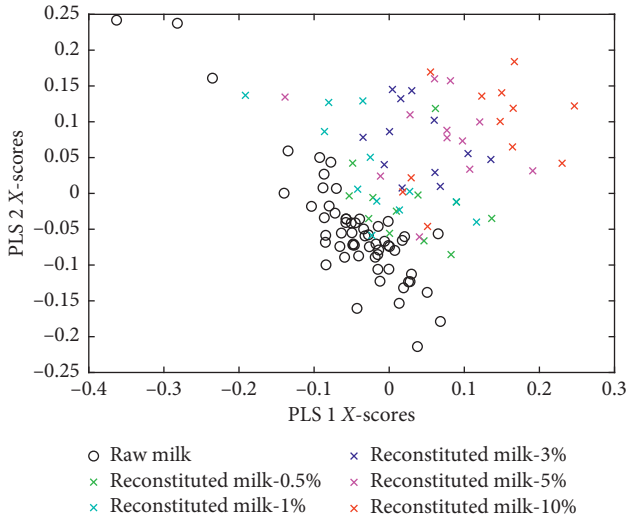

Figure 4: Partial least-squares-discriminant analysis (PLS-DA) scores plot of FTIR fingerprints.

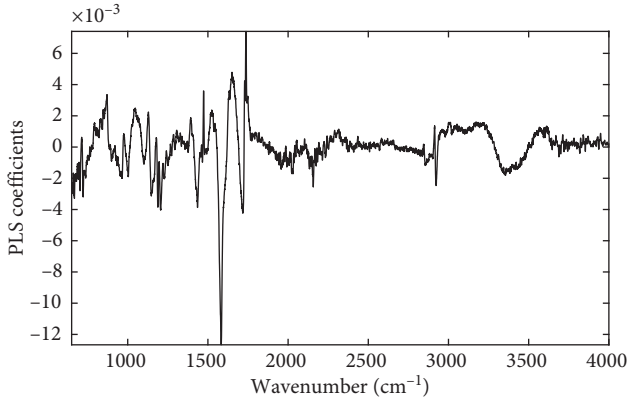

Figure 5: Coefficients of the PLS-DA model.

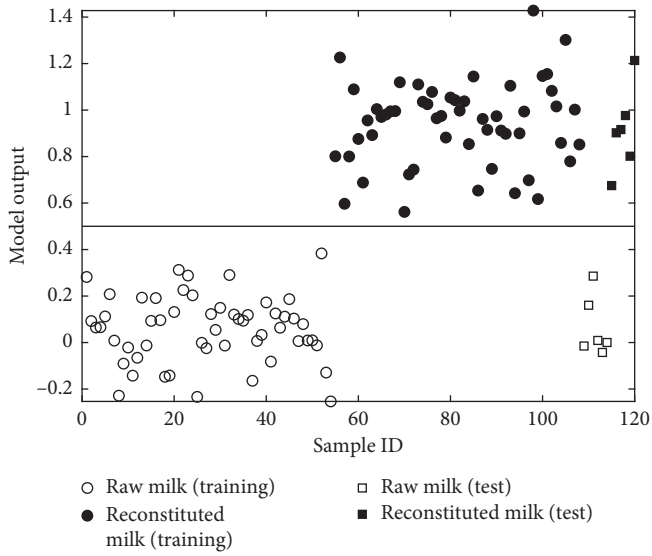

Figure 6: External prediction output of the partial least-squares-model. The line at 0.5 was the criteria of the PLS-DA model to determine the sample type.

applied to model the adulteration level. All other parameters remained the same as PLS-DA. The final external validation yielded a root-mean-squared error of 3.0, indicating an effective quantification of the adulteration level.

Other than a 9 : 1 (training set/test set) split ratio, further evaluations by different split ratios of 2 : 1 and 1 : 1 were performed to prevent model overfitting. Except that, all other calculations remain unchanged. The result was consistent with that from the previous condition. Specifically, only one test sample was misclassified when the split ratio was 1 : 1, and all other predictions were correct. It can be concluded that the MPA modelling approach is robust and still reliable even when half of the data were removed.

4. Conclusion

FTIR spectroscopy combined with chemometrics has been successfully demonstrated to detect possible presence of reconstituted milk in raw milk. This work indicates FTIR spectroscopy has great potentials in quality control of milk and their related products because the PLS-DA model yielded satisfactory separation of the two spectral fingerprints. Noting that, due to the limited sample size and variability, careful selection of liquid and powdered milk in a larger data set may be necessary before practice to assure the universality of the final model. Additionally, simpler methods such as sampling without lyophilization and quantitating the level of adulteration need to be investigated in the future.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

[1] C. D. Calvano, A. Monopoli, P. Loizzo, M. Faccia, and C. Zambonin, "Proteomic approach based on MALDI-TOF MS to detect powdered milk in fresh cow's milk," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 8, pp. 1609–1617, 2012.

[2] M. A. M. Madkour and B. M. I. Moussa, "The determination of milk powder added to whole milk," *Food/Nahrung*, vol. 33, no. 1, pp. 77–82, 1989.

[3] R. Van Renterghem and J. De Block, "Furosine in consumption milk and milk powders," *International Dairy Journal*, vol. 6, no. 4, pp. 371–382, 1996.

[4] A. Montilla, J. Á. Gómez-Ruiz, A. Olano, and M. D. del Castillo, "A GC-FID method for analysis of lysinoalanine," *Molecular Nutrition & Food Research*, vol. 51, no. 4, pp. 415–422, 2007.

[5] G. P. Lin, Y. H. Rau, Y. F. Chen, C. C. Chou, and W. G. Fu, "Measurements of δD and δ18O stable isotope ratios in milk," *Journal of Food Science*, vol. 68, no. 7, pp. 2192–2195, 2003.

[6] T.-C. Lin, I.-J. Chen, and Y.-C. Ko, "The mechanism of determining the adulteration of whole milk with milk powder by spectrophotometry," *Food/Nahrung*, vol. 35, no. 4, pp. 351–358, 1991.

[7] I. Birlouez-Aragon, P. Sabat, and N. Gouti, "A new method for discriminating milk heat treatment," *International Dairy Journal*, vol. 12, no. 1, pp. 59–67, 2002.

[8] H. Yu, J. Wang, and Y. Xu, "Identification of adulterated milk using electronic nose," *Sensors and Materials*, vol. 19, no. 5, pp. 275–285, 2007.

[9] N. Nicolaou and R. Goodacre, "Rapid and quantitative detection of the microbial spoilage in milk using fourier transform infrared spectroscopy and chemometrics," *The Analyst*, vol. 133, no. 10, pp. 1424–1431, 2008.

[10] N. Nicolaou, Y. Xu, and R. Goodacre, "Fourier transform infrared spectroscopy and multivariate analysis for the detection and quantification of different milk species," *Journal of Dairy Science*, vol. 93, no. 12, pp. 5651–5660, 2010.

[11] P. Jaiswal, S. N. Jha, A. Borah, A. Gautam, M. K. Grewal, and G. Jindal, "Detection and quantification of soymilk in cow-buffalo milk using attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)," *Food Chemistry*, vol. 168, pp. 41–47, 2015.

[12] Y. Cheng, Y. Dong, J. Wu et al., "Screening melamine adulterant in milk powder with laser Raman spectrometry," *Journal of Food Composition and Analysis*, vol. 23, no. 2, pp. 199–202, 2010.

[13] J. M. Harnly, P. B. Harrington, L. L. Botros et al., "Characterization of near-infrared spectral variance in the authentication of skim and nonfat dry milk powder collection using ANOVA-PCA, pooled-ANOVA, and partial least-squares regression," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 32, pp. 8060–8067, 2014.

[14] L. L. Botros, J. Jablonski, C. Chang et al., "Exploring authentic skim and nonfat dry milk powder variance for the development of nontargeted adulterant detection methods using near-infrared spectroscopy and chemometrics," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 41, pp. 9810–9818, 2013.

[15] H.-D. Li, Y.-Z. Liang, D.-S. Cao, and Q.-S. Xu, "Model-population analysis and its applications in chemical and biological modeling," *TrAC Trends in Analytical Chemistry*, vol. 38, pp. 154–162, 2012.

[16] M. Carbonaro and A. Nucara, "Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region," *Amino acids*, vol. 38, no. 3, pp. 679–690, 2010.

[17] M. Ye, R. Zhou, Y. Shi, H. Chen, and Y. Du, "Effects of heating on the secondary structure of proteins in milk powders using mid-infrared spectroscopy," *Journal of Dairy Science*, vol. 100, no. 1, pp. 89–95, 2017.

[18] Q. Zhou, S.-Q. Sun, L. Yu, C.-H. Xu, I. Noda, and X.-R. Zhang, "Sequential changes of main components in different kinds of milk powders using two-dimensional infrared correlation analysis," *Journal of Molecular Structure*, vol. 799, no. 1-3, pp. 77–84, 2006.

[19] H. Fabian and M. Werner, "Infrared spectroscopy of proteins," in *Handbook of Vibrational Spectroscopy*, John Wiley & Sons, Ltd, Hoboken, NJ, USA, 2002.

[20] M. J. Wenstrup, M. Plans, and L. E. Rodriguez-Saona, "Effect of a novel induction food-processing device in improving frying oil quality," *International Journal of Food Science & Technology*, vol. 49, no. 10, pp. 2223–2229, 2014.

[21] S. G. Anema and Y. Li, "Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size," *Journal of Dairy Research*, vol. 70, no. 1, pp. 73–83, 2003.

[22] W. Lu, X. Lv, B. Gao, H. Shi, and L. Yu, "Differentiating milk and non-milk proteins by UPLC amino acid fingerprints combined with chemometric data analysis techniques," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 15, pp. 3996–4002, 2015.

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Research Article

Use of Natural Dietary Spices for Reclamation of Food Quality Impairment by Aflatoxin

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Certain natural dietary spices, in addition to their use in food flavoring and preservation, have the ability to detoxify or degrade a wide range of chemical carcinogens. In the present study, aqueous extracts of judiciously selected natural dietary spices were evaluated for their ability to degrade aflatoxin B1 (AFB1). A total of 9 spices including garlic (*Allium sativum*), ginger (*Zingiber officinale*), black cumin (*Nigella sativum*), clove (*Syzygium aromaticum*), sacred basil (*Ocimum basilicum*), lemon grass (*Cymbopogon citratus* L. (DC) *Stapf*)), thyme (*Thymus schimperi*), fenugreek (*Trigonella foenum-graecum*), and lemon traditionally used by the Ethiopian Community for food flavoring and preservation were considered. Aflatoxin degradation efficacy of the spice extracts was studied through determination of the toxin in extract-treated and nontreated samples using LC-MS/MS. The degradation was characterized by electrochemical methods based on the characteristic oxidation peak of phenolic hydroxyl resulting from the degradation of the toxin after treatment with the extracts. Of the various spices, garlic showed the highest 35.8% (30 min) to 61.7% (1 hr) in spiked and 46.7% (30 min) to 68.3% (1 hr) and real-sample (contaminated maize) treatment reduction followed by lemon 34.2% (30 min) to 56.0% (1 hr) in spiked and 41.1% (30 min) to 60.6% (1 hr) in real-sample treatment at 25°C. The level of reduction was higher in real-sample treatment than that in spiked. This suggests adsorptive contribution by the matrix in addition to the chemical degradation. The current findings revealed nonobvious benefits of the use of natural dietary spices as effective solution for AFB1 degradation and decontamination of food.

1. Introduction

Food quality impairment by food-borne pathogens is among major public health problems across the world [1]. Some of these pathogenic problems are associated with mycotoxins that are elaborated by certain species of fungi. Among the most important mycotoxins, aflatoxins (AFs) are considered to be the most toxigenic. AFs are secondary metabolites mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* [2, 3]. These fungi can infect crops before or after harvest and produce aflatoxins. Aflatoxins consist of a group of approximately 20 related compounds, although only aflatoxins B1 (AFB1), B2 (AFB2), G1, and G2 are commonly encountered in feed and food commodities [4]. Among the AFs, aflatoxin B1 (AFB1) is the most toxic and can be a significant risk to human and can cause significant economic losses.

Numerous studies have reported that AFB1 can induce liver cancer in many species of laboratory and wild animals, including subhuman primates, and is a potent hepatocarcinogen in humans [5–9]. The genotoxicity of AFB1 is linked to its epoxidation reaction in the liver of hosting animal. The epoxide form is highly reactive and can bind to DNA and albumin in the blood serum, forming adducts and hence causing DNA damage [10]. Consequently, aflatoxins affect protein synthesis and can lead to suppression of immune responses [10–12]. In addition to the health effects, the economic consequences of aflatoxin are also profound. Crops with unacceptable levels of aflatoxins often have to be destroyed. Alternatively, contaminated food crops are sometimes diverted into animal feed, which in turn can lead to reduced growth rates, illness, and death of the animals. Moreover, animals consuming aflatoxin-contaminated feeds

can produce meat and milk that contain the toxin residues and another toxic biotransformation product, aflatoxin M1 (AFM1) [13].

Considering the socioeconomic impact of aflatoxin, different countries and organizations have stipulated the maximum permissible level of AFs in food and feed commodities. The US Food and Drug Administration (USFDA) sets total aflatoxins below 20 ppb in interstate commerce food and feed and limits AFM1 below 0.5 ppb for the sale of milk [14]. The Commission Regulation of the European Union (EU) No 165/2010 stipulated 8 μg/kg of AFB1 in food subjected to sorting or physical treatment before human consumption, and the corresponding 2 μg/kg of AFB1 for direct human consumption and 0.05 μg/L for AFM1 in milk [15].

Risk of human exposure to aflatoxin contamination of food and feed commodities is a major concern in Ethiopia due to the occurrence of the toxin in in different kinds of grains and dairy products [13, 16–18]. The prevalence of aflatoxin contamination of food and feed commodities in Ethiopia was attributed to the climatic conditions (temperature, humidity, etc.) that are favorable for the proliferation of the aflatoxigenic fungi [16]. Other factors such as the predisposing pre- and postharvest factors including frequent seasonal drought causing soil and water stress, lack of resistant varieties, harvesting methods, and storage facility are also contributing to the common incidence of aflatoxin in this country. The problem is exacerbated by, *inter alia*, lack of awareness about aflatoxins and the risks associated with them in the value chain actors including farmers, traders, and consumers. Farmers often feed left-over moldy grains to livestock and commonly use different mixed concentrate feeds such as brewery by-product ("atela"), wheat bran, noug cake, maize grains, and silage to increase production. However, these products are susceptible to contamination with AFB1 [17, 18]. Thus, humans are exposed since the toxins or their biotransformation products accumulate in the value chain products. That is why the Comprehensive Africa Agriculture Development Programme (CAADP) recently set aflatoxins as a high-priority research area, establishing the Partnership for Aflatoxin Control in Africa (PACA) [19]. Despite the progresses in some areas, very limited scientific information is available on control strategies. Particularly, community-based, low-cost, safer, and easy to implement at a larger scale approaches are yet to be developed. Therefore, conduct of studies aiming at practical solutions to the detoxification of AFs is of paramount importance to design effective strategies for aflatoxin control.

The scientific methods reported to date on AF control have focused on three approaches: prevention of contamination of food and feed by the fungi that elaborate the toxins (mainly *A. flavus* and *A. parasiticus*), decontamination of the toxins from contaminated foods and feeds, and inhibition of aflatoxin absorption in the gastrointestinal tract [20]. Although prevention can be considered as the most rational and economic approach, its implementation is difficult in tropical areas where favorable environmental and climatic conditions promote the fungal growth. In addition, aflatoxins are extremely durable and unavoidable under most conditions of storage, handling, and processing of foods or feeds [21]. In this context, degradation or detoxification of aflatoxin is the most promising route to decontaminate foods already contaminated with the toxins. Since the two key sites for toxicity of AFB1 are a double bond in furan ring and a lactone in coumarin ring, either removal of the double bond or opening up of the lactone ring can be taken as possible sites of degradation and detoxification [20].

Various physical, chemical, and biological methods have been proposed for the detoxification of aflatoxins [22–24]. Nevertheless, all these methods have their own drawbacks. To be successful, however, a detoxification strategy must ensure the Food and Agriculture Organization criteria such as destroy, inactivate or remove the mycotoxins, not produce or leave toxic and/or carcinogenic residue, and not alter significantly the nutritional and technological properties of the product and must be technically and economically feasible [25].

Natural plant extracts are of interest as a source of safer or more effective alternative to biological agents for aflatoxin detoxification. The use of plant products for aflatoxin control has been reported by several authors [24]. However, much emphasis has been given to inhibition of the plant extracts against the growth of the aflatoxigenic fungi. In addition, many of these plants are not suitable to be used in foods, as the resultant products cannot be consumed by humans. In this context, the use of natural dietary spices provides an attractive opportunity as a community-based (suitable for large-scale implementation), safer, cost-effective, and practical method for aflatoxin control.

In the present study, aqueous extracts obtained from judiciously selected dietary natural spices traditionally used by the Ethiopian Community for food flavoring and preservation were evaluated for their ability to degrade AFB1.

2. Materials and Methods

2.1. Chemicals and Materials. All the reagents and standards used in this study were of analytical grade. Aflatoxin B1 standard was purchased from ACROS, New Jersey, USA, in a crystalline form. Stock solution of AFB1 (2000 μg/L) was prepared in methanol and stored at 4°C. The working solutions of AFB1 standard were freshly prepared by diluting the stock solution with phosphate buffer solution (1.7 mM, pH = 7). Methanol and acetonitrile were used for TLC analysis and sample preparation. Mobile phases for LC-MS were of HPLC grade and were purchased from VWR International (Leuwen, Belgium). Monobasic potassium phosphate and sodium phosphate dibasic were obtained from Sigma Aldrich (St. Louis, MO, USA), while acetic acid, ammonia, nitric acid, and potassium bromide were from Merck (Darmstadt, Germany). In all analytical steps, MQ water produced by Direct-Q3 water purification system was used (Millipore, Molsheim, France). Liquid chromatography tandem mass spectrometry (LC-MS/MS) Agilent Technologies 1260 HPLC and Triple Quad 6460 series was used for AFB1 quantification.

2.2. Sample Selection and Collection. Selection of spices samples was based on their large-scale use as edible food flavoring and preserving products and reported phytochemical compositions and ability to inhibit the effects of a wide range of chemical carcinogens [26–28]. A total of 81 (3 samples of each spices × 3 markets × 9 kinds of spices) spices samples were randomly collected from local retail traders around Hawassa city, Southern Nations, Nationalities, and Peoples Region of Ethiopia. Samples were collected during January to March 2017. The collected samples were made up of nine (×9 samples each) different kinds of spices (garlic (*Allium sativum* L.), ginger (*Zingiber officinale Roscoe*), black cumin (*Nigella sativa*), thyme (*Thymus vulgaris* L), fenugreek (*Trigonella foenum-graecum*), clove (*Syzygium aromaticum* L.), lemongrass (*Cymbopogon citrates* (DC.) Stapf.), sacred basil (*Ocimum santcum*), and lemon (*Citrus limon* (L.))) commercialized in three different markets (Aroge Gebeya, Addisu Gebeya, and Dato Gullit) near Hawassa city. The samples were randomly purchased by picking three samples of each spice from each market. Maize samples were collected from local farmers around Hawassa city. All samples were washed with distilled water and dried in an oven at 65°C for 72 hr. Then the dried material of each sample was ground to fine powder and stored in plastic bags at 4°C until the beginning of the analysis.

2.3. Screening and Preparation of Maize and Spice Extracts. Screening of the collected maize and spices samples for the presence or absence of AFs was carried out using TLC against AFs standards and according to the Association of Analytical Communities Official Method of Analysis (AOAC, 1995). Firstly, 25 gm of each of the spices and maize powder samples was added into a 250 ml Erlenmeyer flask. Then, 100 ml of methanol and water (85 : 15) was added, stoppered, and protected with foil. The mixture was vigorously shaken for 30 min and filtered through Whatman No. 1 filter paper. For cleanup, 20 ml of the filtrate solution was transferred into a 125 ml separating funnel containing 20 ml of 10% NaCl solution. Then, 12.5 ml of n-hexane was added and shaken for one minute. The phase was allowed to separate. The upper phase (organic) was discarded, and the lower phase (aqueous) was drained into the second 125 ml Erlenmeyer flask. To the aqueous phase, 12.5 ml of chloroform was added and shaken for one minute. Phases were allowed to separate. The lower phase was allowed to pass through a bed rock of sodium sulphate into the 250 ml Erlenmeyer flask, and the upper phase was discarded. After chloroform extraction, the extract was concentrated by evaporating on a steam bath and dissolved in 10 ml of methanol and shaken very well. Finally, screening was carried out by spotting the samples and AFB1 standard on TLC. Diethyl ether: methanol: water in 96 : 3 : 1 was used as the solvent system for plate development. The spots were visualized under a long wavelength UV lamp (365 nm) to determine the presence or absence of AFB1 in the samples based on the bluish fluorescent characteristics of the toxin. Only clean (not contaminated with aflatoxin) spices were considered for further experiments. However, the contaminated maize samples were considered for the treatments after the determination of initial loads of the toxin using LC/MS/MS. For preparation of aqueous extracts of the spices, 20 g of dry powder plant material from each plant species was soaked in sterile PBS (10 ml of 2 mM PBS/g of plant material) and kept on a shaker for 24 hr. Then, the mixtures were filtered through muslin cloth followed by Whatman No. 41 filter paper to remove leaf debris and obtain a clear filtrate, respectively. The filtrate was kept at 4°C and used for detoxification studies.

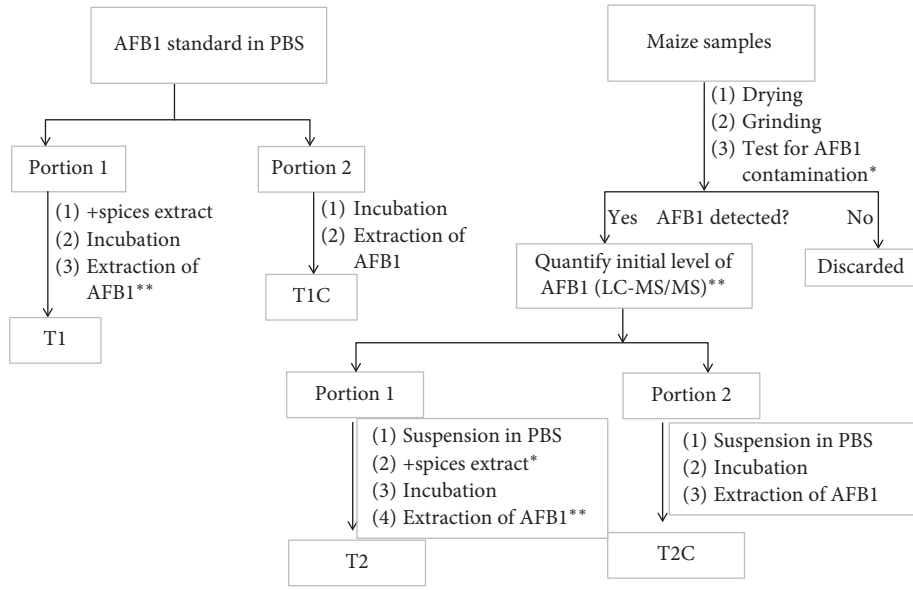
2.4. Testing Aflatoxin Degradation by Spice Extracts. Two types of samples, namely, standard AFB1 samples (T1) and maize samples naturally contaminated with AFB1 (T2), were considered for the treatment as shown in Figure 1. The latter samples are regarded herein as real samples. In the case of T1, 100 ng of AFB1 was added into 500 μl (50 mg/L crude) of plant extract in PBS in a microcentrifuge tube and incubated at 37°C for 24 h in an incubator. A similar procedure was used for real-sample treatment. Prior to the treatment, however, the contaminated maize samples were powdered, homogenized, and divided into two portions—portion 1 and portion 2. From both portions, nearly 17 gm was suspended in a 500 μl of plant extract and incubated under same conditions. After incubation, the aflatoxin in the mixture was extracted with same volume (500 μl) of chloroform. The chloroform fraction was evaporated on a heat block at 60°C, and the residue was dissolved in 10 μl of methanol and analyzed by LC-MS/MS. The mobile phase consisted of water: acetonitrile: methanol: fatty acid (60 : 25 : 15 : 0.1 v/v). The column temperature was maintained at 35°C. The total operation time was 20 min with the flow rate of 0.5 mL·min^{-1}. MS conditions were as follows: capillary current 450 nA, gas flow was 10 L·min^{-1}, and 50 ESI nebulizer used for ionization. The AFB1 levels were determined at the beginning ($t = 0$) and during ($t \# 0$) the exposure time. A brief outline of the overall procedure including the treatments is shown in Figure 1.

The efficacy of each spice extract was evaluated in terms of percent degradation of the toxin in treated (T1 and T2) vis-à-vis nontreated (T1C and T2C) samples using the following formula:

$$\% \text{ Degradation} = \frac{C_0 - C_t}{C_0} \times 100\%, \quad (1)$$

where C_0 is the concentration of AFB1 at the beginning (after mixing the samples with the extract) and C_t is the residual levels of AFB1 corrected for the matrix effect after incubation for a period of time t. Method validation was carried out by spiking known amounts of AFB1 standard in the powdered maize samples.

2.5. Electrochemical Characterization of Degradation. Electrochemical experiment was carried out using BAS CV 50W, which was connected to Dell Pentium personal computer. The standard buffer solution of pH = 12 has been used in this study. Glassy carbon (GC) as working electrode, Ag/AgCl as reference electrode, and platinum rod as counter



*Screening of maize samples for AFB1 contamination was carried out using TLC
**Extraction was carried out according to AOAC

Figure 1: A schematic of the overall procedure followed for the determination of efficacy of the spices against aflatoxin B1.

electrode were used for recording cyclic voltammetry (CV). The GC was polished with alumina suspensions and washed with water prior to measurement. CVs were recorded between 0 and 1.5 V at a scan rate of 50 mVs^{-1}, after incubation of 100 $\mu g{\cdot}L^{-1}$ AFB1 standards with garlic extract for 48 hr at 40°C.

2.6. Phytochemical Basis and Mechanistic Insights. Qualitative screening of various secondary metabolites was performed according to Ngbele [29].

2.6.1. Test for Alkaloid. One milliliter of 1% HCl was added to 3 ml of the extract in methanol in a test tube. The mixture was heated for 20 min, cooled, and filtered. Then, 1 ml of filtrate was tested in 0.5 ml of Dragendorff's reagent. Formation of reddish brown precipitate is marked as positive test.

2.6.2. Test for Flavonoids. Three milliliters of extract was poured to the beaker. A piece of magnesium ribbon and 3 drops of concentrated hydrochloric acid were added to the mixture. A red coloration indicated the presence of flavonoids.

2.6.3. Test for Phenols. To test the presence of phenol, 3 ml of extract was mixed with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

2.6.4. Test for Terpenoids. Five millimeters of the extract was dissolved in methanol with 2 mL of chloroform. Then, 3 mL of concentrated H_2SO_4 was added. A reddish brown coloration indicates positive result for the test.

2.7. Statistical Analysis. All measurements and assays in this study were carried out in triplicate. Statistical analysis of the data was carried out using Student's *t*-test and one-way ANOVA to test whether there is significant difference ($P = 0.05$) between the reduction in the level of AFB1 in the two or more treatments vis-à-vis the presence or absence of extract.

3. Results and Discussion

3.1. Characterization of AFB1 Degradation by the Extracts Using LC-MS/MS. For LC-MS/MS quantification, standard concentrations of AFB1 in the range of 1–10 $\mu g{\cdot}L^{-1}$ were used to construct the calibration curve. For LC-MS/MS quantification, a linear calibration curve with a regression equation of $y = 1244.80x$ and $R^2 = 0.997$ was observed using standard concentrations of AFB1 in the range of 1–10 $\mu g{\cdot}L^{-1}$. The validation of LC-MS/MS data was then carried out with determination of % recoveries and coefficient of variation (% CV). The recoveries were recorded by spiking powdered maize samples 3.5 ng/g of AFB1 standard and determining the recovered amount. Simultaneously, %CV corresponding to triplicate measurements was tabulated. The recorded % recovery ranged between 86.4 and 89.7% with %CV of 2.4%. The residual levels of AFB1 in T1 and T2 were determined in $\mu g{\cdot}L^{-1}$ and $\mu g{\cdot}kg^{-1}$, respectively. The initial level of AFB1 contamination in the contaminated maize samples was 7.47 μg AFB1/kg maize. This level is higher than the limits of the EU. It is interesting to see whether the treatments by the edible spice extracts can reduce this level to below permissible value. For the treatment, 200 $\mu g{\cdot}L^{-1}$ of standard AFB1 (T1)

and 250 μg AFB1/kg maize (nearly 17 gm of maize containing 7.47 μg AFB1/kg in 500 μL) (T2) were prepared in PBS. Figures 2(a) and 2(b) show the residual levels of AFB1 in the two treatments (T1 and T2) as a function of exposure time to 50 mg·L^{-1} and 50 mg·kg^{-1} of the extract, respectively. The reduction in AFB1 levels ranged from 6.5% to 35.8% (T1) and 17.3% to 46.7% (T2) after 30 min exposure at 25°C. Therefore, the 30 min exposure was sufficient to observe a significant ($P = 0.05$) difference between the treated and nontreated samples. Of the various spices, garlic showed the highest 35.8% (30 min) to 61.7% (1 hr) in T1 and 46.7% (30 min) to 68.3% (1 hr) in T2 reduction followed by lemon 34.2% (30 min) to 56.0% (1 hr) in T1 and 41.1% (30 min) to 60.6% (1 hr) in T2 at 25°C. In the current experiment, relatively the least reduction is observed with thyme. Nevertheless, all the studied extracts could reduce AFB1 levels to certain extents compared to nontreated samples after 24 hr exposure at 25°C. Therefore, garlic and lemon were considered for further study on the effect of concentration on the rate of AFB1 decrease. As expected, the rate of decrease of AFB1 levels in both T1 and T2 increased with the concentration of the spice extracts (Figures 2(c) and 2(d)). The efficacy of the extract varied significantly with the type of the spices. Also, a slight difference was observed between the efficacies of the spices in T1 and T2, which could be most likely due to the matrix effect.

This result suggested that dietary spices can be used to effectively control the level of AFB1 in food preparations. The current result is also consistent with the findings of previous studies [30, 31] in which the observed reductions of aflatoxin B1 contents of contaminated foods varied from 56% to 95%. Vijayanandraj et al. [32], showed 58.1, 55.8, 36.4, and 33.6% reduction of AFB1 by *Trigonella foenum-graecum* (fenugreek), *Curcuma longa, Syzygium aromaticum*, and *Ocimum basilicum*, respectively. Rastegar et al. [33] reported up to 93.1% reduction of AFB1 by the roasting process incorporating lemon juice and/or citric acid. Proctor et al. [31] reported that increased spice extract concentration, exposure time, and temperature significantly increased the rate of reduction of AFB1. The observed degradation of AFB1 by the extracts suggested the existence of water soluble phytochemicals like phenolics and alkaloids in the plant materials [27, 32]. The highest activity of garlic could thus be attributed to such chemicals and their relative abundance. More recently, Panda et al. [34] reported 74.7% and 70.2% reduction of AFB1 in vitro and in rice, respectively, through exposure to aqueous extract of *Ocimum tenuiflorum* (sacred basil) at high temperature (85°C/4 h). Direct detoxification was also reported by other authors [35, 36] who showed the detoxification of the toxin by allyl isothiocyanate from oriental mustard. As a result, the current findings are consistent with the existing literature and add values to it by lending credit to the indigenous knowledge on the use of dietary spices for aflatoxin control in line with their application to food flavoring and preservation.

3.2. Characterization of AFB1 Degradation by the Spices. Whether the observed decrease of AFB1 after treatment with spice extracts is due to adsorptive removal or degradation to a different compound is verified using LC/MS/MS. In this case, the fate of a characteristic peak on the chromatogram and molecular base ion in the mass spectrum specific to AFB1 is used as a basis to decipher the mechanism of AFB1 reduction by the spice extracts. Briefly, 100 ng of AFB1 standard was mixed with 100 ml of aqueous extract of garlic and incubated at 25°C. After overnight exposure, the AFB1 level in the mixture was analyzed by lc-ms/ms. Figures 3(a) and 3(b) show the selected ion chromatogram and molecular base ion peak in the mass spectrum, respectively, of AFB1 obtained before treatment and Figures 3(c) and 3(d) after treatment with aqueous extract of garlic. In the AFB1 mass spectrum, the most abundant fragment eluting at 7.5 min in the positive ionization mode was the protonated molecule $[M + H]^+$ at m/z 313 specific to AFB1 [37]. When AFB1 was incubated with the aqueous extract of garlic, the peak on the chromatogram and molecular base ion peak at m/z 313 specific for AFB1 had disappeared confirming almost complete degradation of AFB1 by the extract. Similar result has been reported by previous authors [32].

The aflatoxins toxicity data demonstrated that the presence of double bond in the terminal furan and lactone rings are key factors for the toxic and carcinogenic activities of AFB1. The disappearance of AFB1 in response to treatment by natural spice extracts thus indicated degradation of the toxin, and this could be most likely due to cleavage of the lactone ring to phenolic and acidic groups.

3.3. Electrochemical Characterization of AFB1 Degradation. The disappearance of characteristic peaks in the chromatogram and selected base ion peak in the mass spectrum led us to propose structural changes in the AFB1 occurring most likely in the lactone ring leading to phenolic and acidic groups. Since a degradation product bearing the phenolic group is electroactive unlike aflatoxin B1, it can serve as a basis for electrochemical characterization of the degradation. The cyclic voltammetric behavior of a solution of AFB1 standard in 0.1 M phosphate buffer (pH = 12) before and after treatment with citric acid and garlic extract was studied. When AFB1 in PBS was treated with citric acid, a discernible anodic peak (curve (B) in Figure 4(a)) was observed between 800 and 1000 mV. The absence of this peak in other control solutions (AFB1 in 0.1 M KNO_3 (A), citric acid in 0.1 M KNO_3 (C), and PBS (D)) suggested the peak to be attributed to the product of the treatment. Similarly, when AFB1 in PBS was treated with garlic extract anodic peak, though diminished, curve (C) in Figure 4(b)) was observed between in the same region. The negative scan did not show any peak. These results reinforced our hypothesis and confirmed the successful application of cyclic voltammetry for characterization of AFB1 detoxification.

The findings are in agreement with the reports by other authors. In 1976, Cucullu and his coworkers identified the ammoniation product of aflatoxin B1 as dihydro-4-hydroxy-6-methoxyfuro [38, 39] benzofuran and nonfluorescent phenol similar to aflatoxin D1 that lacks the cyclopentenone ring with a molecular weight of 206 [40]. The conversion of AFB1 into AFD1 in the presence of citric acid was also

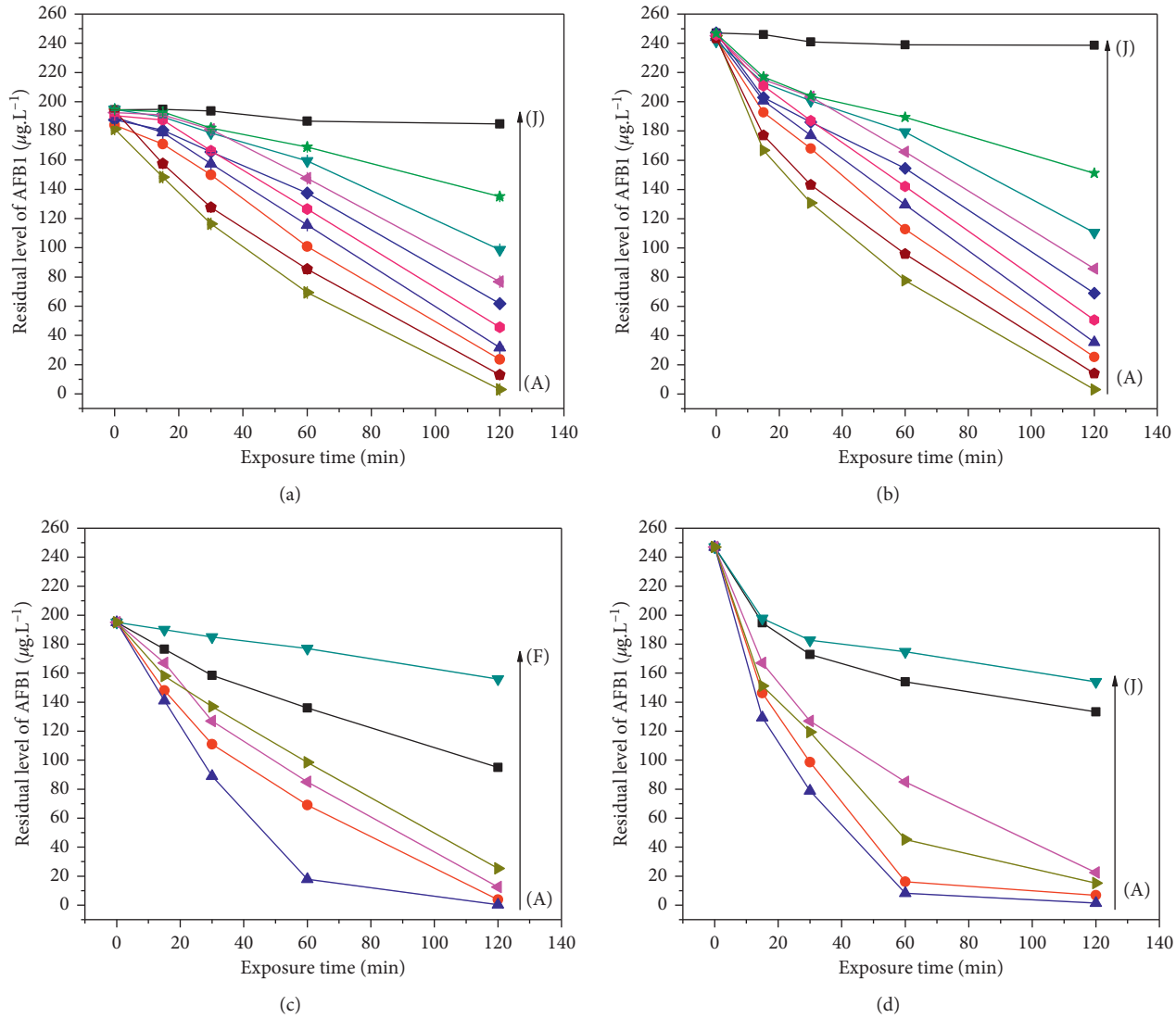


Figure 2: Effect of exposure time when the different aqueous extracts (A: garlic, B: lemon, C: sacred basil, D: black cumin, E: lemongrass, F: ginger, G: clove, H: fenugreek, I: thyme, and J: control) are incubated with standard AFB1 (a) and AFB1-contaminated maize sample (b); effect of concentration (A: 50 mg./L, B: 10 mg/L, and C: 1 mg/L garlic and D: 50 mg/L, E: 10 g/L, and F: 1 mg/L lemon) on standard (c) and AFB1-contaminated maize samples (d).

reported [41]. The reduced toxicity of AFD1 has been associated with the open lactone ring in AFD1 structure [42].

3.4. Correlation between Efficacy against AFB1 and the Aflatoxigenic Fungi. Most of the previous studies on the use of herbal, medicinal, and aromatic plants extracts for aflatoxin control focused on the ability to inhibit the growth of the principal aflatoxigenic fungi (*A. flavus* and *A. parasiticus*), and consequently, the production of aflatoxins in culture media and food products. In the present study, the spices were tested for their ability to degrade aflatoxin, not against its production by the aflatoxegenic fungi. We examined whether a good correlation exists between the efficacy of selected spices against aflatoxin (current study) and production of AFB1 by *A. flavus and A. parasiticus*. The result is shown in Table 1.

Although a good correlation can be observed with certain spices such as garlic, lemon, and ginger, it is difficult to draw a general conclusion. Thyme, for instance, was found to be the most efficient growth-inhibiting agent of the two fungi as reported by several authors [42], but it had the least activity in reducing AFB1 as observed in the current study.

3.5. Phytochemical Basis and Mechanistic Insights. In order to support the insights regarding molecular basis for the degradation of AFB1 by plant extracts, we conducted qualitative screening of the studied spices for classes of compounds they elaborate. The results are indicated in Table 2. The presence of the envisaged classes of compounds including alkaloids and other compounds containing amine or hydroxyl functional groups strongly supported the proposed mechanism that the degradation is attributed to the

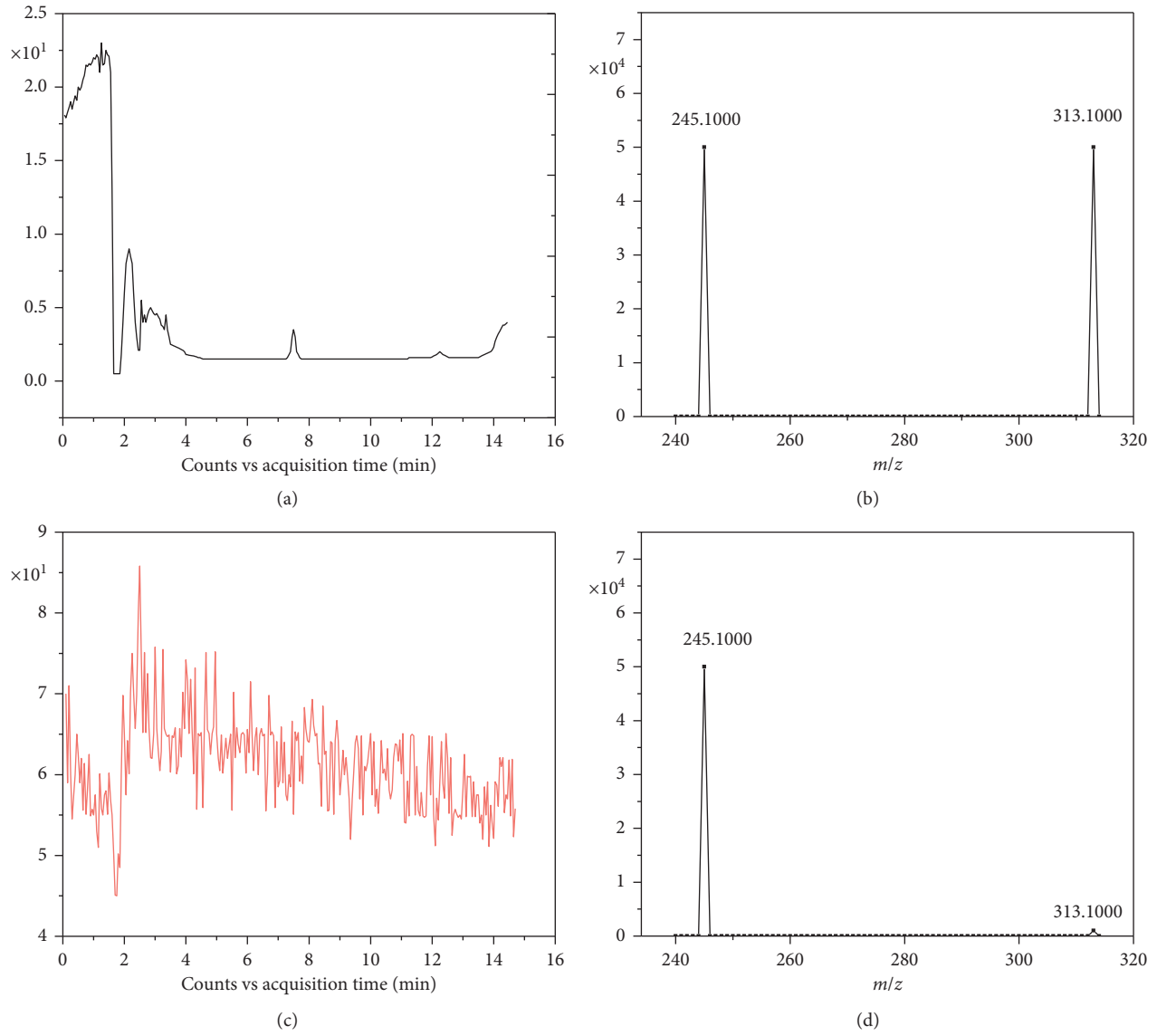


Figure 3: (a) Chromatogram of AFB1-contaminated sample before treatment, (b) mass spectrum of selected ion (molecular base ion peak) of AFB1 before treatment, (c) chromatogram of AFB1-contaminated sample after treatment with garlic extract for 24 h·min at 25°C, and (d) mass spectrum of selected ion (molecular base ion peak) of AFB1 after treatment.

presence of such water-soluble compounds in the plant extracts as active principles. Several authors reported a similar finding on detoxification of aflatoxin by plant products [32, 38, 47]. Vijayanandraj et al. [32] reported alkaloids as active principles for the observed detoxification of aflatoxin by the leaf extract of *A. vasica*. Alkaloids were suggested as active principles because other potential classes of compounds such as phenolics and terpenoids were not detected in the leaf extract. In other words, phenolics and terpenoids may also have the detoxification activity if present in the plant extract. Other studies on the degradation of aflatoxin by chemical agents revealed structural alterations in aflatoxin molecules after detoxification. Ciegler et al. [39, 48] observed hydroxydihydro-aflatoxin B1 as the product of detoxification. The same authors identified the ammoniation product of aflatoxin B1 as dihydro-4-hydroxy-6-methoxyfuro [2,3-b] benzofuran, a nonfluorescent phenol similar to aflatoxin D1 that lacks the cyclopentenone ring with a molecular weight of 206. Velazhahan et al. [38] suggested the modification of lactone ring structure of AFG1 as the mechanism of detoxification of aflatoxin G1 by the seed extract of ajwain (T. *ammi*). Other workers reported molecular breeding of crops with an ability to degrade the aflatoxin as an alternative strategy for the management of aflatoxin contamination in agricultural commodities [49, 50]. Molecular breeding of the natural dietary spicy plant can be even more interesting. Hence, it can be concluded that natural dietary spices can be used as alternative agents for degradation of aflatoxin.

4. Conclusions

The efficient degradation of AFB1 by aqueous extracts of selected natural dietary spices has been successfully

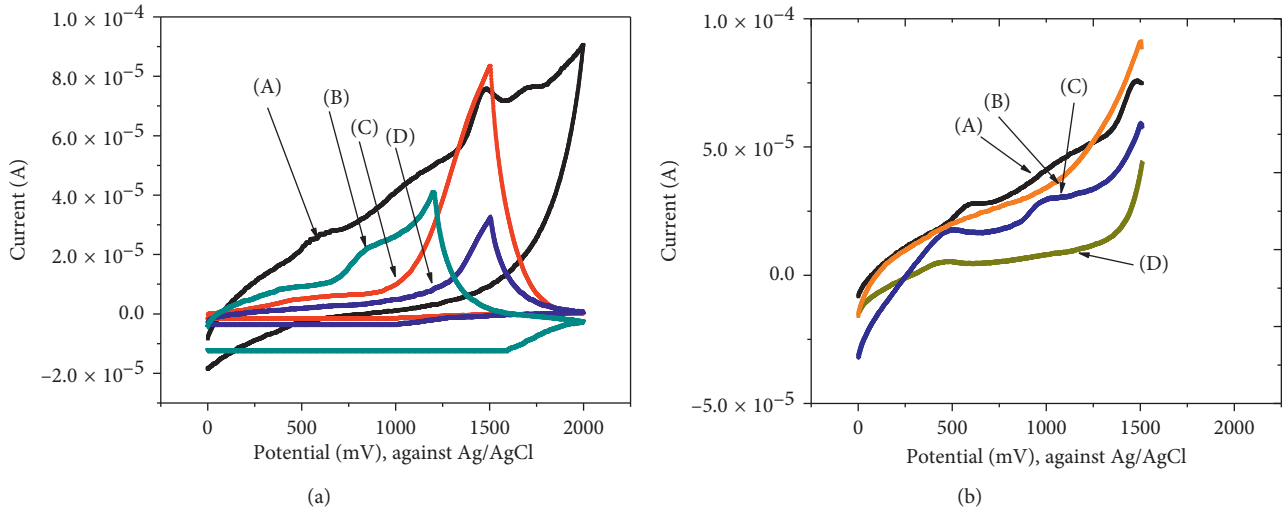


Figure 4: (a) Cyclic voltammogram of (A) AFB1 in 0.1 M KNO_3, (B) AFB1 incubated with citric acid for 60 min at 28°C, (C) citric acid in 0.1 M KNO_3, and (D) PBS (scan rate of 50 mV/s; phosphate buffer pH = 12). (b) Cyclic voltammogram of (A) AFB1 in methanol, (B) garlic extract in 0.1 M KNO_3, (C) AFB1 incubated with garlic extract for 60 min at 28°C, and (D) phosphate buffer (scan rate of 50 mV/s; phosphate buffer pH = 12).

Table 1: Comparison between different reported plant extracts for detoxification of aflatoxin with current work.

Plants name	Scientific name	Current result (% reduction of AFB1)	Reported result (inhibition of AFB1 production)	Reference
Cumin	*Cuminum cyminum* (L.)	—	89.0 (ELISA)	[32]
Clove	*Syzygium aromaticum* (L.)	49.54 (LC/MS/MS)	36.4 (ELISA)	[32]
Basil	*Ocimum basilicum*	—	90.14 (HPLC)	[32]
Lemongrass	*Cymbopogon citratus*	53.84 (LC/MS/MS)	91.5% (HPLC)	[43]
Lime	*Citrus aurantifolia* (L.)	—	66 (HPLC)	[44]
Lemon	*Citrus lemon* (L.)	90.02 (LC/MS/MS)	—	
Ajwain	*Trachyspermum ammi*	—	91.98 (ELISA)	[45]
Vasaka	*Adhatoda vasica* nees	—	98.3 (ELISA)	[41]
Garlic	*Allium sativum*	93.20 (LC/MS/MS)	54.2 (HPLC)	[46]
Thyme	*Thymus vulgaris*	51.22 (LC/MS/MS)	79.1 (HPLC)	[46]
Ginger	*Zingiber officinalis*	57.75 (LC/MS/MS)	79.1 (HPLC)	[46]
Black cumin	*Nigella sativa*	40.32 (LC/MS/MS)	—	
Fenugreek	*Trigonella foenum-graecum*	55.72 (LC/MS/MS)	—	

Table 2: Phytochemical test results of eight tested spices.

Spices	Garlic	Ginger	Clove	Fenugreek	Thyme	Black cumin	Lemon	Lemongrass	Sacred basil
Alkaloids	+	+	−	+	−	+	+	+	+
Flavonoids	−	+	+	−	+	−	+	+	+
Phenols	+	−	+	+	+	+	+	+	−
Terpenoids	+	+	+	+	−	−	−	+	+

demonstrated using LC-MS/MS. The mechanism of reduction of AFB1 by the spice extracts was investigated using LC-MS/MS and electrochemical methods. Among the studied spices, garlic showed the maximum (61.7%) degradation of AFB1 followed by lemon (56.0%) during 1 hr exposure of AFB1 standard to the spice extracts at 25°C. Chemical transformation of the toxin parent compound to another compound, most likely lacking lactone ring, is proposed as a possible mechanism of AFB1 degradation based on the results from LC-MS/MS and electrochemical characterization of the mechanism of AFB1 reduction by the plant extracts. The present study has thus lent scientific credit to the nonobvious benefits of indigenous knowledge of using natural spices for food flavoring and preservation to control aflatoxin. Further studies on the degradation products and structural elucidation of the active principle in the efficient spices and other similar plant products for aflatoxin reduction are needed.

Data Availability

The experimental data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] World Health Organization, International Agency for Research on Cancer, "Some naturally occurring substances: food items and constituents: heterocyclicaromatic amines and mycotoxins," *IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans*, Vol. 56, World Health Organization, International Agency for Research on Cancer, Lyon, France, 1993.

[2] L. S. Hong, N. M. Yusof, and H. M. Ling, "Determination of aflatoxins B1 and B2 in peanuts and corn based products," *Sains Malaysiana*, vol. 39, pp. 731–735, 2010.

[3] S. Ahsan, I. A. Bhatti, M. R. Asi, H. N. Bhatti, and M. A. Sheikh, "Occurrence of aflatoxins in maize grains from central areas of Punjab, Pakistan," *International Journal of Agriculture and Biology*, vol. 12, no. 4, pp. 571–575, 2010.

[4] WHO, World Health Organization, *Environmental Health Criteria, Safety Evaluation of Certain Food Additives*, WHO, World Health Organization, Geneva, Switzerland, 1979.

[5] G. N. Wogan, T. W. Kensler, and J. D. Groopman, "Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review," *Food Additives & Contaminants: Part A*, vol. 29, no. 2, pp. 249–257, 2012.

[6] S. H. Henry, F. X. Bosch, and J. C. Bowers, "Aflatoxin, hepatitis and worldwide liver cancer risks," in *Mycotoxins and Food Safety*, J. W. DeVries, M. W. Trucksess, and L. S. Jackson, Eds., vol. 504, pp. 229–233, Springer, Boston, MA, USA, 2002.

[7] J. H. Williams, T. D. Phillips, P. E. Jolly, J. K. Stiles, C. M. Jolly, and D. Aggarwal, "Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions," *American Journal of Clinical Nutrition*, vol. 80, no. 5, pp. 1106–1122, 2004.

[8] E. O. Farombi, "Aflatoxin contamination of foods in developing countries:implications for hepatocellular carcinoma and chemopreventive strategies," *African Journal of Biotechnology*, vol. 5, no. 1, pp. 1–14, 2006.

[9] X. Zhao, D. W. Schaffner, and T. Yue, "Quantification of aflatoxin risk associated with Chinese spices: point and probability risk assessments for aflatoxin B1," *Food Control*, vol. 33, no. 2, pp. 366–377, 2013.

[10] V. M. Raney, T. M. Harris, and M. P. Stone, "DNA conformation mediates aflatoxin B1-DNA binding and the formation of guanine N7 adducts by aflatoxin B1 8,9-exo-epoxide," *Chemical Research in Toxicology*, vol. 6, no. 1, pp. 64–68, 1993.

[11] C. Mollea and F. Bosco, "Mycotoxins: are they perceived as a seriousthreatfor human health," *Chemical Engineering Transactions*, vol. 44, pp. 133–138, 2015.

[12] K. A. Guindon, L. L. Bedard, and T. E. Massey, "Elevation of 8-hydroxydeoxyguanosine in DNA from isolated mouse lung cells following in vivo treatment with aflatoxin B1," *Toxicological Sciences*, vol. 98, no. 1, pp. 57–62, 2007.

[13] T. Shigute and A. P. Washe, "Reduction of aflatoxin M1 levels during Ethiopian traditional fermented milk *(Ergo)* production," *Journal of Food Quality*, vol. 2018, Article ID 4570238, 10 pages, 2018.

[14] National Grain and Feed Association, *FDA Mycotoxin Regulatory Guidance. A Guide for Grain Elevators, Feed Manufacturers, Grain Processors and Exporters*, National Grain and Feed Association, Arlington, VA, USA, 2011, http://www.ngfa.org.

[15] European Commission (EC), "Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs," *Official Journal of the European Communities*, vol. L364, p. 5e25, 2006.

[16] H. Fuffa and K. Urga, "Survey of aflatoxin contamination in Ethiopia," *Ethiopian Journal of Heals Sciences*, vol. 1, pp. 17–25, 2001.

[17] N. M. Chauhan, A. P. Washe, and T. Minota, "Fungal infection and aflatoxin contamination in maize collected from Gedeo zone, Ethiopia," *SpringerPlus*, vol. 5, no. 1, pp. 753–760, 2016.

[18] D. Gizachew, B. Szonyi, A. Tegegne, J. Hanson, and D. Grace, "Aflatoxin contamination of milk and dairy feeds in the Greater Addis Ababa milk shed, Ethiopia," *Food Control*, vol. 59, pp. 773–779, 2016.

[19] E. N. K. Sowley, "Aflatoxins: a silent threat in developing countries," *African Journal of Biotechnology*, vol. 15, no. 35, pp. 1864–1870, 2016.

[20] http://www.aflatoxinpartnership.org.

[21] B. Kabak, A. D. W. Dsoobn, and I. Var, "Strategies to prevent mycotoxin contamination of food and animal feed: a review," *Critical Reviews in Food Science and Nutrition*, vol. 46, no. 8, pp. 593–619, 2006.

[22] L. M. Martins, A. S. Sant'Ana, B. T. Iamanaka, M. I. Berto, J. I. Pitt, and M. H. Taniwaki, "Kinetics of aflatoxin degradation during peanut roasting," *Food Research International*, vol. 97, pp. 178–183, 2017.

[23] J. M. Wagacha and J. W. Muthomi, "Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies," *International Journal of Food Microbiology*, vol. 124, no. 1, pp. 1–12, 2008.

[24] F. B. Campagnollo, K. C. Ganev, A. Mousavi Khaneghah et al., "The occurrence and effect of unit operations for dairy-products processing on the fate of aflatoxin M1: a review," *Food Control*, vol. 68, pp. 310–329, 2016.

[25] S. Arzandeh and S. Jinap, "Effect of initial aflatoxin concentration, heating time and roasting temperature on aflatoxin reduction in contaminated peanuts and process optimisation using response surface modelling," *International Journal of Food Science and Technology*, vol. 46, no. 3, pp. 485–491, 2011.

[26] M. Jalili, "A review on aflatoxins reduction in food," *Iranian Journal of Health, Safety and Environment*, vol. 3, no. 1, pp. 445–459, 2015.

[27] A. V. Vipin, R. K. Raksha, N. K. Kurrey, K. A. AnuAppaiah, and G. Y. J. Venkateswaran, "Protective effects of phenolics rich extract of ginger against Aflatoxin B1-induced oxidative stress and hepatotoxicity," *Biomedicine and Pharmacotherapy*, vol. 91, pp. 415–424, 2017.

[28] Y. J. Surh, "Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review," *Food and Chemical Toxicology*, vol. 40, no. 8, pp. 1091–1097, 2002.

[29] J. Ngbele, R. A. Yakubu, and D. A. Nyam, "Phytochemical screening for active compounds in canariumschweinfurthii (atile) leaves from jos North, Plateau State Nigeria," *Research Journal of Biological Sciences*, vol. 11, no. 39, pp. 1076–1078, 2008.

[30] A. D. Prudente and J. M. King, "Efficacy and safety evaluation of ozonation to degrade aflatoxin in corn," *Journal of Food Science*, vol. 67, no. 8, pp. 2866–2872, 2002.

[31] T. Proctor, K. Hua Tan, and K. Fuse, "Cracking the incremental paradigm of Japanese creativity," *Creativity Innovation Management*, vol. 13, no. 4, pp. 207–215, 2004.

[32] S. Vijayanandraj, R. Brinda, K. Kannan et al., "Detoxification of aflatoxin B1 by an aqueous extract from leaves of *Adhatoda vasica* Nees," *Microbiological Research*, vol. 169, no. 4, pp. 294–300, 2014.

[33] H. Rastegar, S. Shoeibi, H. Yazdanpanah et al., "Removal of aflatoxin B1 by roasting with lemon juice and/or citric acid in contaminated pistachio nuts," *Food Control*, vol. 71, pp. 279–284, 2017.

[34] P. Panda, V. Aiko, and M. Alka, "Effect of aqueous extracts of *Menthaarvensis* (mint) and Piper betle (betel) on growth and citrinin production from toxigenic *Penicilliumcitrinum*," *Journal of Food Science and Technology*, vol. 52, no. 6, pp. 3466–3474, 2015.

[35] G. M. Azaiez, L. Manyes, and M. Fernández-Franzón, "Antifungal activity of gaseous allyl, benzyl and phenyl isothiocyanates in vitro and their use for *fumonisins* reduction in bread," *Food Control*, vol. 32, no. 2, pp. 428–434, 2013.

[36] G. Meca, F. B. Luciano, T. Zhou, R. Tsao, and J. Mañes, "Chemical reduction of the mycotoxin beauvericin using allyl isothiocyanate," *Food and Chemical Toxicology*, vol. 50, pp. 1755–1762, 2012.

[37] R. W. Detroy and C. W. Hesseltine, "Aflatoxicol: structure of a New transformation product of aflatoxin B1," *Canadian Journal of Biochemistry*, vol. 48, no. 7, pp. 830–832, 1970.

[38] R. Velazhahan, S. Vijayanandraj, A. Vijayasamundeeswari et al., "Detoxification of aflatoxins by seed extracts of the medicinal plant, *Trachyspermumammi* (L.) Sprague ex turrill–structural analysis and biological toxicity of degradation product of aflatoxin G1," *Food Control*, vol. 21, no. 5, pp. 719–725, 2010.

[39] A. F. Cucullu, L. S. Lee, W. A. J. Pons, and J. B. Stanley, "Ammoniation of aflatoxin B1: isolation and characterization of a product with molecular weight 206," *Journal of Agricultural and Food Chemistry*, vol. 24, no. 2, pp. 408–410, 1976.

[40] M. Jemmali, "Decontamination and detoxification of mycotoxins," *Pure and Applied Chemistry*, vol. 52, no. 1, pp. 175–181, 1979.

[41] S. S. Hajare, S. H. Hajare, and A. Sharma, "Aflatoxin inactivation using aqueous extract of ajowan (*Trachyspermumammi*) seeds," *Journal Food Science*, vol. 70, no. 1, pp. 29–34, 2005.

[42] A. Kumar, R. Shukla, P. Singh, C. S. Prasad, and N. K. Dubey, "Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post-harvest fungal infestation of food commodities," *Innovative Food Science and Emerging Technologies*, vol. 9, no. 4, pp. 575–580, 2008.

[43] W. Iram, T. Anjum, M. Iqbal, A. Ghaffar, M. Abbas, and A. M. Khan, "Structural analysis and biological toxicity of aflatoxins B1 and B2 degradation products following detoxification by *Ocimum basilicum* and *Cassia fistula* aqueous extracts," *Frontiers in Microbiology*, vol. 7, p. 1105, 2016.

[44] A. Janardhan, K. A. Praveen, Y. Sivaprasad, G. D. V. R. Sai, and D. Subramanyam, "Inhibition of *Aspergillus flavus* growth and detoxification of aflatoxin B1 by the medicinal plant leaf extract-*cymbopogoncitratus*," *International Journal of Clinical and Biological Sciences*, vol. 1, no. 1, pp. 11–23, 2016.

[45] E. B. M. Kabbashi, S. E. A. Ali, and N. A. Abdelrahman, "Lime (*Citrus aurantifolia* L.) juice a potent treatment for the virulent hepatocarcinogen aflatoxin B1 in peanut paste," *Journal of Molecular Pathophysiology*, vol. 5, no. 2, pp. 18–22, 2016.

[46] A. R. M. Abd El-Aziz, M. R. Al-Othman, S. A. Al-Sohaibani, M. A. Mahmoud, and K. Murugan, "Prevention of aflatoxin contamination of maize by *Aspergillus flavus* through aqueous plant extracts in Saudi Arabia," *African Journal of Microbiology Research*, vol. 6, no. 41, pp. 6931–6935, 2012.

[47] R. Sandosskumar, M. Karthikeyan, S. Mathiyazhagan, M. Mohankumar, G. Chandrasekar, and R. Velazhahan, "Inhibition of *Aspergillus flavus* growth and detoxification of aflatoxin B1 by the medicinal plant zimmu (*Allium sativum* L. × *Allium cepa* L.)," *World Journal of Microbiology and Biotechnology*, vol. 23, no. 7, pp. 1007–1014, 2007.

[48] A. Ciegler and R. E. Peterson, "Aflatoxin detoxification: hydroxydihydro-aflatoxin B1," *Journal of Applied Microbiology*, vol. 16, pp. 665-666, 1968.

[49] B. Poppenberger, F. Berthiller, D. Lucyshyn et al., "Detoxification of the Fusarium mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*," *Journal of Biological Chemistry*, vol. 278, no. 48, pp. 47905–47914, 2003.

[50] N. Takahashi-Ando, S. Ohsato, T. Shibata, H. Hamamoto, I. Yamaguchi, and M. Kimura, "Metabolism of zearalenone by genetically modified organisms expressing the detoxification gene from *Clonostachys rosea*," *Applied and Environmental Microbiology*, vol. 70, no. 6, pp. 3239–3245, 2004.

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Research Article

Methods for Extraction of Muscle Proteins from Meat and Fish Using Denaturing and Nondenaturing Solutions

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The objective of the present study was to test two extraction methods including solutions with different ionic strengths on the extraction and separation of the myofibrillar proteins from meat and fish muscles of different species. Meat samples from *longissimus thoracis* muscle of beef and lamb, *pectoralis major* muscle of chicken, and dorsal white muscle of fish from sole (*Solea solea*), European hake (*Merluccius merluccius*), and sea bass (*Dicentrarchus labrax*) were analyzed. The extraction method using nondenaturing solution led to a major extraction of high molecular-weight proteins as myosin heavy chain, α-actinin, and desmin; on the contrary, the denaturing method provided a good protein extractability of proteins and fragments with low molecular-weight as actin, troponin-T, tropomyosin, and myosin light chain 1 and 2 proteins for the most meat and fish samples. The nondenaturing extraction method showed several advantages resulting in time and labour saving and in minimizing the use of toxic and polluting agents.

1. Introduction

Muscle proteins are grouped into three categories based on location in the skeletal muscle and solubility as sarcoplasmic, stromal, and myofibrillar proteins. Myofibrillar proteins are the main component of the skeletal muscle accounting for about 50% of total proteins and are mainly constituted by myosin and actin, involved in muscle contraction. Due to their structure and localization [1], myofibrillar proteins require denaturing conditions, e.g., high ionic strength solution to be solubilized and extracted [2]. Sarcoplasmic proteins, localized in the sarcoplasm of the muscle fibers, are recognized to be soluble in water or in solutions of low ionic strength, whereas stromal proteins, such as collagen and elastin, are reported to remain insoluble in high-salt solutions [3].

Proteomics techniques have been extensively applied to separate, characterize, and identify proteins in animal food products [4, 5]. Sample preparation and extraction are the most crucial steps in the electrophoretic analysis for obtaining reliable results [6]. The choice of the extraction method of muscle proteins is essential for obtaining samples with high protein concentration and free of salt and other disturbing factors, such as lipids, that could interfere with the electrophoretic analysis. The most commonly used procedures for myofibrillar protein extraction involves denaturing solutions containing urea, thiourea, reducing agents (DTT, beta-mercaptoethanol), detergents (SDS, sodium dodecyl sulfate), and salts [7, 8]. However, it has to be considered that the use of these reagents is regarded as toxic and highly polluting and require proper disposer procedures. Chen et al. [9] reported the use of water or low ionic strength media for the extraction and solubilization of myofibrillar proteins from skeletal muscle. To the best of our knowledge, no research is available on the comparison of the extraction capacity of denaturing and nondenaturing solutions. In the light of this consideration, the objective of the present study was to provide a comparison between two methods for the extraction and separation of myofibrillar proteins including solutions with different ionic strength in meat and fish muscles.

2. Materials and Methods

2.1. Chemicals and Reagents. All reagents used in the experiment were of analytical grade. Potassium chloride, disodium phosphate, monopotassium phosphate, urea, thiourea, dithiothreitol, cholamidopropyl dimethyl hydroxy propanesulfonate (CHAPS), IGEPAL® CA-630 NP 40, glycerol, and tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acrylamide, bis-acrylamide, ammonium persulfate (APS), N,N,N,N-Tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), tris(hydroxymethyl)-aminomethane, glycine, bromophenol blue, β-mercaptoethanol, and Coomassie Brilliant Blue G-250 were purchased from Bio-Rad Laboratories (Hercules, CA).

Phosphate buffer (pH 7, 0.003 M), KCl phosphate buffer (pH 7.5), and Tris-HCl (pH 8, 20 mM) were freshly prepared. Ultrapure water was obtained in the laboratory using a Water Purification System Barnstead™ Pacific TII (ThermoFisher Scientific, USA).

2.2. Samples Collection and Preparation. Meat samples from *longissimus thoracis* muscle of beef and lamb; *pectoralis major* muscle of chicken; and dorsal white muscle of fish from sole (*Solea solea*), European hake (*Merluccius merluccius*), and sea bass (*Dicentrarchus labrax*) were purchased from a local market and immediately transferred under refrigeration to the laboratory. For each species, a total of fifteen animals were included in the experiment. Adipose and connective tissues were removed from meat samples, while bones, scales, and fat were discarded from fish samples. All fresh samples were finely minced prior to protein extractions.

2.3. Protein Extraction Methods. The flowchart of the extraction of muscle protein fractions from different species analyzed is shown in Figure 1. Meat and fish proteins were fractionated based on different solubility. Samples were homogenized with 0.03 M phosphate buffer (pH 7) containing a protease inhibitors cocktail (P2714, Sigma-Aldrich, St. Louis, MO) on ice for 2 min using an Ultra-Turrax T18 basic (IKA, Wilmington, Germany). The homogenate was centrifuged at 8,000 $\times g$ (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) for 20 min at 4°C. After centrifugation, the supernatant (sarcoplasmic proteins) was discarded, and the extraction of myofibrillar proteins were obtained as follows.

Two different extraction methods were carried out for myofibrillar proteins using denaturing and nondenaturing solutions. The extraction of myofibrillar proteins with nondenaturing solution is based on the method reported by Hashimoto et al. [10] with the modifications reported as follows: the pellet recovered was resuspended in 10 volumes of KCl phosphate buffer pH 7.5 (0.45 M KCl, 15.6 mM Na_2HPO_4, 3.5 mM KH_2PO_4) and vortexed for 2 min. The vortexing step was introduced to optimize the homogenization of the pellet and to prevent the formation of a mellow complex. The mixture was centrifuged twice at 5,000 $\times g$ (Eppendorf 5810R, Eppendorf AG, Germany) for 15 min at 4°C. After centrifugation, the supernatant containing the myofibrillar proteins was recovered, aliquoted, and frozen at −80°C.

For comparison, myofibrillar proteins were extracted using denaturing solution according to Marino et al. [11]. Briefly, the pellet was resuspended in a solution (8.3 M urea, 2 M thiourea, 64 mM dithiothreitol (DTT), CHAPS 2% (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate), IGEPAL 2%, glycerol 10%, and 20 mM Tris-HCl, pH 8) and incubated overnight at 4°C in an orbital shaker. Subsequently, samples were centrifuged at 15,000 $\times g$ (Eppendorf 5810R, Eppendorf AG, Germany) for 20 min at 10°C. After centrifugation, the supernatant containing myofibrillar proteins was recovered, aliquoted, and frozen at −80°C until further protein analysis to avoid calpain protease activation.

For each species, all myofibrillar extracts obtained with the different methods were quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Absorbance was measured at 580 nm by the spectrophotometric assay (Power Wave XS, Biotek, UK), with a bovine serum albumin (BSA; >98% pure, Sigma-Aldrich) standard curve.

2.4. SDS-PAGE Analysis. The fifteen myofibrillar extracts of each species obtained by the denaturing or nondenaturing method were pooled and resolved by SDS-polyacrylamide gel electrophoresis in a gradient gel 8–18% [11]. Gels were loaded with 50 μg of proteins and run with a Protean II xi vertical slab gel unit (Bio-Rad Laboratories, Hercules, CA). Coomassie Brilliant Blue G-250 was used to visualize bands of interest. Gels were destained in an aqueous solution of acetic acid and methanol (10% v/v, and 7% v/v, respectively) and acquired by the ChemiDoc EQ system (Bio-Rad Laboratories, Hercules, CA). The relative quantity of each band was determined as percentage of the signal intensity of the defined band in a lane with the Quantity One software (Bio-Rad Laboratories, Hercules, CA). Identification of the protein molecular weight was done by comparison with the precision plus protein standard-broad range (Bio-Rad Laboratories, Hercules, CA).

2.5. Statistical Analysis. Protein concentration and electrophoretic data were analyzed using the GLM procedure of the SAS statistical software [12]. The tested effect was the extraction methods on the myofibrillar fraction of muscle proteins from beef, lamb, chicken, sole, hake, and sea bass. When significant differences were found (at $P < 0.05$), the Student t-test was used to locate significant differences among means.

3. Results and Discussion

3.1. Protein Extractability. Solubility is an indicator of protein extractability; indeed, a solubilized protein could be easily extracted into a solution from muscle fibers or myofibrils [9]. The amount of myofibrillar proteins extracted using denaturing and nondenaturing solutions from beef,

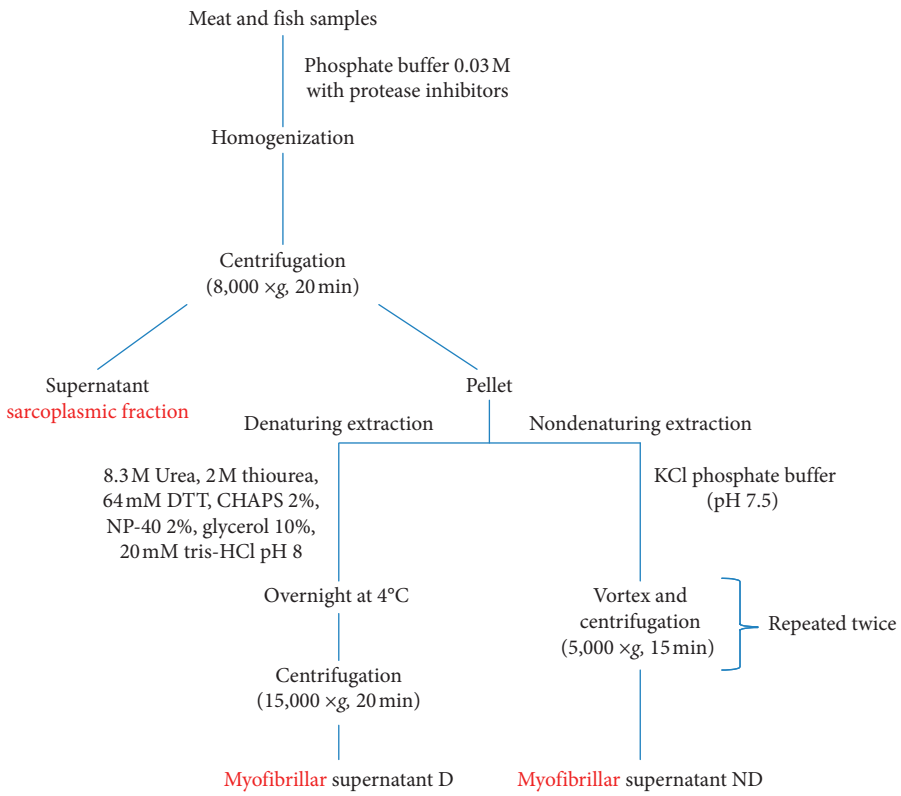

Figure 1: Flowchart of the extraction of sarcoplasmic and myofibrillar protein fractions from different species.

lamb, chicken, sole, European hake, and sea bass is reported in Figure 2. No differences were found in the protein extractability of beef, European hake, and sea bass when the different extraction methods were tested, evidencing that nondenaturing extraction method led to successful protein extraction as denaturing extraction method. The physical force applied by repeated centrifugations damaged the structures of myofibrillar proteins partly allowing the dissolution of myofibrillar proteins in water.

On the contrary, the extraction capacity of the denaturing solution seemed to be more efficient in lamb, chicken, and sole with an amount of myofibrillar proteins extracted of about 30% in lamb and of about 10% in chicken and sole higher than nondenaturing solution. It is known that the extraction of protein from skeletal muscles is a complex phenomenon that is influenced by the parameters of the extraction, by the tissue structure, and by the *post-mortem* changes that occur during the transformation of muscle [13]. The greater extractability of myofibrillar proteins by the denaturing solution in lamb, chicken, and sole samples could be due to the type and structure of muscle [14], suggesting that the power of solubilization could be species-specific.

3.2. Myofibrillar Fraction. The densitometric profile and SDS-PAGE of myofibrillar fraction extracted with denaturing and nondenaturing solutions from meat and fish species are showed in Figures 3 and 4, respectively. Both extraction methods provided an adequate separation of

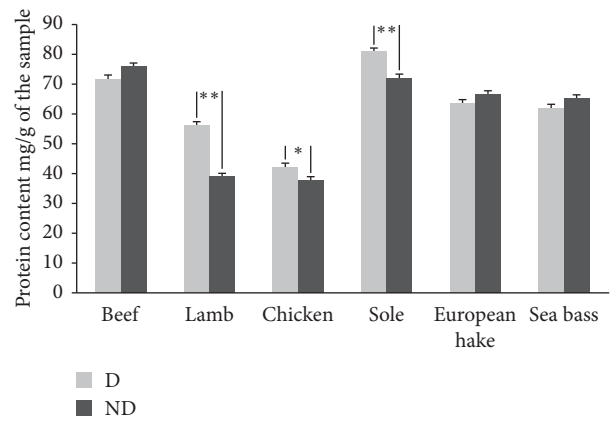

Figure 2: Protein solubility of myofibrillar proteins from the muscle of beef, lamb, chicken, sole, European hake, and sea bass using denaturing and nondenaturing solution (D = denaturing; ND = nondenaturing; $^{*}P < 0.05$; $^{**}P < 0.01$) (means ± SEM).

myofibrillar proteins and derived fragments as showed by the electrophoretic profile with well-defined bands and the absence of any contaminants (e.g., lipids). In meat samples, the main protein bands identified in the range of molecular weights from 250 to 10 kDa were myosin heavy chain (MHC), α-actinin (α-act), desmin, actin (ACT), troponin T (TnT), tropomyosin (TPM), myosin light chains 1 (MLC1), troponin C (TnC), and myosin light chains 2 (MLC2). On the contrary, the electrophoretic profile of fish samples revealed the absence of troponin complex. However, all

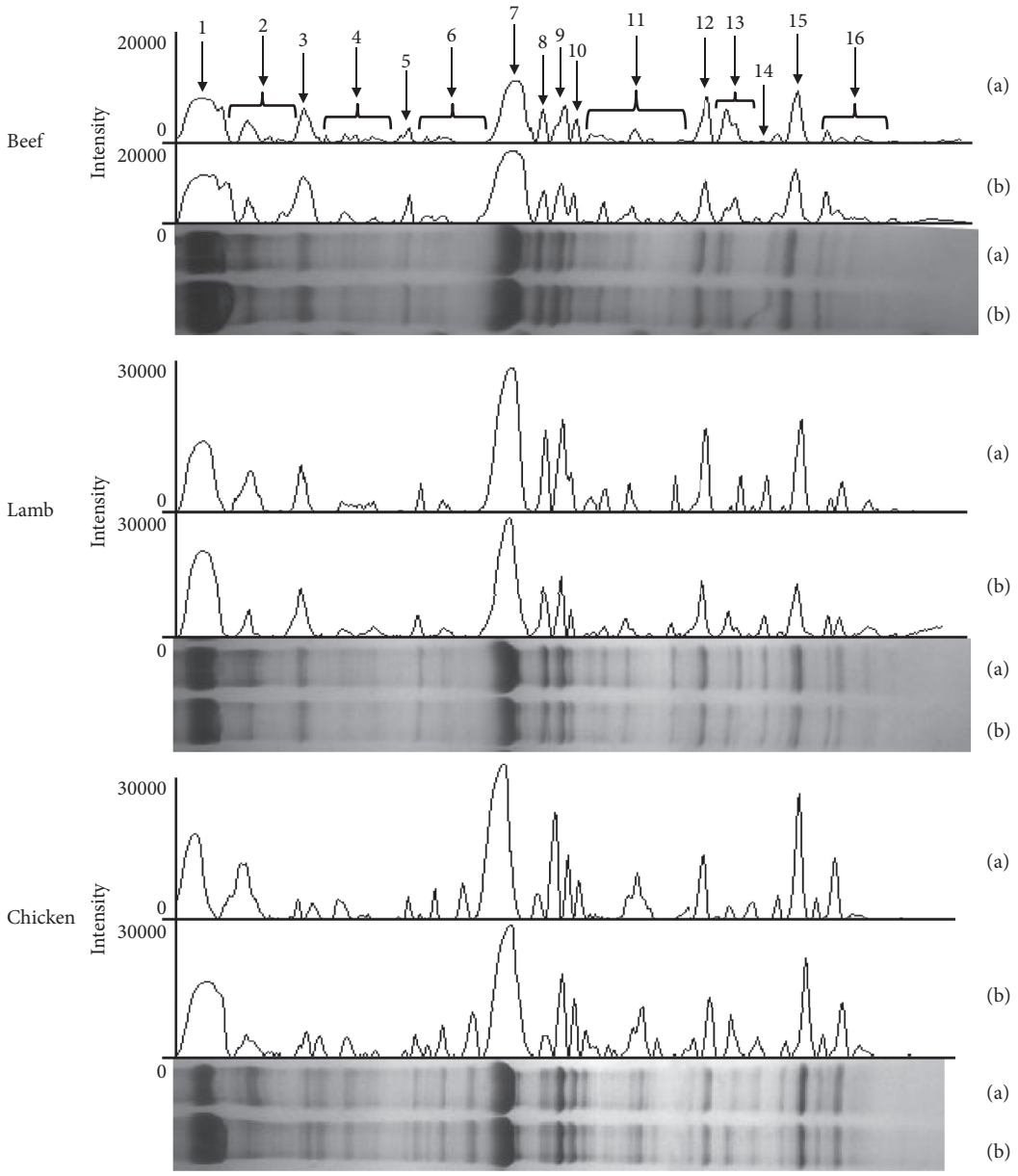

Figure 3: Densitometric profile and SDS-PAGE of a pool of 15 myofibrillar extracts obtained from beef, lamb, and chicken samples extracted with denaturing (a) and nondenaturing (b) solutions (1 = myosin heavy chain; 2 = 180–110 kDa; 3 = α-actinin; 4 = 95–55 kDa; 5 = desmin; 6 = 51–47 kDa; 7 = actin; 8 = 39 kDa; 9 = troponin T; 10 = tropomyosin; 11 = 33–23 kDa; 12 = myosin light chain 1; 13 = 21–18 kDa; 14 = troponin C; 15 = myosin light chain 2; 16 = 14–10 kDa).

species analyzed showed protein fragments in the molecular weight ranging from 180 to 110 kDa, from 95 to 55 kDa, from 51 to 47 kDa, from 40 to 38 kDa, from 33 to 23 kDa, from 21 to 18 kDa, and from 14 to 10 kDa and bands at 39 and 16 kDa.

The densitometric profile of SDS-PAGE revealed that the use of the denaturing solution led to a more complex profile in terms of number of bands and fragments extracted (30, 32, and 32 vs 26, 27, and 28 bands in the nondenaturing profile of meat samples and 35, 29, and 30 vs 31, 26, and 28 bands in the nondenaturing profile of fish samples) while the use of the nondenaturing solution revealed a major intensity for most of the myofibrillar protein analyzed.

The percentage of the main myofibrillar proteins extracted using nondenaturing and denaturing solutions from meat and fish species are reported in Figures 5 and 6, respectively. All samples extracted with the nondenaturing solution showed the highest values of MHC ($P < 0.001$ in beef, lamb, chicken, sole, and European hake; $P < 0.01$ in sea bass), α-actinin ($P < 0.001$ in lamb; $P < 0.01$ in beef, chicken, and sole; $P < 0.05$ in European hake), and desmin ($P < 0.001$ in beef; $P < 0.01$ in sea bass; $P < 0.05$ in lamb, not detected in European hake).

It is known that myosin mainly contributes to the tensile strength of the muscle, while α-actinin and desmin are cytoskeletal proteins responsible for the maintenance of

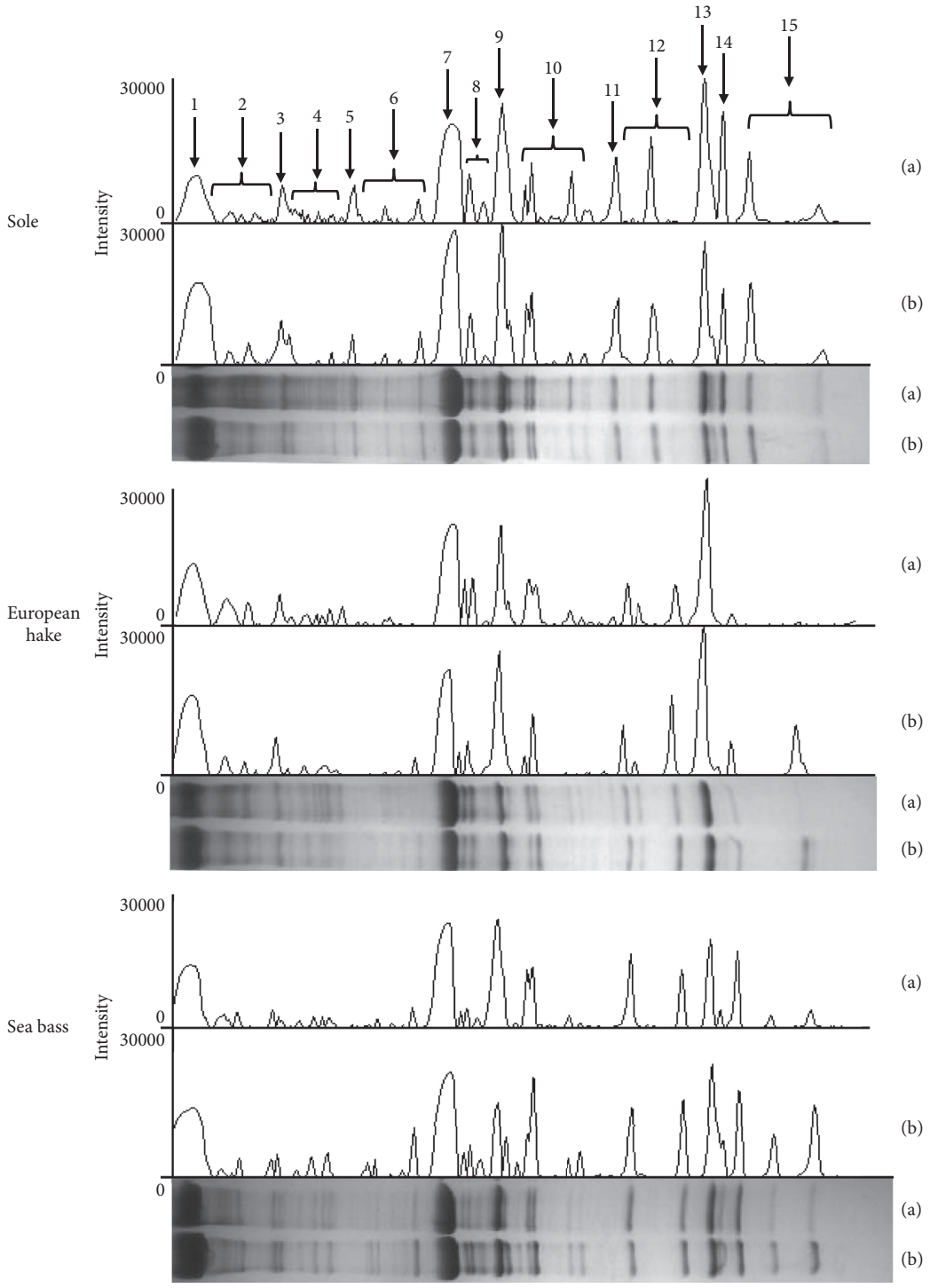

Figure 4: Densitometric profile and SDS-PAGE of a pool of 15 myofibrillar extracts obtained from sole, European hake, and sea bass samples extracted with denaturing (a) and nondenaturing (b) solutions (1 = myosin heavy chain; 2 = 180–110 kDa; 3 = α-actinin; 4 = 95–55 kDa; 5 = desmin; 6 = 51–47 kDa; 7 = actin; 8 = 40–38 kDa; 9 = tropomyosin; 10 = 33–23 kDa; 11 = myosin light chain 1; 12 = 21–18 kDa; 13 = 16 kDa; 14 = myosin light chain 2; 15 = 14–10 kDa).

structural and mechanical integrity of actin filaments in the Z-disk [15]. In any case, whether the reduced relative quantity of all these proteins is due to proteolysis, denaturation, or a combination of both, desmin has also been considered a marker of freshness in some fish species [16]. In the present study, the use of a solution with low ionic strength, in the nondenaturing extraction method, led to a major extraction of these proteins with high molecular weight.

The pH value of the solution in the salt-soluble method seemed to be favourable for protein extraction. Accordingly, Chen et al. [2] reported a greater solubilization of myosin

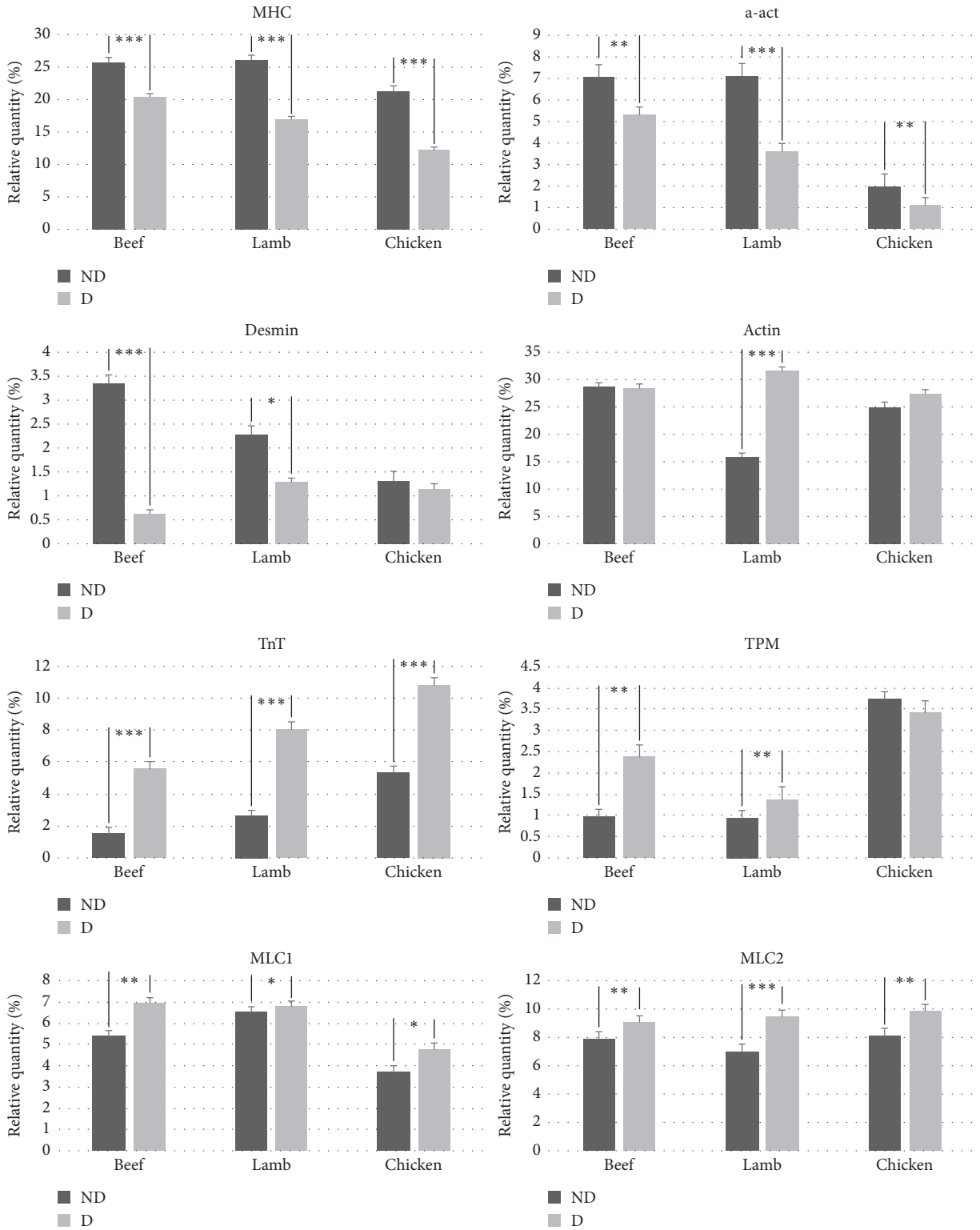

Figure 5: Percentage of the main myofibrillar proteins from beef, lamb, and chicken samples extracted with denaturing and nondenaturing solutions (D = denaturing; ND = nondenaturing; MHC = myosin heavy chain; a-act = α-actinin; TnT = troponin T; TPM = tropomyosin; MLC1 = myosin light chain 1; MLC2 = myosin light chain 2; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) (means ± SEM).

using a 0.6 M KCl solution pH 6.0 due to myosin filament dissociation induced by low ionic strength of the buffer solution. The reduction of salt content in the nondenaturing solution could have led to a modification of the physiological conditions of protein due to change of pH improving the solubility of proteins with high molecular weight.

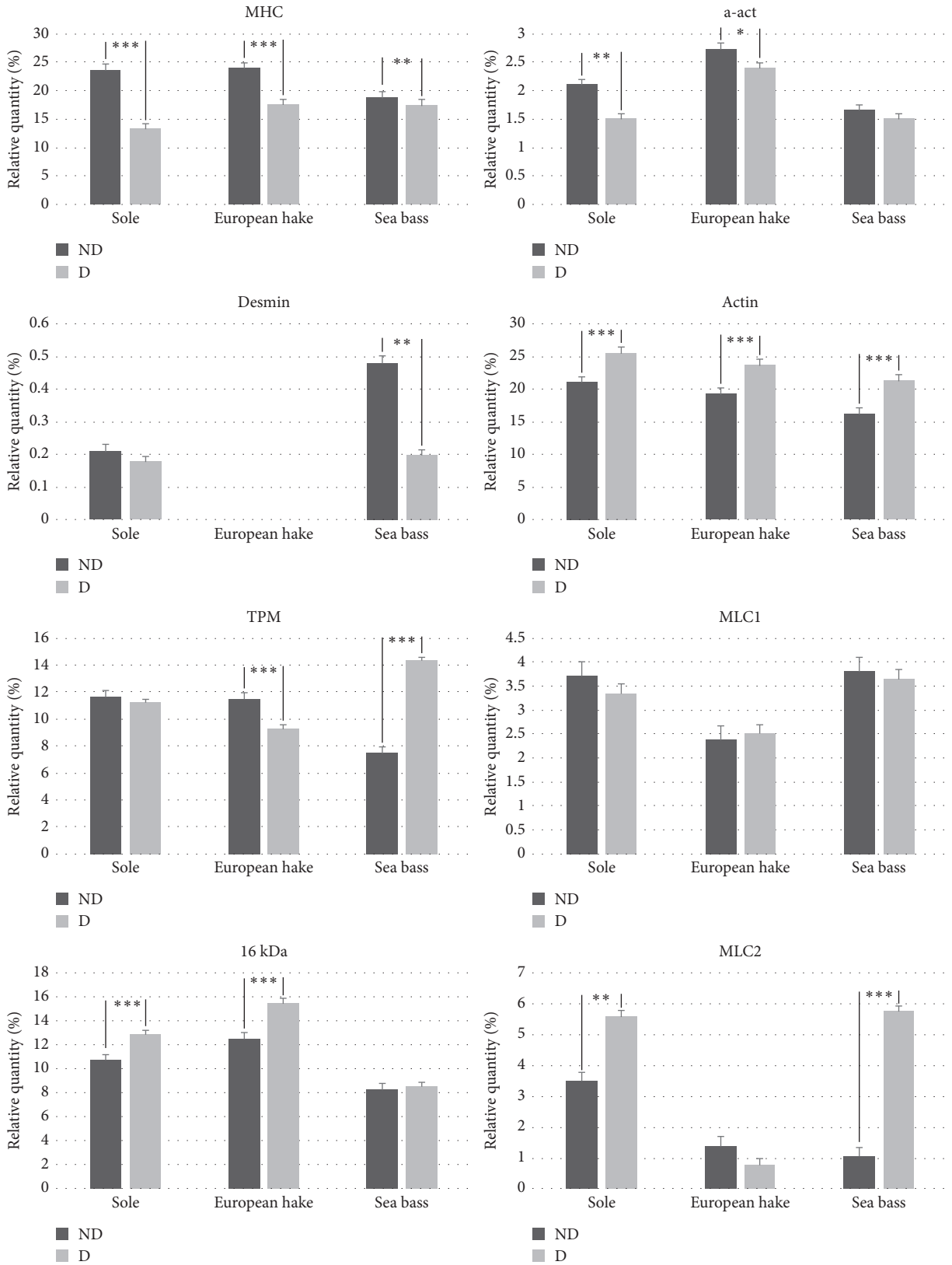

Figure 6: Percentage of the main myofibrillar proteins from sole, European hake, and sea bass samples extracted with denaturing and nondenaturing solutions (D = denaturing; ND = nondenaturing; MHC = myosin heavy chain; a-act = α-actinin; TPM = tropomyosin; MLC1 = myosin light chain 1; MLC2 = myosin light chain 2; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) (means ± SEM).

The use of denaturing solutions led to a major extractability of myofibrillar proteins with low molecular weight (under 45 kDa) as actin ($P < 0.001$ in lamb, European hake, and sea bass), troponin T ($P < 0.01$ in beef, lamb and chicken, European hake, and sea bass), tropomyosin ($P < 0.01$ in beef and lamb; $P < 0.001$ in European hake and sea bass), MLC1 ($P < 0.01$ in beef and $P < 0.05$ in lamb and chicken), and MLC2 ($P < 0.001$ in lamb and sea bass; $P < 0.01$ in beef, chicken, and sole) proteins. No significant differences were found between the two extraction methods in MLC1 of fish samples.

These results could be due to the compounds such as urea, thiourea, CHAPS, and DTT of the denaturing solution. It is known that urea is a chaotropic agent, efficient in the rupture of hydrogen bonds, denaturing proteins by breaking the noncovalent and ionic links between amino-acid residues [17]. Thiourea, indeed, breaks hydrophobic interactions leading to an increase in the solubilization of membrane proteins [18]. Previous studies [19, 20] reported that the combination of urea and thiourea exhibit a superior solubilizing power and increase dramatically the extraction of proteins. In addition, CHAPS and DTT affect protein solubilization because it prevents hydrophobic interaction and promote the reoxidation of disulphide bonds avoiding the lack of proteins by aggregation or precipitation [21]. The presence of denaturing compounds in the extraction solution led to an increase of extraction of myofibrillar proteins with low molecular weight probably due to differences in protein molecular size, conformation, and inter- and intramolecular bonds, resulting in more sensitivity to the strength of extraction of the denaturation method.

4. Conclusion

Nondenaturing and denaturing extraction methods were efficient to solubilize the main muscle proteins. Proteomic analysis revealed a good separation of proteins with well-defined bands without any contamination for all samples analyzed. The extraction method using nondenaturing solution lead to a major extraction of myofibrillar proteins with high molecular weight; on the contrary, the denaturing method provided good extractability of proteins and fragments with low molecular weight for the most meat and fish samples.

The nondenaturing extraction method showed several advantages such as easy to carry out, less invasive, and minimal use of toxic and polluting agents.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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References

[1] Y. Au, "The muscle ultrastructure: a structural perspective of the sarcomere," *Cellular and Molecular Life Sciences*, vol. 61, no. 24, pp. 3016–3033, 2004.

[2] X. Chen, Y. F. Zou, M. Y. Han et al., "Solubilization of myosin in a solution of low ionic strength L-histidine: significance of the imidazole ring," *Food Chemistry*, vol. 196, pp. 42–49, 2016.

[3] A. Listrat, B. Lebret, I. Louveau et al., "How muscle structure and composition influence meat and flesh quality," *The Scientific World Journal*, vol. 2016, Article ID 3182746, 14 pages, 2016.

[4] J. Z. Han and Y. B. Wang, "Proteomics: present and future in food science and technology," *Trends in Food Science and Technology*, vol. 19, no. 1, pp. 26–30, 2008.

[5] B. Picard, L. Lefaucheur, C. Berri, and M. J. Duclos, "Muscle fibre ontogenesis in farm animal species," *Reproduction Nutrition Development*, vol. 42, no. 5, pp. 415–431, 2002.

[6] M. Fountoulakis, "Proteomics: current technologies and applications in neurological disorders and toxicology," *Amino Acids*, vol. 21, no. 4, pp. 363–381, 2001.

[7] M. J. Anderson, S. M. Lonergan, and E. Huff-Lonergan, "Myosin light chain 1 release from myofibrillar fraction during postmortem aging is a potential indicator of proteolysis and tenderness of beef," *Meat Science*, vol. 90, no. 2, pp. 345–351, 2011.

[8] B. C. Bowker, T. M. Fahrenholz, E. W. Parokzay, J. S. Eastridge, and M. B. Solomon, "Effect of hydrodynamic pressure processing and ageing on the tenderness and myofibrillar proteins of beef strip loins," *Journal of Muscle Foods*, vol. 19, no. 1, pp. 74–97, 2008.

[9] X. Chen, R. K Tume, X. Xu, and G. Zhou, "Solubilization of myofibrillar proteins in water or low ionic strength media: classic techniques, basic principles and novel functionalities," *Critical Reviews in Food Science and Nutrition*, vol. 15, no. 15, pp. 3260–3280, 2015.

[10] K. Hashimoto, S. Watabe, M. Kono, and K. Shiro, "Muscle protein composition of sardine and mackerel," *Bulletin of the Japanese Society of the Science of Fish*, vol. 45, no. 11, pp. 1435–1441, 1979.

[11] R. Marino, M. Albenzio, A. della Malva, A. Santillo, P. Loizzo, and A. Sevi, "Proteolytic pattern of myofibrillar protein and meat tenderness as affected by breed and aging time," *Meat Science*, vol. 95, no. 2, pp. 281–287, 2013.

[12] SAS Institute, *SAS/STAT User's Guide (Version 9.2)*, Statistical Analysis System Inst, Cary, NC, USA, 2011.

[13] M. Eady, D. Samuel, and B. Bowker, "Effect of pH and postmortem aging on protein extraction from broiler breast muscle," *Poultry Science*, vol. 93, no. 7, pp. 1–9, 2014.

[14] Y. M. Choi, S. H. Lee, J. H. Choe et al., "Protein solubility is related to myosin isoforms, muscle fiber types, meat quality traits, and postmortem protein changes in porcine longissimus dorsi muscle," *Livestock Science*, vol. 127, no. 2-3, pp. 183–191, 2010.

[15] A. Lana and L. Zolla, "Proteolysis in meat tenderization from the point of view of each single protein: a proteomic perspective," *Journal of Proteomics*, vol. 147, pp. 85–97, 2016.

[16] V. Verrez-Bagnis, J. Noel, C. Sautereau, and J. Fleurence, "Desmin degradation in postmortem fish muscle," *Journal of Food Science*, vol. 64, no. 2, pp. 240–242, 1999.

[17] M. M. Shaw and B. M. Riederer, "Sample preparation for two-dimensional gel electrophoresis," *Proteomics*, vol. 3, no. 8, pp. 1408–1417, 2003.

[18] T. Rabilloud, "Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis," *Electrophoresis*, vol. 19, no. 5, pp. 758–760, 1998.

[19] L. L. Chan, S. C. L. Lo, and I. J. Hodgkiss, "Proteomics study of a model causative agent of harmful red tide. Prorocentrum triestinum I: optimization of sample preparation methodologies for analyzing with two-dimensional electrophoresis," *Proteomics*, vol. 2, no. 9, pp. 1168–1186, 2002.

[20] M. P. Molloy, B. R. Herbert, B. J. Walsh et al., "Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis," *Electrophoresis*, vol. 19, no. 5, pp. 837–844, 1998.

[21] C. B. Malafaia, M. L. Guerra, T. D. Silva et al., "Selection of a protein solubilization method suitable for phytopathogenic bacteria: a proteomics approach," *Proteome Science*, vol. 13, no. 1, pp. 1–7, 2015.

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Research Article

Combined Use of Trisodium Citrate and Transglutaminase to Enhance the Stiffness and Water-Holding Capacity of Acidified Yak Milk Gels

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In this research, the synergistic effect of trisodium citrate (TSC) and microbial transglutaminase (TGase) treatment on the textural properties of acidified yak skim milk gels was investigated. TSC was added to yak skim milk to concentrations of 0, 20, and 40 mmol/L, followed by adjusting the pH to 6.7. The samples were incubated with TGase for the cross-linking reaction, after which the samples were acidified with 1.4% (w/v) gluconodelta-lactone (GDL) at 42°C for 4 h to form gels. The stiffness and water holding capacity (WHC) of gels exhibited higher values at 20 or 40 mmol/L than without TSC. The final storage modulus (G′) of yak milk gels was positively related to the concentration of TSC prior to TGase treatment. Cryoscanning electron microscopy observations showed that the gel networks were more rigid with higher TSC concentrations. Overall, TSC dissociated particles in yak milk into smaller ones. The newly formed particles in yak skim milk could form acid-induced gels with greater stiffness and higher WHC in the presence of TGase.

1. Introduction

Yak (*Bos grunniens*) milk is produced in the China Qinghai-Tibet Plateau area at an altitude of 3000 m [1]. It has become a new source of dairy products in China, due to its higher nutritional properties, less allergenicity, and better digestibility than cow milk [2–4]. Of the yak milk-based products, yogurt is the fastest growing one. However, textural defects including fragile structure (low stiffness) and syneresis (low water holding capacity) (WHC) usually occur in the yak yogurt gels, during storage or after mechanical damage. These defects significantly reduce the consumer acceptance of yak yogurt. Therefore, it is necessary to develop yak yogurt gels with higher stiffness and WHC.

The stiffness and WHC of yogurt gels are determined by their gel network structures, whose primary building blocks are caseins and whey proteins. In yak milk at native pH of 6.5–7.2, the casein molecules are noncovalent cross-linked by calcium phosphate, forming particles named casein micelles (about 200 nm in diameter) [5, 6]. On acidification, dissociation of the micelles happens, resulting in the aggregation of caseins and thus forming a weak network structure through noncovalent interactions at pH~4.6 [7–10]. Whey proteins can be denatured and interacted with caseins after heated (higher than 70°C), which could further improve the textural properties of acid-induced milk gels [11, 12].

Introducing covalent bonds is an effective mean to improve the textural property of yogurt gels [13–15]. TGase has been widely used to generate covalent bonds among protein molecules [16–18]. TGase could catalyze the acyl transfer reaction between γ-carboxyl groups and ε-amino groups among protein molecules, leading to the intramolecular or intermolecular cross-linking of proteins [19, 20]. This could significantly improve the gel stiffness and WHC of yogurt gels [21]. Unfortunately, although lots of studies have been performed on the use of TGase to enhance the textural properties of yogurt, the defects have not been totally inhibited [22–25].

The calcium ions chelating agents, including trisodium citrate (TSC), ethylene diamine tetraacetic acid, and sodium

phosphates, were found to disrupt the casein micelles in milk [26–29]. This might alter the gel formation of TGase-treated caseins [18]. In this case, the cross-link bonds among milk proteins before or during acidification would be altered. Therefore, the TGase-treated milk proteins might create different gel network structures compared with the gels prepared from acidified TGase-treated native milk proteins.

In this research, with the aim to improve the textural properties of yak yogurt, we investigated the effect of TSC prior TGase treatment on the properties of acid-induced gel prepared from heated yak skim milk. Our finding will be beneficial to improve the textural properties of yak yogurt.

2. Materials and Methods

2.1. Materials. Yak milk was obtained from the Maqu grassland on the Qinghai-Tibetan Plateau in northwest China. The altitude of pastures is about 3450 m, while the temperature is 9.4 ± 3.7°C. The ash, dry matter, protein, fat, and lactose in the yak milk were 0.81%, 17.38%, 5.14%, 5.47%, and 5.09% (w/v), respectively. To prevent bacterial growth, 0.04% (w/v) sodium azide was added into the yak milk. Calcium-independent TGase (200 U/g) was obtained from C&P Group GmbH (Rosshaupten, Germany). GDL and TSC were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of Acid-Induced Yak Milk Gels. The fat in yak milk was removed by centrifugation at 4200 g at 25°C for 30 min, followed by denaturing the whey proteins at 80°C for 20 min. The skim milk was added with different amount of TSC powder to a concentration of 0, 20, and 40 mmol/L. After magnetic stirred for 10 min, the pH of heated yak skim milk was adjusted to 6.7 with 0.5 mol/L HCl. After adding the TGase to 10 U/g milk proteins, the yak skim milk was further magnetic stirred for 10 min. Then, all samples were stored at 42°C for 60 min, followed by acidification with 1.4% GDL (w/v) at 42°C for 4 h. The final pH of all the samples was between 4.3 and 4.6 [30]. Finally, all the gels were stored at 4°C for 6 h for further use.

For comparison, heated yak skim milk treated with 0, 20, and 40 mmol/L TSC (in the absence of TGase) was also investigated. The other procedures were performed as described in the above section.

2.3. Characterization of the Treated Milk or Gels. The particle size distribution was measured with a Zetasizer (Model Nano-ZS3600, Malvern, UK) at 25°C [31]. The skim milk was diluted 300-fold with ultrapure water.

The acid-induced gelation processes of yak skim milk were determined with a rheometer (AR1500ex, TA Instruments, USA). GDL was added into TSC- and TGase-treated samples to 1.4% (w/v). The samples were then transferred into the concentric cylinder. The acid-induced yak skim gels were oscillated at 0.1 Hz and with 1% of applied strain. The determination temperature was 42°C.

The stiffness of the acid-induced gels was determined by the analyzer (TMS-Pro, Food Technology Corp., Sterling, USA). The acid gels prepared in 2.2 were placed at room temperature for 60 min before determination. A cylinder probe (25 mm in diameter) moving into the gel to a distance of 10 mm at 30 mm/min was used for the penetration test.

The WHC of the gels was measured according to a modified procedure [32]. Forty millimeters of the TSC and TGase-treated yak skim milk were acidified at 42°C for 4 h in 50 mL centrifuge tubes, followed by centrifugation at 1500 g at 25°C for 15 min. The WHC was defined as the percentage of the weight of gels remaining in the centrifuge tubes to their initial weight.

The gel microstructures were observed by the cryo-oscanning electron microscopy (S-3000N, Hitachi Co., Tokyo, Japan), based on the reported literatures [26].

2.4. Statistical Analysis. The experiments were performed in at least triplicate. Data analysis of variance (ANOVA) was used to check the significance of differences between means with $P < 0.05$ indicating significance.

3. Results and Discussion

3.1. Particle Size. The size distribution curves by number fraction of particles in heated yak skim milk are shown in Figure 1. In the absence of TGase, when the added TSC concentrations in the yak skim milk were 0, 20, and 40 mmol/L, the corresponding particle peaking diameters were 145.1 ± 7.0, 56.9 ± 14.9, and 34.2 ± 4.6 nm (mean ± SD), respectively. In the presence of TGase, when the TSC concentrations in yak skim milk were 0, 20, and 40 mmol/L, the corresponding particle peaking diameters were 151.5 ± 10.8, 61.4 ± 15.4, and 29.2 ± 9.7 nm (mean ± SD), respectively. This suggested that the particles in yak skim milk became smaller with increasing TSC concentrations. It is well established that calcium-chelating agents can disrupt the micellar framework by removing calcium from the micelles, leading to the dissociation of casein micelles. Therefore, it was expected that the citrate ions would dissociate the particles in yak skim milk into smaller particles.

3.2. Gelation Kinetics. The storage modulus (G') evolution of heated yak skim milk (after GDL addition) is shown in Figure 2. It can be clearly observed that both TSC and TGase had significant influence on the acid-induced gelation kinetics of yak milk. Gelation time of yak skim milk was positively related to the TSC concentrations, whether or not the TGase is in the presence. However, the influence of TSC on the final storage modulus (G') of gels was heavily dependent on the TGase. In the presence of TGase, the final G' of gels was higher at 20 or 40 mmol/L^{-1} TSC than at 0 mmol/L^{-1} TSC. As proved above, TSC favors the dissociation of particles in the yak skim milk into smaller particles. This could enhance the flexibility of the newly formed casein particles [10, 26] and thus favored the

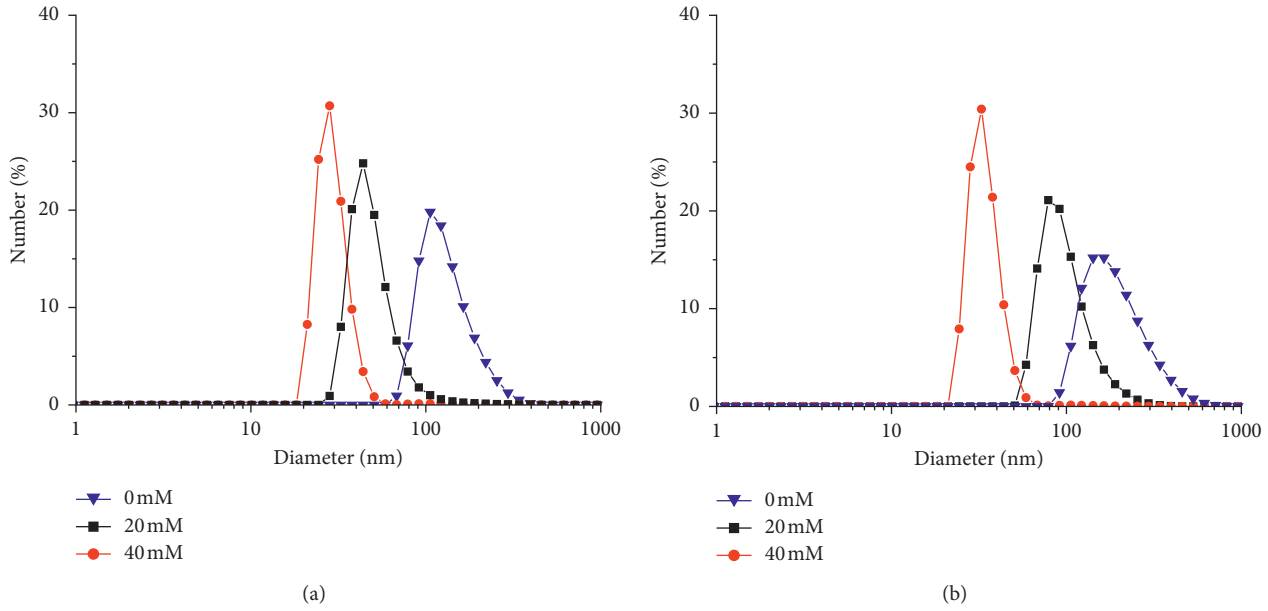


Figure 1: Particle size distribution curves of heated yak skim milk treated with different concentrations of TSC without (a) and with (b) TGase treatment.

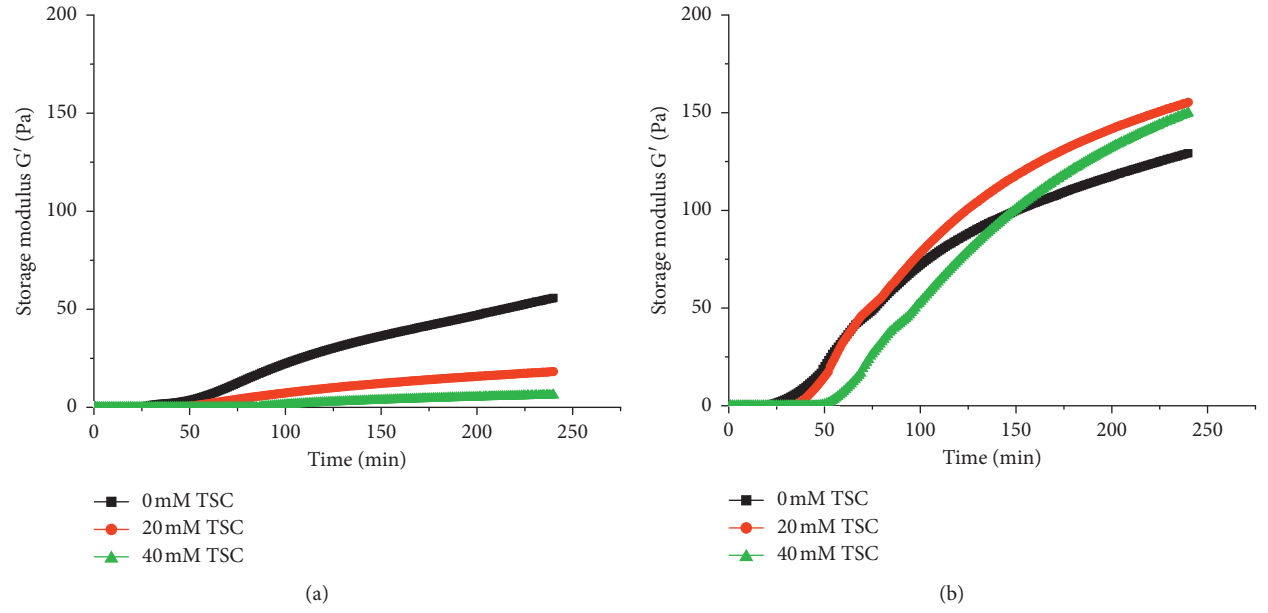


Figure 2: Effects of trisodium citrate on the storage modulus of acid-induced yak milk gels without (a) and with (b) TGase.

adequate rearrangement of caseins during gelation. This made the newly formed particles to be more susceptible to TGase cross-linking [33].

3.3. Stiffness and WHC. The stiffness of the acid-induced yak skim milk gels is shown in Figure 3. In the samples without TGase, it can be seen that the addition of TSC reduced the stiffness of acid-induced casein gels, which was consistent with the G′ results. It was observed that the stiffness of gels prepared in the presence of TGase was higher at 20 or 40 mmol/L^{-1} TSC than those without TSC addition. The reasons for this can also be explained as described in the storage modulus (G′) of gels. It can also be observed that, in the same TSC concentrations, the stiffness of gels prepared in the presence of TGase was significantly higher than those in the absence of TGase. This indicated that the TGase cross-linking played an important role in the gel stiffness.

The effect of TSC on the WHC of acid-induced yak skim milk gels is shown in Figure 4. In the same TSC concentration, the WHC of gels prepared in the presence of

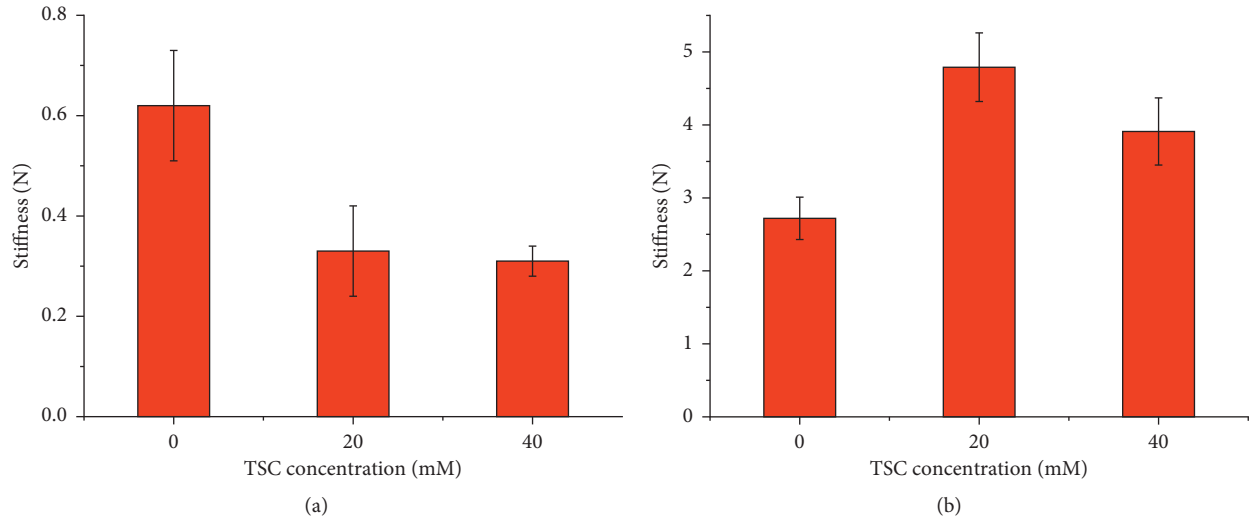


Figure 3: Effects of trisodium citrate on the stiffness (*N*) of acid-induced yak milk gels without (a) and with (b) TGase.

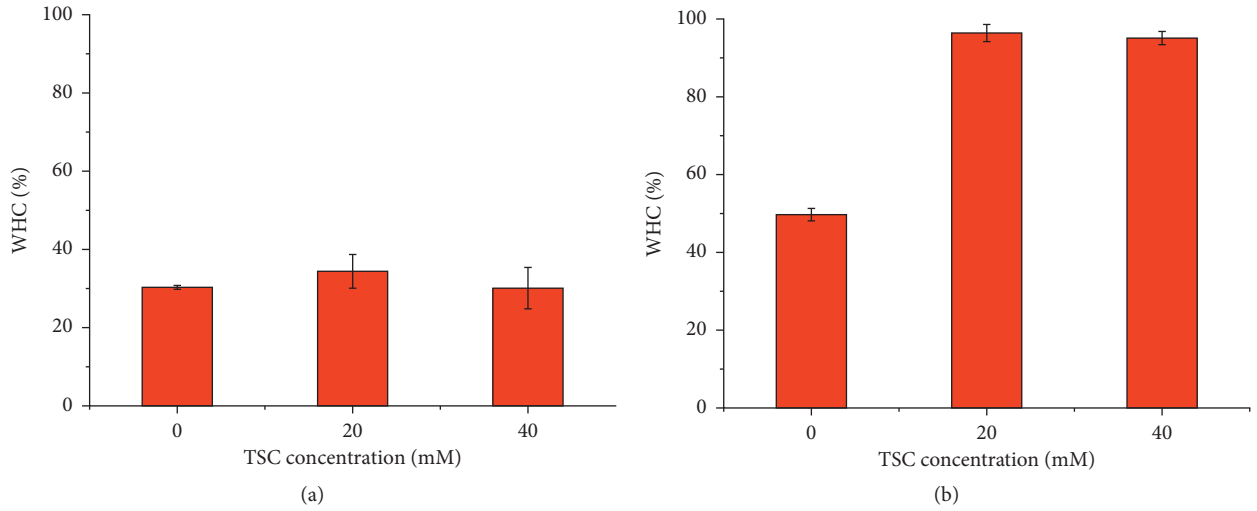


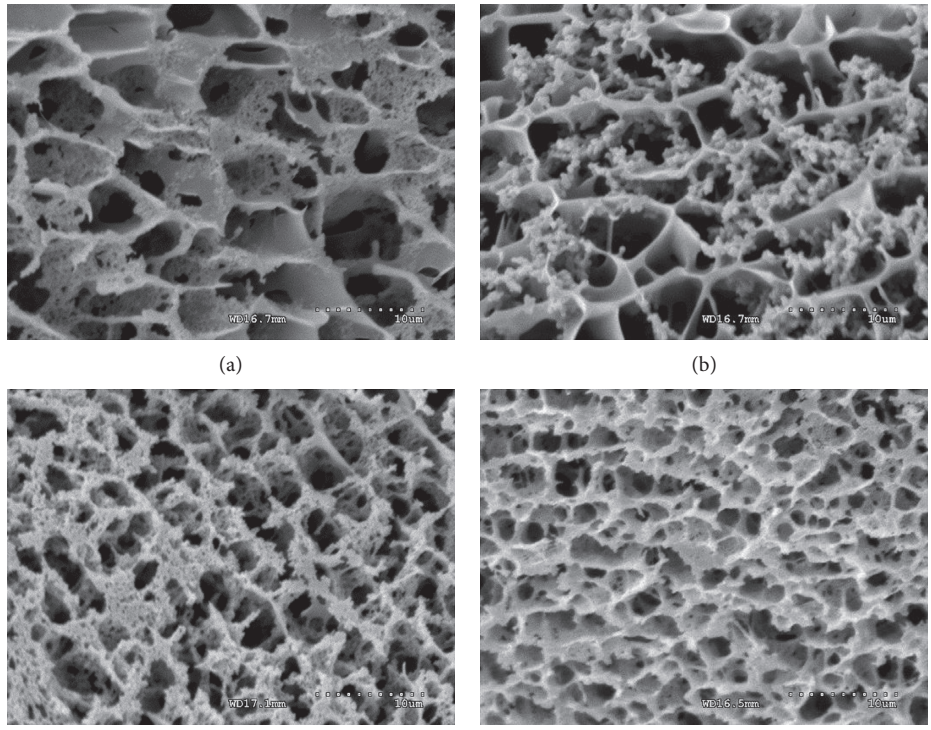
Figure 4: Effects of trisodium citrate on the water holding capacity of acid-induced yak milk gels without (a) and with (b) mTGase.

TGase was significantly higher than those in the absence of TGase. This indicated that the TGase cross-linking played a crucial role in improving the WHC. It can be seen that, in the presence of TGase, when the concentration of TSC was 20 or 40 mmol/L, little water in the final gels prepared was expelled after centrifugation. This indicated that the gels prepared from the newly formed smaller particles exhibited greater water retention in the presence of TGase. However, in the absence of TGase, more than 60% of water was expelled. This was identical with the results of gel stiffness because weak gels from noncovalent bonds are easier to shrinkage and subsequent expulsion of water.

3.4. Microstructure. The microstructures of acidified yak skim milk gels are exhibited in Figure 5. In the samples, in the presence of TGase, it can be observed that network structures were more rigid with TSC addition than without TSC addition. This was consistent with the previous textural property results.

4. Conclusion

In this study, the effect of TSC on the stiffness of acid-induced, TGase-treated yak milk gels was investigated. Yak milk first treated by TSC (20 or 40 mmol/L) and then cross-linked with TGase resulted in gels with higher stiffness, WHC, and storage modulus (G′). TSC could dissociate the casein micelles in yak milk into smaller particles. In the presence of TGase, the newly formed particles formed acid-induced gels with higher stiffness, higher WHC, and storage modulus (G′).



(a) (b) (c) (d)

Figure 5: Effects of trisodium citrate on the microstructure of acid-induced yak milk gels without ((a) 0 mmol/L^{-1} TSC; (b) 30 mmol/L^{-1} TSC) and with ((c) 0 mmol/L^{-1} TSC; (d) 30 mmol/L^{-1} TSC) mTGase.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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References

[1] G. X. Cui, F. Yuan, A. A. Degen et al., "Composition of the milk of yaks raised at different altitudes on the Qinghai-Tibetan Plateau," *International Dairy Journal*, vol. 59, pp. 29–35, 2016.

[2] H. Li, Y. Ma, J. Xiang et al., "Comparison of the immunogenicity of yak milk and cow milk," *European Food Research and Technology*, vol. 233, no. 4, pp. 545–551, 2011.

[3] Q. Sheng, J. Li, M. S. Alam, X. Fang, and M. Guo, "Gross composition and nutrient profiles of Chinese yak (Maiwa) milk," *International Journal of Food Science and Technology*, vol. 43, no. 3, pp. 568–572, 2008.

[4] H. Zhang, J. Xu, J. Wang et al., "A survey on chemical and microbiological composition of kurut, naturally fermented yak milk from Qinghai in China," *Food Control*, vol. 19, no. 6, pp. 578–586, 2008.

[5] P. Wang, H. Liu, P. Wen, H. Zhang, H. Guo, and F. Ren, "The composition, size and hydration of yak casein micelles," *International Dairy Journal*, vol. 31, no. 2, pp. 107–110, 2013.

[6] Y. Zhang, Y. Li, P. Wang, Y. Tian, Q. Liang, and F. Ren, "Rennet-induced coagulation properties of yak casein micelles: a comparison with cow casein micelles," *Food Research International*, vol. 102, pp. 25–31, 2017.

[7] D. S. Horne, "Formation and structure of acidified milk gels," *International Dairy Journal*, vol. 9, no. 3, pp. 261–268, 1999.

[8] G. H. Meletharayil, H. A. Patel, L. E. Metzger, C. Marella, and T. Huppertz, "Influence of partially demineralized milk proteins on rheological properties and microstructure of acid gels," *Journal of Dairy Science*, vol. 101, no. 3, pp. 1864–1871, 2018.

[9] J.-L. Mession, S. Roustel, and R. Saurel, "Interactions in casein micelle - pea protein system (part II): mixture acid gelation with glucono-δ-lactone," *Food Hydrocolloids*, vol. 73, pp. 344–357, 2017.

[10] T. Ozcan, J. A. Lucey, and D. S. Horne, "Effect of tetrasodium pyrophosphate on the physicochemical properties of yogurt gels," *Journal of Dairy Science*, vol. 91, no. 12, pp. 4492–4500, 2008.

[11] G. Liu, T. C. Jæger, S. B. Nielsen, C. A. Ray, and R. Ipsen, "Physicochemical properties of milk protein ingredients and their acid gelation behaviour in different ionic environments," *International Dairy Journal*, vol. 85, pp. 16–20, 2018.

[12] I. C. Torres, J. M. Amigo, J. C. Knudsen, A. Tolkach, B. O. Mikkelsen, and R. Ipsen, "Rheology and microstructure of low-fat yoghurt produced with whey protein microparticles as fat replacer," *International Dairy Journal*, vol. 81, pp. 62–71, 2018.

[13] T. Huppertz and C. G. de Kruif, "Ethanol stability of casein micelles cross-linked with transglutaminase," *International Dairy Journal*, vol. 17, no. 5, pp. 436–441, 2007.

[14] D. Jaros, U. Schwarzenbolz, N. Raak, J. Lobner, T. Henle, and H. Rohm, "Cross-linking with microbial transglutaminase: relationship between polymerisation degree and stiffness of acid casein gels," *International Dairy Journal*, vol. 39, no. 2, pp. 345–347, 2014.

[15] S. J. Jiang and X. H. Zhao, "Transglutaminase-induced cross-linking and glucosamine conjugation of casein and some functional properties of the modified product," *International Dairy Journal*, vol. 21, no. 4, pp. 198–205, 2011.

[16] L. Chen, Y. Li, J. Han, D. Yuan, Z. Lu, and L. Zhang, "Influence of transglutaminase-induced modification of milk protein concentrate (MPC) on yoghurt texture," *International Dairy Journal*, vol. 78, pp. 65–72, 2018.

[17] S. M. T. Gharibzahedi and I. S. Chronakis, "Crosslinking of milk proteins by microbial transglutaminase: utilization in functional yogurt products," *Food Chemistry*, vol. 245, pp. 620–632, 2018.

[18] N. F. N. Silva, F. Casanova, F. Gaucheron et al., "Combined effect of transglutaminase and sodium citrate on the microstructure and rheological properties of acid milk gel," *Food Hydrocolloids*, vol. 82, pp. 304–311, 2018.

[19] C. Kleemann, I. Selmer, I. Smirnova, and U. Kulozik, "Tailor made protein based aerogel particles from egg white protein, whey protein isolate and sodium caseinate: influence of the preceding hydrogel characteristics," *Food Hydrocolloids*, vol. 83, pp. 365–374, 2018.

[20] E. Lam, C. Holt, P. Edwards et al., "The effect of transglutaminase treatment on the physico-chemical properties of skim milk with added ethylenediaminetetraacetic acid," *Food Hydrocolloids*, vol. 69, pp. 329–340, 2017.

[21] L. Zhang, L. Zhang, H. Yi et al., "Enzymatic characterization of transglutaminase from *Streptomyces mobaraensis* DSM 40587 in high salt and effect of enzymatic cross-linking of yak milk proteins on functional properties of stirred yogurt," *Journal of Dairy Science*, vol. 95, no. 7, pp. 3559–3568, 2012.

[22] K. Hinz, T. Huppertz, and A. L. Kelly, "Susceptibility of the individual caseins in reconstituted skim milk to cross-linking by transglutaminase: influence of temperature, pH and mineral equilibria," *Journal of Dairy Research*, vol. 79, no. 4, pp. 414–421, 2012.

[23] T. Huppertz and C. G. de Kruif, "Structure and stability of nanogel particles prepared by internal cross-linking of casein micelles," *International Dairy Journal*, vol. 18, no. 5, pp. 556–565, 2008.

[24] T. Huppertz, M. A. Smiddy, and C. G. de Kruif, "Biocompatible micro-gel particles from cross-linked casein micelles," *Biomacromolecules*, vol. 8, no. 4, pp. 1300–1305, 2007.

[25] C. Schorsch, H. Carrie, and I. T. Norton, "Cross-linking casein micelles by a microbial transglutaminase: influence of cross-links in acid-induced gelation," *International Dairy Journal*, vol. 10, no. 8, pp. 529–539, 2000.

[26] H. Li, C. Yang, C. Chen et al., "The Use of trisodium citrate to improve the textural properties of acid-induced, transglutaminase-treated micellar casein gels," *Molecules*, vol. 23, no. 7, p. 1632, 2018.

[27] T. Ozcan-Yilsay, W. J. Lee, D. Horne, and J. A. Lucey, "Effect of trisodium citrate on rheological and physical properties and microstructure of yogurt," *Journal of Dairy Science*, vol. 90, no. 4, pp. 1644–1652, 2007.

[28] P. Thomar and T. Nicolai, "Dissociation of native casein micelles induced by sodium caseinate," *Food Hydrocolloids*, vol. 49, pp. 224–231, 2015.

[29] P. Wang, C. Chen, H. Guo, H. Zhang, Z. Yang, and F. Ren, "Casein gel particles as novel soft Pickering stabilizers: the emulsifying property and packing behaviour at the oil-water interface," *Food Hydrocolloids*, vol. 77, pp. 689–698, 2017.

[30] P. Wang, N. Cui, J. Luo et al., "Stable water-in-oil emulsions formulated with polyglycerol polyricinoleate and glucono-δ-lactone-induced casein gels," *Food Hydrocolloids*, vol. 57, pp. 217–220, 2016.

[31] P. Wang, S. Jin, H. Guo, L. Zhao, and F. Ren, "The pressure-induced, lactose-dependent changes in the composition and size of casein micelles," *Food Chemistry*, vol. 173, pp. 468–474, 2015.

[32] M. Jacob, S. Nöbel, D. Jaros, and H. Rohm, "Physical properties of acid milk gels: acidification rate significantly interacts with cross-linking and heat treatment of milk," *Food Hydrocolloids*, vol. 25, no. 5, pp. 928–934, 2011.

[33] C. H. Lee and C. Rha, "Microstructure of soybean protein aggregates and its relation to the physical and textural properties of the curd," *Journal of Food Science*, vol. 43, no. 1, pp. 79–84, 1978.