

DETECTION OF PORK, HORSE OR DONKEY MEAT ADULTERATION IN BEEF-BASED FORMULATIONS BY FOURIER TRANSFORM INFRARED SPECTROSCOPY

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ABSTRACT

Fourier Transform Infrared (FTIR) Spectroscopy with chemometrics was employed to discriminate pork, horse or donkey meat substitution in beef. Wavenumber range of 1480-1360 cm⁻¹ (94.97% of variance is explained by the first two principal components) and fingerprint region (FR) (90.08%) clearly differentiated beef, pork and beef-pork mixtures in principal component analysis (PCA). For beef-donkey mixtures, 1760-1710 cm⁻¹ (99.31%) and FR (96.03%) provided discrimination. For beef-horse meat mixtures; a grouping was obtained for 1290-1210 cm⁻¹ (90.41%), FR (84.83%) and whole spectrum (88.61%). In hierarchical cluster analysis (HCA), the region between 1480-1425 cm⁻¹ was able to separate all donkey adulterated mixtures, 100% beef and 100% donkey meat from each other with 100% sensitivity and specificity while 2980-2880 cm⁻¹, whole spectrum and FR provided differentiation for beef-horse mixtures. 1760-1710 and 1210-1190 cm⁻¹ regions provided classification between 100% beef, 100% pork and pork-beef mixtures (except for 5% of substituted) with 100% sensitivity and specificity.

Keywords: Beef, donkey meat, horse meat, pork, adulteration, FTIR spectroscopy

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SIĞIR ETİ KARIŞIMLARINDA DOMUZ, AT VE EŞEK ETİ TAĞŞIŞININ FOURIER DÖNÜŞÜMLÜ KIZILÖTESİ SPEKTROSKOPİSİ İLE BELİRLENMESİ

ÖZ

Sığır eti karışımlarında domuz, at ve eşek etleri tağşışının belirlenmesinde Fourier Dönüşümlü Kızılötesi (FTIR) spektroskopisi, kemometri ile birlikte kullanılmıştır. Temel bileşenler analizinde (PCA) sığır, domuz ve sığır-domuz eti karışımları 1480-1360 cm^{-1} (varyansın %94.97'si ilk iki temel bileşen tarafından açıklanmıştır) dalga sayısı aralığında ve parmak izi bölgesinde (PB) (%90.80) ayırt edilmiştir. Sığır-eşek eti karışımlarının ayırımında, 1760-1710 cm^{-1} dalga sayısı aralığı (%99.31) ve PB (%96.03) başarılı olurken; sığır-at eti karışımlarında 1290-1210 cm^{-1} (%90.41) aralığı, PB (%84.83) ve tüm spektrumda (%88.61) gruplama sağlanmıştır. Hiyerarşik kümeleme analizinde (HCA), 1480-1425 cm^{-1} dalga sayısı aralığı tüm eşek eti karışımlarının, %100 eşek eti ve %100 sığır etinin; 2980-2880 cm^{-1} dalga sayısı aralığı, tüm spektrum ve PB ise sığır-at karışımlarının, %100 at ve %100 sığır örneklerinin ayırımında %100 duyarlılık ve özgülükte kullanılabilir bölgeler olarak belirlenmiştir. 1760-1710 ve 1210-1190 cm^{-1} arasındaki bölgeler %100 sığır, %100 domuz ve domuz-sığır karışımlarının (%5'lik karışım hariç) %100 duyarlılık ve özgülük ile kümelendiğini sağlamıştır.

Anahtar kelimeler: Sığır eti, domuz eti, at eti, eşek eti, tağşış, FTIR spektroskopisi

INTRODUCTION

Food quality and safety have been of significant public concern all over the world for a significant number of years. One of the most important food quality and safety issues worldwide is food fraud or economically motivated adulteration which was defined by United States Food and Drug Administration as “the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production for economic gain” (Federal Register, 2009). These fraudulent practices negatively affect the reputation and fair trade of food businesses and consumer rights, being a significant problem for people with ethical or religious concerns (Nunes et al., 2016; Spink, 2016).

Notwithstanding the fact that adulteration of food products has been of concern all over the world since ancient times, it surfaced again and became a significant threat along the supply chain after the latest cases where beef burgers were found to contain horse meat in Europe (BBC, 2013a), donkey meat was detected in beef products in South Africa (BBC, 2013b), and donkey meat was adulterated with fox meat in China (Reuters, 2014).

Consumption of foods containing pork is forbidden in Judaism and Islam, and horse and donkey meats are not conventional sources of meats in most countries of the world because eating these meats is taboo or prohibited due to religious beliefs (Farouk, 2013; Regenstein et al., 2003). On the other hand, donkey meat is particularly preferred by consumers as a highly expensive meat type like in some regions of China (Blakeway, 2014). There have been many reported incidences of undeclared utilization of these meats in food product formulations. This is not necessarily a food safety issue; however, it is considered an important challenge for traceability in the food supply chain.

In order to develop control mechanisms, authentication to identify animal species in meat products has become a crucial concern for food authorities and consumer's groups. A variety of standard analytical methods, i.e., histological tests, electrophoretic separation of proteins, immunological procedures, DNA based techniques, chromatography, and spectroscopy available for the identification and authentication of raw meat mixtures (Cuadros-Rodríguez et al., 2016; Safdar et al., 2014; Sentandreu and Sentandreu, 2014). However, most of them are characterized by being time consuming, invasive and expensive, and also require sophisticated

laboratory procedures with tedious sample preparation steps.

Among the techniques used for authentication, DNA based methods are of interest and reliable for meat species identification. However, the integrity of DNA could be affected by factors such as storage conditions and cooking. It was reported that meat cooked at high temperatures resulted in an overall low DNA yield, which would cause unsuccessful results in an amplification assay (Vlachos et al. 2016). Thus, due to the drawbacks of the well-known DNA-based methods and other elaborated techniques, the research on the spectroscopic methods is now gaining acceptance due to rapidness and minimum preprocessing requirements. Among the spectroscopic methods, Fourier Transform Infrared (FTIR) spectroscopy in meat authentication has drawn considerable attention due to its properties as a fingerprint technique which can be used for qualitative and quantitative analyses. FTIR provides a fast look to the structure of a sample, and at the same time, consolidates simultaneously a number of sample structures. FTIR spectroscopy has been applied quite recently in a few studies on meat species identification such as pork in meatball by Rohman et al. (2011), pork in ham sausage by Xu et al. (2012), turkey meat in minced beef by Alamprese et al. (2013), beef offal in beef burger by Zhao et al. (2014), and rat meat in meatball by Rahmania et al. (2015). In some of these studies, pre-sample preparation steps were used which caused extended experimental time. In others, spectral data were differentiated based on only whole spectrum and fingerprint regions without conducting a detailed spectral analysis. In our laboratory, FTIR spectroscopy was used as a tool to identify adulterated beef in raw meat mixtures incorporated with chicken or turkey meat (Deniz et al., 2018). The results showed that this technique is a reliable one to detect adulteration of beef mixtures by chicken or turkey meat. Therefore, the current study was designed to identify intentional substitution of pork, horse or donkey meat at different concentrations in beef mixtures using FTIR spectroscopy and as well as chemometrics by focusing on obtaining data from

detailed spectral analyses of different characteristic regions in addition to the whole spectrum and the fingerprint regions in the FTIR spectra.

MATERIALS AND METHODS

In the present study, the methods used by Deniz et al. (2018) were followed for sample preparation, FTIR Spectroscopy measurements and chemometric analyses

Preparation of Meat Mixtures

Beef and pork were purchased from local butchers in Ankara and in İstanbul, respectively. Horse and donkey meats were kindly supplied by Darica Zoo and Konya Zoo, respectively. *Longissimus dorsi* muscles from beef, pork, horse, or donkey obtained in different months for the three replications were individually ground and used as materials in this study. The meats from four different species were individually ground before preparing the adulterated mixes. In the current study, beef was the main meat type and it was separately substituted with horse, pork or donkey meat at 0, 5, 10, 20, 40 and 100% (wt/wt) ratios. Eighteen mixtures for each type of substituted meat (6 different ratios x 3 replications), in total 36 different meat mixtures (18x3 adulterant meat types) were prepared in 200 g portions based on the ratios given above. Since small amounts of samples were used in FTIR measurement, in order to ensure thoroughly mixed samples, BKK 1160 model chopper (Beko, Turkey) was utilized for homogenization of raw meat mixtures. These 36 formulations were then, lyophilized in a Millrock Freeze Drier (Ultra Tainer, Kingston, USA). Each lyophilized mixture was shredded by an Aromatic Model Blender (Fakir™, Germany) and used for FTIR spectroscopic measurements.

Spectral Measurements

FTIR spectra in the mid-infrared region between 4000-850 cm^{-1} wavenumbers were obtained on a Bruker Tensor 27 FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany) equipped with an attenuated total reflectance (ATR) ZnSe crystal (Pike Miracle ATR Cell). Interferograms were accumulated for 16 scans at 4 cm^{-1} resolution at a controlled ambient temperature of

22°C (Ayhan, 2013). Before each measurement, a new reference air background subtraction was performed. Three replications for each meat type were used for each mixture with six technical replicates for scanning (n=18).

Data Analysis and Chemometrics

Recorded spectra were base-lined and averaged by a “spectrum calculator” tool, and then normalized by vector normalization for further data analysis. Data obtained from intensity values of characteristic bands of different meat mixtures were subjected to analysis of variance, and means were separated with Duncan’s Multiple Comparison Test at 5% level of probability using SPSS software (Version 17.0 for Windows, SPSS Inc., USA).

For the classification of meat mixtures, whole spectrum (4000-850 cm^{-1}), fingerprint region (1500-900 cm^{-1}), six characteristic regions (region 1 at 2980-2800 cm^{-1} , region 2 at 1760-1710 cm^{-1} ; region 3 at 1480-1360 cm^{-1} , region 4 at 1290-1210 cm^{-1} , region 5 at 1210-1140 cm^{-1} , and region 6 at 1140-1020 cm^{-1}), and specific peaks were used in hierarchical cluster analysis (HCA) with OPUS software, and principal component analysis (PCA) using a Chemostat Standalone Package (Helfer et al., 2015). For PCA, baselined spectra were used and each region was evaluated after using various preprocessing methods in combination. Normalization by max and by range, multiplicative standard correction (MSC) and standard normal variate (SNV) transformations, and first and second derivatizations were used as spectral preprocessing methods in PCA. The best results obtained from the different preprocessing methods were discussed in this study. HCA dendrograms were generated by using Euclidean distance and Ward’s algorithm with five different preprocessing types as follows: second derivatization, second derivatization+vector normalization, first derivatization, first derivatization+vector normalization, and vector normalization in addition to no-preprocessing.

RESULTS AND DISCUSSION

In the FTIR spectra of the mixtures, there were no visually noticeable differences between the different meat species used in the study. For this reason, the spectral view was zoomed to better differentiate the peaks or shoulders corresponding to the stretching and bending vibrations of structural or functional groups present in the evaluated meat mixtures. The zoomed views of absorption spectra obtained from mixtures of beef and pork, horse or donkey meat are given in Figure 1 for characteristic regions 1 and 2, and Figure 2 for characteristic regions 3, 4, 5 and 6. In total, 12 peaks were detected in the spectra of mixed samples whereas 5 shoulders were observed in the mixtures containing only beef.

In region 1, at wavenumbers between 2980 and 2800 cm^{-1} , four peaks were detected (Figure 1). Similar peaks were also reported at these wavenumbers in extracted fat samples from beef meatball, pork and/or beef fat by Rahmania and Rohman (2015), Rohman et al., (2011) and Kurniawati et al., (2014), respectively. It was shown that CH, CH₂ and CH₃ bonds of phospholipids, cholesterol and creatine exhibit characteristic signals at 2916-2919 cm^{-1} wavenumber, and this region was defined in the literature as characteristic for lipids (Stuart, 2004). Asymmetric stretching vibration of CH₂ of alkyl chains (lipids) was reported to be responsible for the signals around 2922 cm^{-1} while C-H stretching vibrations of CH₂, lipids and fatty acids are related to the signals around 2850 cm^{-1} (Movasaghi et al., 2008; Shetty et al., 2006). In region 2, one peak was observed at about 1740 cm^{-1} wavenumber which could be originated from lipids (Figure 1). This peak was also detected by Rahmania and Rohman (2015) in beef meatball, by Rohman et al. (2011) and Kurniawati et al. (2014) in beef and pork fat, and most recently by Deniz et al (2018) in beef, chicken and turkey meat mixtures.

Regarding signals of the peaks in these two regions, the intensities of the peaks 2, 4 and 5 decreased significantly ($p < 0.05$) with the increasing ratios of pork, horse or donkey meat. These peaks could be very decisive in order to

detect distinctions between the different meat species. Adding even a small amount of pork, horse or donkey to the beef-based mixtures they could be detected using peaks 2, 4 and 5. Similar to the alterations in peak 5 in the current study, Zhao et al. (2014) indicated that perceptible changes in authentic and offal adulterated beef burger samples depending on formulation were observed at around 1744 cm^{-1} wavenumber. In another study, Meza-Márquez et al. (2010) determined a slight shoulder at 1740 cm^{-1} in lean beef while this shoulder was not noticeable for horse meat. In the current study, however, a peak at this wavenumber (peak 5) was detected in the

zoomed view for beef as well as horse meat with lower intensity values ($p < 0.05$) for horse meat. This is a clear indication that the present study with zoomed view provided considerable distinction of the specific peaks from beef as well as from other mixtures in terms of intensity values. One shoulder peak at 1728 cm^{-1} (S1) in region 2 was observed only for the mixtures containing beef (Figure 1) where intensities were greater with increasing content of beef in the mixtures, indicating that this shoulder is characteristic for beef samples (Deniz et al. 2018).

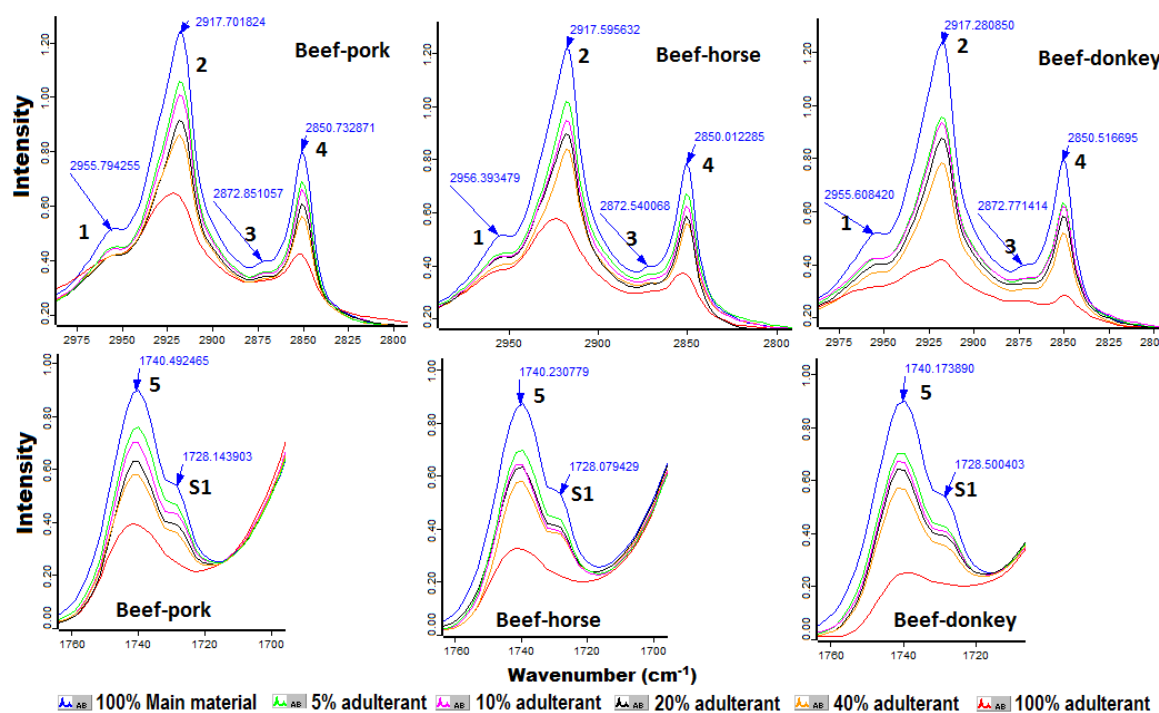


Figure 1. Zoomed view of normalized IR spectrum for characteristic region 1 (above) and 2 (below) (S1: Shoulder peak 1)

In this study, the spectra from the other four characteristic regions “3, 4, 5 and 6” which in the literature are generally examined within the “fingerprint region” are displayed in Figure 2. Beef samples exhibited four shoulder peaks (S2, S3, S4 and S5) which are distinctive only for beef except for S3 which was also detected in pork mixtures. This shoulder was also observed for both pork and beef fats in the studies by Kurniawati et al. (2014) and Rohman et al. (2011).

In the fingerprint region, the most noticeable peaks that could be used for classification were peak 6 in region 3 (at 1366 cm^{-1} wavenumber), peak 8 in region 4 (at 1240 cm^{-1} wavenumber), and peaks 9 and 10 in region 5 (at 1195 and 1176 cm^{-1} wavenumbers, respectively). The absolute intensity values for these peaks showed a decrease ($p < 0.05$) with higher ratios of adulterant meats (pork, horse and donkey). It is noteworthy to mention that peak 9 was only detected in the

spectra of beef samples, which was also previously reported by Deniz et al. 2018, but not observed in the spectra of 100% meat from pork, donkey or horse. Two peaks at 1115 and 1096 cm^{-1} wavenumbers were detected in beef samples in region 6 which were attributed to different nature and composition of lipids which might result from stretching vibrations of C=O in triacylglycerols (Kurniawati et al., 2014), C–H bending vibration and C–H deformation vibrations of fatty acids, respectively (Rohman et al., 2011).

Classification of Mixtures by Chemometric Methods

After identification and comparison of the specific peaks and shoulders detected in different characteristic regions of the FTIR spectra, the

various meat mixtures were classified using chemometric methods such as PCA and HCA. PCA is a widely-used data reduction method in spectral analyses while HCA is commonly used classification method in FTIR spectroscopy. The whole spectrum (4000-850 cm^{-1}) and the fingerprint region (1500-900 cm^{-1}) which are commonly mentioned in the literature (Kurniawati et al., 2014; McElhinney et al., 1999; Rahmania and Rohman, 2015; Rohman et al., 2011) were analyzed. In addition, a classification based on individual characteristic regions with different preprocessing methods was conducted. By using multivariate unsupervised analyses, meats of different species and adulterated samples (considering all percentages as a one group) were discriminated from each other.

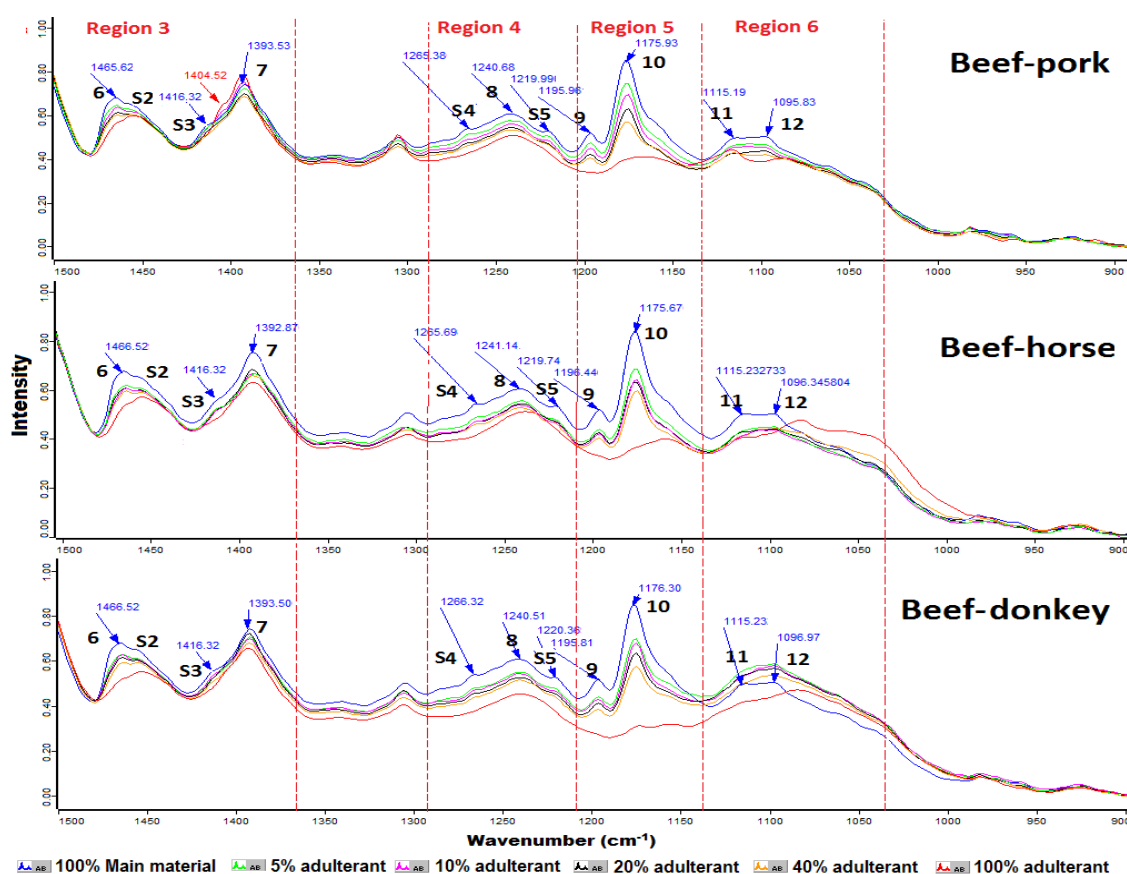


Figure 2. Zoomed view of normalized IR spectrum for fingerprint region (1500-900 cm^{-1}) and for the characteristic regions 3, 4, 5 and 6 (S2: Shoulder peak 2; S3: Shoulder peak 3; S4: Shoulder peak 4; S5: Shoulder peak 5)

The regions which provided grouping of samples in PCA are as follows and their percentages of data variances explained by the first and second principal components are written in parentheses. In PCA, beef, pork and beef-pork mixtures were grouped separately by using region 3 (1480-1360 cm^{-1}) (94.97%) and fingerprint region (90.08%) while region 2 (1760-1710 cm^{-1}) (99.31%) and fingerprint region (96.03%) enabled to differentiate 100% beef and donkey and beef-donkey mixtures. For horse meat adulteration, region 4 (1290-1210 cm^{-1}) (90.41%), whole

spectrum (88.16%) and fingerprint region (84.83%) provided differentiation of 100% horse, beef and their mixtures (Figure 3). PCA and its combination with various discrimination methods which was applied for classification of different meat types in previous studies based on MIR spectroscopy is very useful for analyzing of spectral data sets (Deniz et al. 2018, Rahmania and Rohman 2015, Kurniawati et al., 2014, Rohman et al., 2011, Meza-Márquez et al. 2010, Al-Jowder et al., 2002).

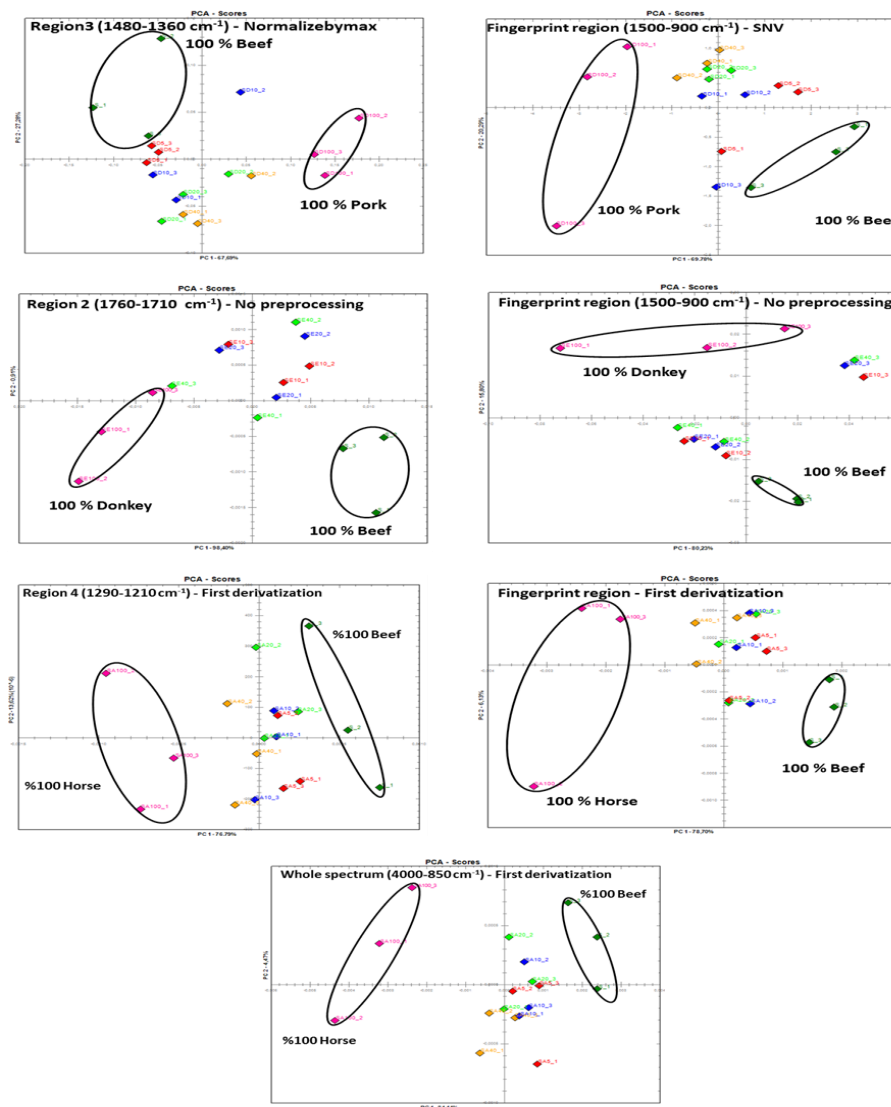
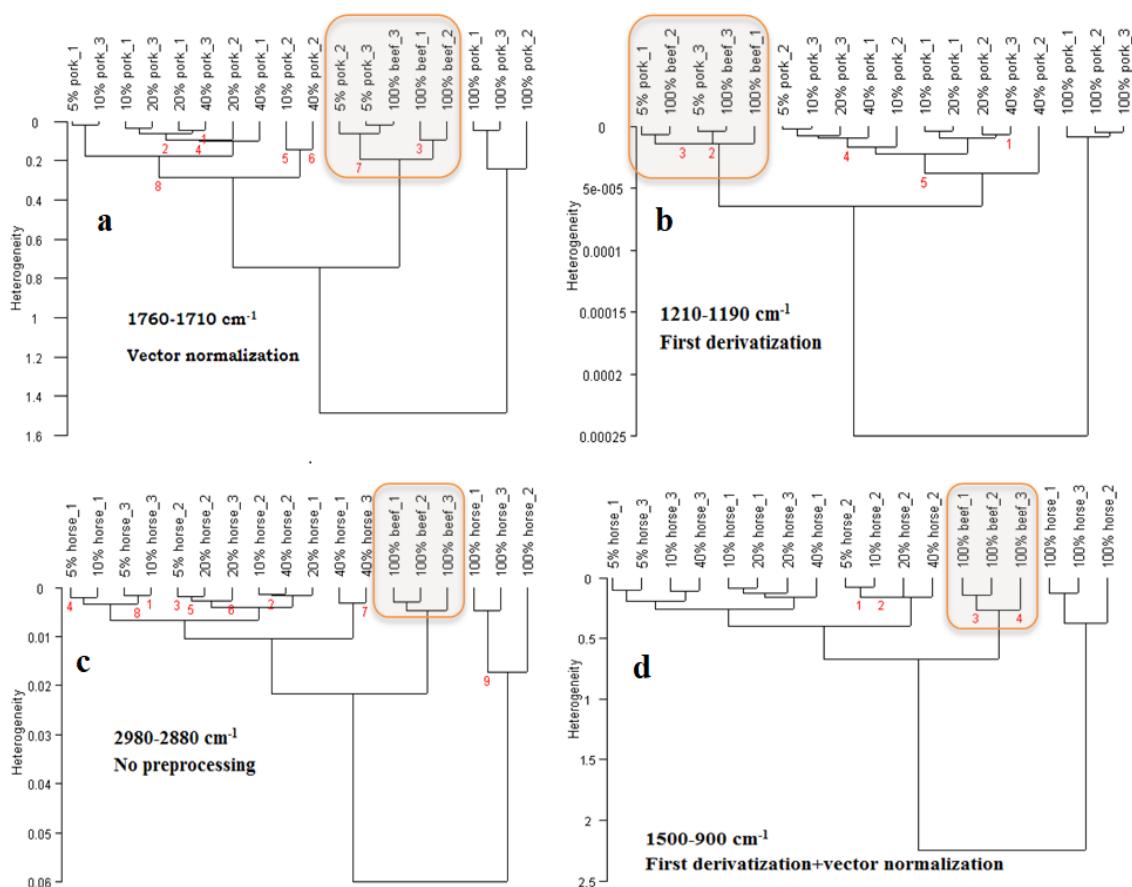


Figure 3. PCA-scores of spectra obtained from beef-pork, beef-horse and beef-donkey mixtures for various wavenumber ranges

Similar to PCA, HCA was also performed using the wavenumbers of each characteristic spectral region and peak, as well as whole spectrum and the fingerprint region with different preprocessing types as mentioned before. In order to avoid confusions, only the sample clusters that provided the best classifications are discussed and given in Figure 4. In beef-pork mixtures, region 2 (1760-1710 cm^{-1}) and peak-9 (1210-1190 cm^{-1}) could be used for differentiation of pork meat adulteration level above 5% with 100% sensitivity and specificity because two samples of 5% pork meat were located in the same cluster branch as 100% pure beef resulting in low sensitivity of 33% (Figure 4, a and b). In these dendrograms, 100% pure pork samples, 100% pure beef and adulterated mixtures were grouped in three separate clusters. To distinguish horse meat from beef, 2980-2880 cm^{-1} (peak 1 and

peak 2, non-preprocessing), whole spectrum and fingerprint region were identified as giving the best results which were able to discriminate all the adulterated mixtures from 100% beef with 100% sensitivity and specificity, as shown in the representative dendrograms in Figure 4 (a-g). HCA was successfully used for differentiation with three different preprocessing methods (first derivatization, first derivatization+vector normalization and vector normalization) based on the whole spectrum and the fingerprint region. In donkey-beef mixtures, peak 6 (1480-1425 cm^{-1}) with vector normalization provided the best differentiation in HCA by classifying 100% beef, 100% donkey meat and their mixtures in separate groups with 100% sensitivity and specificity (Figure 4, h).



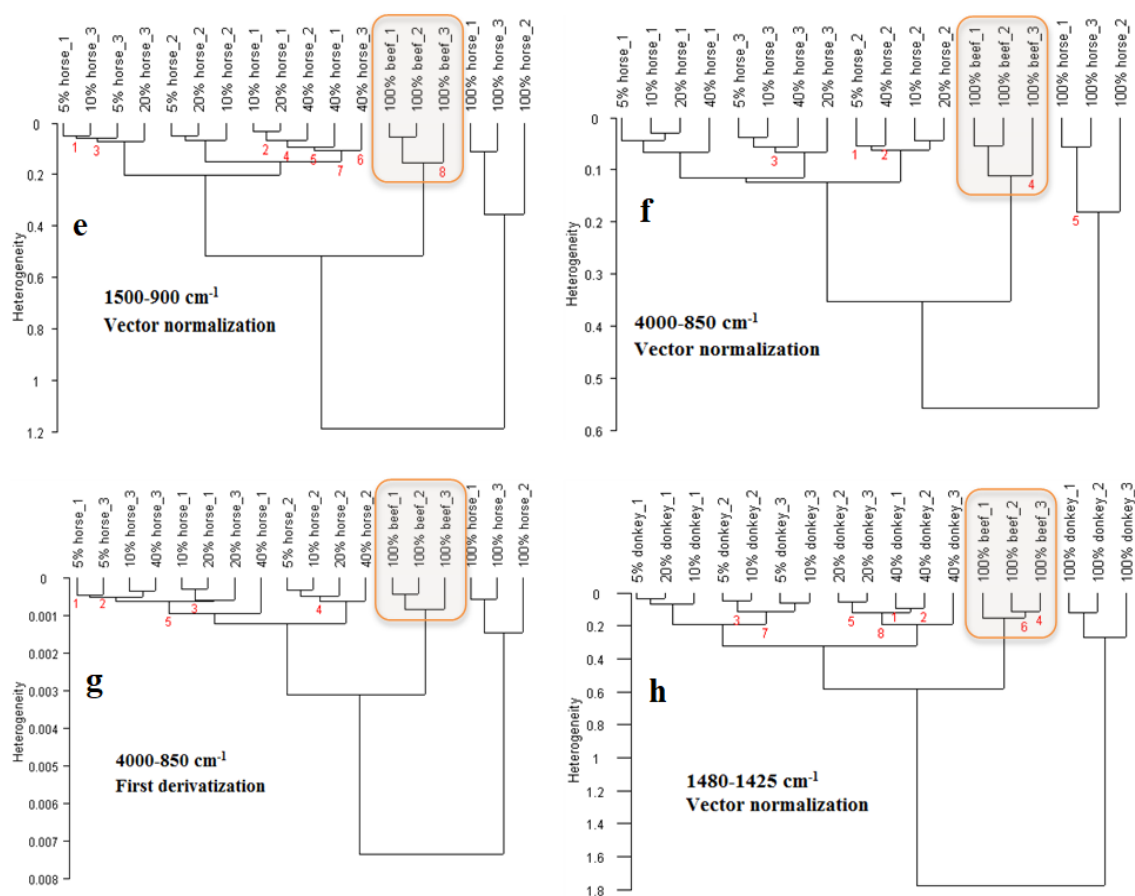


Figure 4. Dendrograms of the mean spectra of beef-pork (a, b), beef-horse (c, d, e, f, g) and beef-donkey (h) mixtures with different preprocessing for various wavenumber ranges

CONCLUSIONS

In the FTIR spectra of the meat mixtures prepared with beef and pork, donkey or horse meat, 12 peaks and 5 shoulders were detected. Alterations in the intensities of the peaks were observed depending on the percentage of adulterant meat used. Zoomed view of the FTIR spectra provided significant distinction of the specific peaks from beef as well as from other mixtures in terms of intensity. Differentiation of all adulterated mixtures from pure samples was accomplished by PCA based on fingerprint region. Moreover, 1480-1360 cm^{-1} ; 1760-1710 cm^{-1} ; 1290-1210 cm^{-1} and whole spectrum also provided better discrimination for beef-pork, beef-donkey and beef-horse mixtures, respectively, using PCA. While HCA with different preprocessing types could be as effective

as PCA in distinguishing beef-donkey or beef-horse meat mixtures, it could be used for differentiation of pork meat adulteration only above 5% level based on our results. Although we did not observe significant discrimination between adulteration levels (5-10-20-40% groups), our results are still promising since the presence of the adulteration can be detected considering all mixture percentages as one group in PCA and HCA. The results obtained from this study demonstrated that FTIR spectroscopy is a promising technique for the detection of beef mixtures adulterated with pork, horse or donkey meat and deserves further study. The distinctive signals detected in the present study from FTIR spectral data could be successfully used to establish biomarkers for identification of fraudulent meat mixtures.

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