



Analytical Methods

Raman spectroscopy in determination of horse meat content in the mixture with other meats

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ABSTRACT

A new method based on FT-Raman measurements that allows to determine the content of horse meat in its mixture with beef has been proposed. In the analysis of the Raman spectra of the meat mixtures, the integral intensity ratios of the 937/1003, 879/1003, 856/1003, 829/1003, and 480/1003 cm^{-1} pairs of bands have been determined the intensities of which were related to the reference intensity of the band at 1003 cm^{-1} . The reasonable results that show good fitting between the spectroscopic parameters and chemical content of the studied samples have been obtained. The analytical equations between these parameters have been proposed.

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1. Introduction

The history of foodstuff adulteration has accompanied mankind since food became a commodity for sale. Since the nineteenth century, the Industrial Revolution intensified the problem of counterfeiting of food. It was nothing unusual that the bread bakers added chalk and brewers replaced part of malt with beet sugar during the brewing process.

Historically, meat was difficult to adulterate because it was usually sold as fresh and in a non-processed form (Hargin, 1996).

Currently, a large proportion of meat is delivered to consumers in the form of processed meat such as minced meat, meat fillings, etc. During the production of minced meat morphological characteristics of the muscles that allow identification of the species and the part of the body from which it was cut out are destroyed. For this reason, minced meat can be easily forged by an addition of lower quality meat or meat derived from other species of animals for slaughter (Meza-Márquez, Gallardo-Velázquez, & Osorio-Revilla, 2010).

In 2013, the coordinated EU-wide testing for horse meat DNA and phenylbutazone requested, and co-financed, by the European Commission in the wake of the horse meat scandal has revealed that less than 5% of the tested products had horse DNA and that about 0.5% of the equine carcasses tested were found to be contaminated with phenylbutazone (europa.eu, 2013).

There are a number of techniques to identify the origin of species present in sold meat. These are the research methods in

the field of immunology, enzymology, proteomics and molecular biology (Gasior-Glogowska et al., 2013). Most of these techniques are characterized by high reliability and sensitivity in detecting forgeries of meat products. However, these methods are expensive and time consuming because they require prior preparation of the samples by extraction of proteins, metabolites, or DNA, from the tested samples of meat (Ballin, 2010; Meza-Márquez et al., 2010).

In the muscle, glucose from blood and glycogen accumulated in muscles are the main source of glucosyl units for glycolysis. The amount of glycogen in muscles depends on the species and the type of muscle. Glycogen concentration in the dorsal muscles of cattle (longissimus muscle) ranges from 60 to 100 mmol/kg (wet) tissue but in horses the concentration is usually 1.5 times higher than that in cattle (Pösö & Puolanne, 2005). Glycogen as a metabolite plays a key role in anaerobic glycolysis in the muscles after death of the animal. It is the primary substrate for the synthesis of lactic acid and consequently the acidification of the muscle. It has been found that 80–90 mmol of lactic acid is sufficient for maximal lowering of the pH value of 1 kg of (wet) muscle mass, from 7.2 to 5.5. This lactic acid concentration corresponds to 40–45 mmol of glycogen per 1 kg of (wet) muscle mass (Immonen, Schaefer, Puolanne, Kauffman, & Nordheim, 2000).

Near-infrared and Fourier transform Raman (NIR, FT-Raman) spectroscopy methods are well-suited to the analysis of biological materials because they are non-destructive and no chemical pre-treatment is required (Gasior-Glogowska et al., 2013). Currently, on the increasing availability of the equipment, Raman spectroscopy is widely used, among others, in tissue research, microbiology analysis of food and agricultural products (Argyria et al., 2013).

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The application of Raman spectroscopy for quality assessment of meat was reviewed in the paper by [Herrero \(2003\)](#). In this work several papers taking into account the results obtained by this technique were cited and its usefulness was confirmed by comparison of these results to those obtained by different traditional methods such as protein solubility, apparent viscosity, water holding capacity, instrumental texture determination, dimethylamine content, peroxide values and fatty acid composition. The changes in the Raman spectra of proteins, water and lipids of muscle in fish and meat were registered for the food substances subjected to various conditions during handling, processing and storage.

The application of various IR and Raman spectroscopic methods to the analysis of valuable plant substances and quality parameters in horticultural and agricultural crops was reviewed by [Schulz and Baranska \(2007\)](#). These measurements can be performed on plant tissues or fractions isolated from these biological materials and the obtained results allow to characterize these samples using the key bands of their components. Such methods as the ones applied to the plant species were also used to food due to their rapid and non-destructive properties ([Li-Chan, 1996](#); [Ozaki, Cho, Ikegaya, Muraishi, & Kawauchi, 1992](#)). The Raman technique is particularly suitable because no sample preparation is required and the measurements can be done utilizing fiber optics. It is important in these studies that the influence of water on Raman spectra is almost negligible in contradiction to IR spectra. The number of the articles related to the application of Raman spectroscopy in food analysis has been particularly increasing in recent years ([Beattie, Bell, Farmer, Moss, & Patterson, 2004](#); [Wold, Marquardt, Dable, Robb, & Hatlen, 2004](#)). In food science these studies were focused on the analysis of pure oils and fats ([Barthus & Poppi, 2001](#); [Sadeghi-Jorabchi, Hendra, Wilson, & Belton, 1990](#)), their cis/trans isomer ratio ([Bailey & Horvat, 1972](#); [Sadeghi-Jorabchi, Wilson, Belton, Edwards-Webb, & Coxon, 1991](#)) as well as the content of fatty acids in milk fat ([Meurens, Baeten, Yan, Mignolet, & Laron-delle, 2005](#)). The Raman spectra were successfully used for the characterization of the fatty acid unsaturation of salmon ([Afseth, Wold, & Segtnan, 2006](#)). It should be noted that the number of works which use Raman and IR techniques in the studies of other than adipose components of fish and animal tissues is still limited.

In the present paper we used Fourier transform Raman method to quantify protein features like the amino-acids content in the meat samples. The aim of this work is to show that the Raman technique can also be used in the studies of horse meat content in a meat mixture that consists of other types of meat, e.g. beef in our case. The IR spectra of the studied samples were also measured for the purpose of comparison. The studied by us samples have a wide range of the protein composition variation, and clear spectral characteristics of the type of amino-acids content. In our work a set of meat samples with naturally varying protein composition was analyzed. There are two main objectives of the study. First, measuring IR and Raman spectra of ox and horse back muscles as well as a series of their mixtures of different compositions. Second, acquisition of their spectral bands and unification of their intensities for the qualitative evaluation of the main amino acids content in the studied samples. The analytical relationships between the integral band intensities and the content of horse meat in the mixture were developed. Such a and similar approach could be used as an analytical tool that allows to detect and fix the content of horse meat in its mixtures with other types of meat.

2. Materials and methods

Fresh samples of ox and horse back muscles (each approximately 400 g) were purchased from a local butcher. The procedure of the sample preparation was standardized i.e. it was the same for

all studied samples. The homogenization and spectroscopic studies of the samples of fresh meat were performed the same day. The samples were homogenized using one step homogenizer within 30 s and next kept cold at about 2 °C before the spectral measurements. Approximately 40 g of beef and horse meat were frozen at –80 °C and freeze-dried overnight using a Labconco FreeZone laboratory freeze dryer 4.5 L.

In this paper the meat mixture was prepared from horse meat and beef of the composition 1:4, 1:2, 3:4, respectively. The series of the studied samples was completed by the clear beef and horse meat, i.e. its numerical strength was five and for all sample three identical compositions were used in the spectroscopic studies.

IR spectra at room temperature were measured in the spectral range 400–4000 cm^{–1} using a Thermo Scientific Nicolet 6700 FT-IR spectrometer with 2 cm^{–1} resolution. The ATR-diamond ATR equipment was used for recording the reflection spectra.

Raman spectra were measured with a Bruker RFS 100/S FT-Raman spectrometer (Bruker, Karlsruhe, Germany). A diode-pumped Nd:YAG laser at 1064 nm with an output of 400 mW was used as the excitation source. The spectral resolution was 2 cm^{–1} and 250 scans were collected.

Three replicate IR and Raman spectra were recorded for every sample. The procedures for spectroscopic measurements were standardized. The same spectral parameters, the power of excitation source, the acquisition and accumulation were applied to all studied samples.

3. Data analysis

All IR and Raman spectra were processed in the same way prior to the used statistical analysis. In the first step, the replicate spectra were compared and analyzed by the commercial computer software (Origin Pro 5, OriginLab). The analysis included a background subtraction and deconvolution of the experimental bands into the Lorentz components. To eliminate an accidental intensity variation in the general intensity level all integral intensities of the observed bands were standardized using the statistical R^2 coefficient of determination. Because in our case it concerns each simple linear regression, then it is the squared correlation between the outcomes and the values of the single regressor used for prediction, or for the number of explanators more than one, then it is the square of the coefficient of multiple correlation. The R^2 values are automatically determined as output of the Origin software. We used GraphPad Prism 4 software to estimate the mean and standard deviation (SD).

4. Discussion of the results

[Figs. 1 and 2](#) present the IR and Raman spectra of horse meat and beef both in the wet and dehydrated stage. [Table 1](#) lists the wavenumbers of the observed bands together with their assignments to the respective normal modes characteristic for the biochemical components of the meats.

The comparison of the IR spectra of wet and dehydrated beef and horse meat allows to derive some essential conclusions on the differences between the studied samples. Dehydrated samples exhibit clear lowering of the intensity of the broad contour at about 3250 cm^{–1} that corresponds to the hydrogen bonds formed between the OH and NH₂ groups of glycogen and aminoacid components of the meat. Clear increase of the band intensity is observed for the series of the bands in the range 1000–1250 cm^{–1} for beef. These bands correspond to the characteristic bands of proteins present in the meats (see assignment in [Table 1](#)). However, these differences do not have any analytical meaning, because for

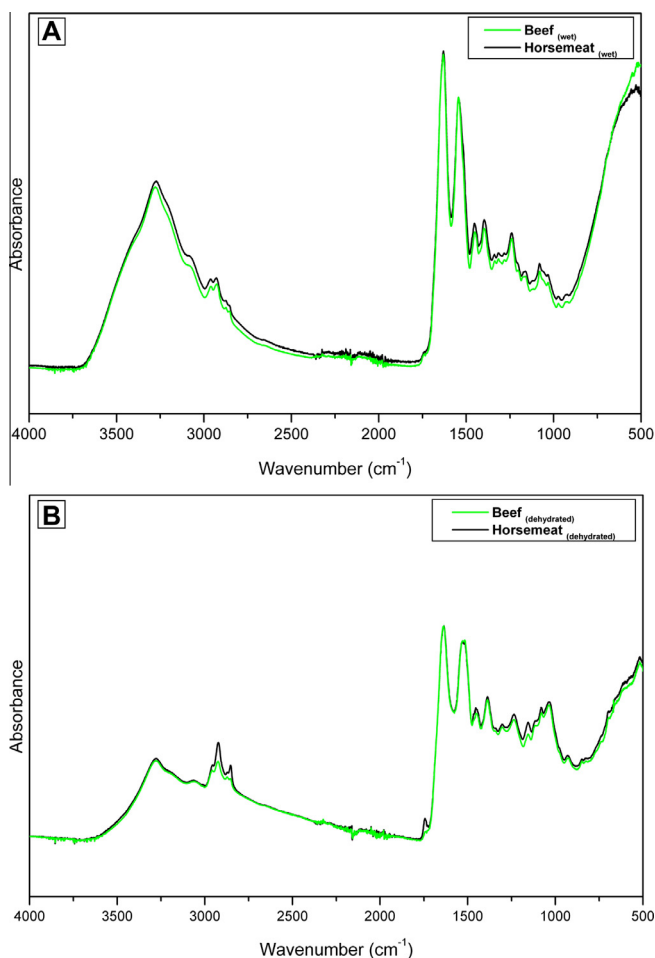


Fig. 1. ATR-FT-IR spectra of the wet (A) and dehydrated (B) beef and horse meat samples.

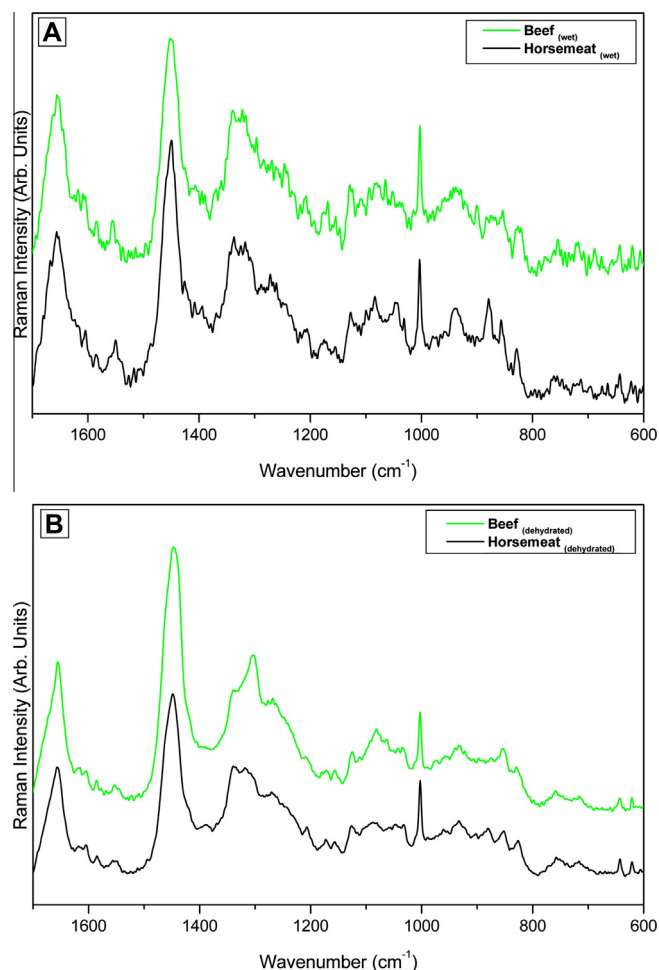


Fig. 2. FT-Raman spectra of the wet (A) and dehydrated (B) beef and horse meat samples.

our analysis the bands the intensity of which is higher for horse meat are important.

Several lines observed in the Raman spectra can have analytical importance in identification of horse meat in the mixture with other types of meat. The comparison of the spectra in Fig. 2 allows to distinguish a series of bands at 937, 879, 856, 829 and 480 cm^{-1} that can be used for the identification of horse meat in its mixture with beef.

The assignment of these bands to the respective vibrations of amino acids is possible when the following data are taken into account:

1. Several chemical parameters like proximate composition, pH and water activity (a_w) in several types of meats from farm and wild animals have been measured and reported. (Paleari, Moretti, Beretta, Mentasti, & Bersani, 2003). Among them horse and bovine meats were compared and the contents of cholesterol, fatty acids and amino acids were studied. For raw horse meat the following parameters were obtained: moisture 76.4%, proteins 20.3%, fat 2.1% and ash 1.3%. Respective data for bovine meat were: moisture 73.6%, proteins 22.2%, fat 2.9% and ash 1.3%. The content of these components changes for cured products, taking the values; for horse meat: moisture 49.2%, proteins 39.7%, fat 3.4% and ash 7.7% and for bovine meat: moisture 55.4%, proteins 34.6%, fat 4.9% and ash 5.1%

(Paleari et al., 2003). Such results prove that in the Raman spectra of wet and dehydrated fresh horse and bovine meats, the most intense bands should be assigned to the proteins.

2. The content of free amino acids in cured meats was measured by Paleari et al. (2003). Among nineteen studied samples the highest content was reported for glutamic acid (Glu), lysine (Lys), leucine (Leu), alanine (Ala), valine (Val), phenylalanine (Phe) and isoleucine (Ile). The content of these amino acids in horse meat was as follows: Glu – 303, Lys – 251, Leu – 241, Ala – 199, Val – 164, Phe – 140 and Ile – 122 mg/100 g. These values for bovine meat were Glu – 167, Lys – 162, Leu – 116, Ala – 177, Val – 93, Phe – 51 and Ile – 73 mg/100 g. These amino acids due to their beneficial content in meat proteins should influence the IR and Raman band intensities of the studied here meat samples most.
3. Raman spectra of amino acids were recorded by several authors. The collection of these data was presented by Zhu et al. for 18 kinds of L-amino acids in solid state and aqueous solutions (Zhu, Zhu, Fan, & Wan, 2011) and by Culka et al. for 13 amino acids comparing the spectra recorded by different spectrometers (Culka, Jehlička, & Edwards, 2010). They showed the Raman spectra, the data on wavenumbers of Raman bands appearing in the spectra as well as the description of the band intensities. The assignment of the most characteristic bands of these compounds to the vibrational normal modes was also proposed.

Table 1

Wavenumbers of the bands observed in the IR and Raman spectra of the studied meat samples together with the proposed assignments of the bands.

Peak position (cm ⁻¹)	Assignments ^a	References
480		Glycogen marker Castillo, Lo, Kuck, and Yu (1992)
510–545	$\nu(\text{SS})$	Cysteine Konorov, Schulze, Piret, Turner, and Blades (2011)
829	$\gamma(\text{COO})$	Phenylalanine Leucine Valine Lactic acid Glutamic acid Alanine Valine Lysine Zhu, Zhu, Fan, and Wan (2011)
856	$\nu(\text{C-CN})$	Glutamic acid Alanine Valine Lysine Zhu et al. (2011)
879	$\nu(\text{C-C-N})$	Glutamic acid Lysine Culka et al. (2010)
937	$\nu(\text{C-CN})$	Lysine Glutamic acid Zhu et al. (2011)
1003	phe ν -ring	Phenylalanine Castillo et al. (1992) El-Hagrasy, Shimizu, Saito, Yamaguchi, and Tamiya (2012) Xu et al. (2011) Gasiór-Głogowska et al. (2013) Schmidt, Scheier, and Hopkins (2013) Konorov, Schulze, Piret, Turner, and Blades (2011) Beattie, Bell, Farmer, Moss, and Patterson (2004) Zhu et al. (2011) Culka et al. (2010)
1031	phe δ -ring	Phenylalanine Zhu et al. (2011)
1082	$\nu(\text{CC})$	Lipids El-Hagrasy et al. (2012) Lyndgaard, Sørensen, van den Berg, and Engelsen (2012) Gasiór-Głogowska et al. (2013)
1126	$\nu(\text{CN})$ $\nu(\text{CC})$	Proteins Lipids El-Hagrasy et al. (2012) Xu et al. (2011) Lyndgaard, Sørensen, van den Berg, and Engelsen (2012) Gasiór-Głogowska et al. (2013)
1156	$\nu(\text{CN})$ $\nu(\text{CC})$	Proteins El-Hagrasy et al. (2012)
1208	phe δ -ring	Phenylalanine Zhu et al. (2011)
1247	Amide III	Proteins β -helix Xu et al. (2011) El-Hagrasy et al. (2012)
1304	Amide III	Proteins α -helix Xu et al. (2011)
1317	$\delta(\text{CH})$	Proteins Lipids Xu et al. (2011)
1337	$\delta(\text{CH})$	Proteins Lipids Xu et al. (2011)
1450	$\delta(\text{CH}_2, \text{CH}_3)$	Proteins Lipids Gasiór-Głogowska et al. (2013) Xu et al. (2011)
1551	Amide II	Proteins El-Hagrasy et al. (2012)
1604	$\nu(\text{C=O})$	Phenylalanine Zhu et al. (2011)
1655	Amide I, C=O stretch and N-H wag	Proteins α -helix Beattie et al. (2004)
1667	Amide I, C=O stretch and N-H wag	Proteins β -helix Beattie et al. (2004)
2934	$\nu(\text{CH}_2, \text{CH}_3)$	Proteins Xu et al. (2011)
3200–3600	$\nu(\text{NH})$, $\nu(\text{OH})$	Lipids Amide A,B, water Gasiór-Głogowska et al. (2013)

^a ν – Stretching; γ – out-of-plane bending; δ – in-plane bending.

The discussed above data were applied by us for identification of horse meat content in its mixture with bovine meat on the basis of their Raman spectra. It was performed for the chosen Raman bands that appeared to be the most characteristic for the studied meats and their mixtures. The bands with the highest intensities among those observed in the Raman spectra of amino acids (Culka et al., 2010; Zhu et al., 2011) should give the highest contribution into the spectra of horse and bovine meat. We propose the following description of these bands:

- The Raman band at 937 cm⁻¹ originates from the $\nu(\text{C-CN})$ vibrations of Lys (945 cm⁻¹) and glutamic acid (941 cm⁻¹).
- The band at 879 cm⁻¹ should be assigned to the overlapping $\nu(\text{C-C-N})$ vibrations of the Glu (873 cm⁻¹) and Lys (877 cm⁻¹).

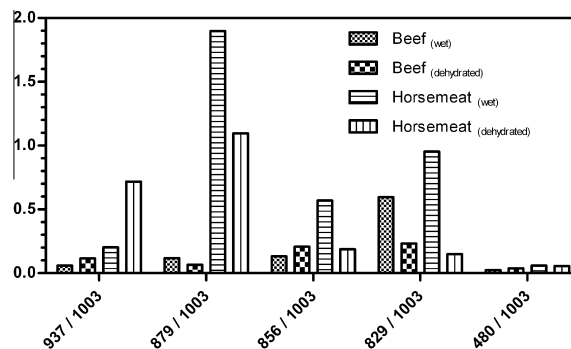


Fig. 3. Illustration of relative intensities of the Raman bands at 937, 879, 856, 829 and 480 compared to the integral intensity of the band at 1003 cm⁻¹ for the wet and dehydrated beef and horse meat ($N = 1$).

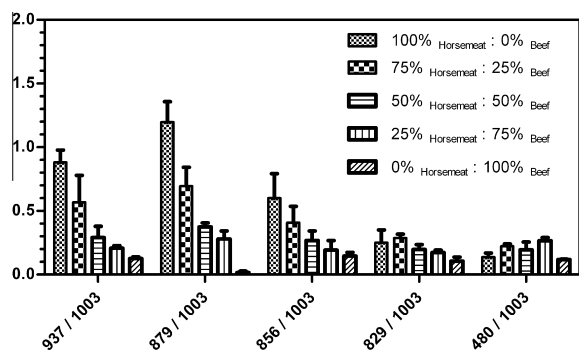


Fig. 4. Relative intensities of the Raman bands at 937, 879, 856, 829 and 480 compared to the integral intensity of the band at 1003 cm^{-1} for the mixtures containing changing amount of horse meat (Mean; SD; $N = 3$).

- The wavenumber 856 cm^{-1} corresponds to the $\nu(\text{C-CN})$ coupled vibrations of Glu (849 cm^{-1}) + Ala (852 cm^{-1}) + Val (850 cm^{-1}) + Lys (845 cm^{-1}).
- The band at 829 cm^{-1} originates from the out-of-plane $\gamma(\text{COO})$ modes of the lactic acid (830 cm^{-1}), Phe (821 and 835 cm^{-1}), Leu (836 cm^{-1}) and Val (825 cm^{-1}).
- The line at 480 cm^{-1} could be assigned to the out-of-plane of the skeleton in glycogen marker (Konorov, Schulze, Piret, Turner, & Blades, 2011).

The given in parentheses wavenumbers were reported in the cited above papers (Zhu et al., 2011) as having strong and very strong intensity in the Raman spectra of respective amino acids in the solid and aqueous solutions.

In the analysis of the Raman spectra of the meat mixtures the integral intensity ratios of the following pairs of bands were

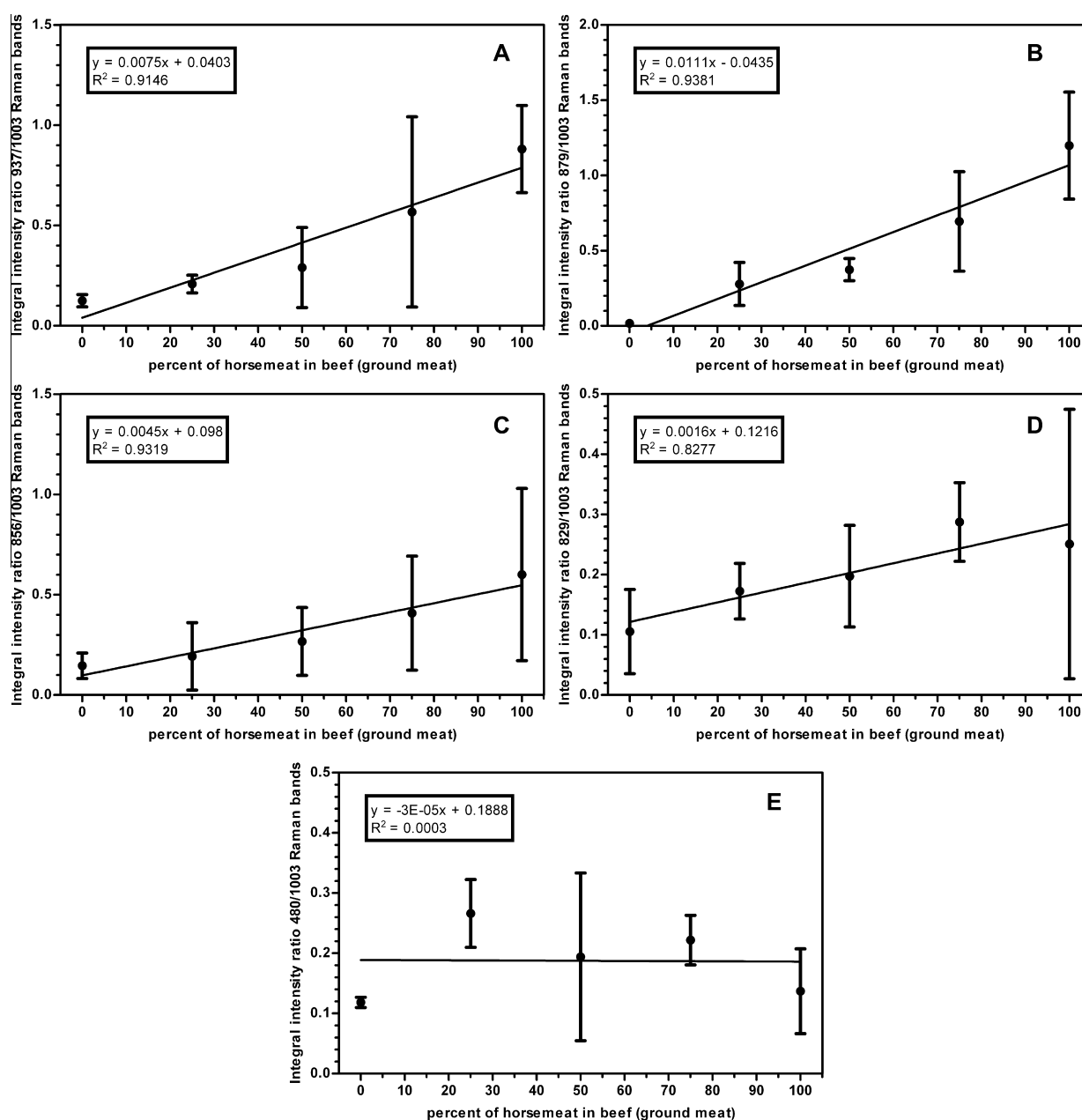


Fig. 5. Linear relationships between the peak area of the Raman bands at 937 (A), 879 (B), 856 (C), 829 (D) and 480 cm^{-1} (E) and percentage content of horse meat in the mixture with beef (Mean; SD; $N = 3$).

considered: 937/1003, 879/1003, 856/1003, 829/1003 and 480/1003 cm^{-1} whose intensities were related to the reference intensity of the band at 1003 cm^{-1} . It corresponds to one of the most stable stretching vibrations of the v-ring of the proteins that do not change their Raman bands intensity (Xu, Han, Fei, & Zhou, 2011). Figs. 3 and 4 illustrate the relations between these intensity ratios and the percentage of horse meat in the mixture with bovine meat for the wet and dehydrated samples. Fig. 5 presents the linear relationships between the peak area of the Raman bands at 937, 879, 856, 829 and 480 cm^{-1} and percentage of horse meat in the mixture with beef.

The equations that describe these dependencies can be expressed in the analytical form:

- $I_{937}/I_{1003} = 0.0075 C_{H/V} + 0.0403$ ($R^2 = 0.9146$).
- $I_{879}/I_{1003} = 0.0111 C_{H/V} - 0.0435$ ($R^2 = 0.9381$).
- $I_{856}/I_{1003} = 0.0045 C_{H/V} + 0.098$ ($R^2 = 0.9319$).
- $I_{829}/I_{1003} = 0.0016 C_{H/V} + 0.1216$ ($R^2 = 0.8277$) - the band at 829 cm^{-1} corresponds mainly to the lactic acid vibration. The lactate concentration is a physiological variable.
- $I_{480}/I_{1003} = -3E-05 C_{H/V} + 0.1888$ ($R^2 = 0.0003$) - no dependency is observed where $C_{H/B}$ denotes the percentage of horse meat in the mixture with bovine meat.

5. Conclusions

The aim of this study was to propose a new method based on the FT-Raman measurements that allows to determine the content of horse meat in its mixture with beef. Using this method we obtained reasonable results that show good fitting between the spectroscopic parameters and chemical content of the studied samples.

The proposed method has a number of advantages over other techniques. It is fast and could be used straight to analyze the fresh meat mixtures. In contrast to the IR spectroscopic method the baseline of the Raman spectra does not play a crucial role in the use of developed analytical relationships because the relations between the integral intensities of the respective Raman bands standardize the obtained results. The good fitting between the experimental chemical content of the studied meat mixtures (the content of the specific for both meats amino-acids) and integral intensities of the Raman bands characteristic for these amino-acids makes this method seem to be reasonable.

We believe that proposed by us a new approach to the determination of the content of some type of meat in the mixture with other class meat will be useful and accepted by the researchers in the field of food sciences.

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