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Original Article

Raman spectroscopy coupled with chemometric methods for the discrimination of foreign fats and oils in cream and yogurt

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ABSTRACT

The adulteration of milk fat in dairy products with cheaper non-milk based fats or oils is frequently encountered in the dairy industry. In this study, Raman spectroscopy with chemometric was used for the discrimination of foreign fats and oils in milk cream and yogurt. Firstly, binary mixtures of cream and oils (corn and sunflower oil), and vegetable fat blends which are potentially or currently used by the dairy industry were prepared. All fat or oil samples and their binary mixtures were examined by using Raman spectroscopy. Then, fat content of skim milk was adjusted to 3% (w/w) by the milk fat, external oils or fats, and binary mixtures, and was used in yogurt production. The lipid fraction of yogurt was extracted and characterized by Raman spectroscopy. The spectral data were then pre-processed and principal component analysis (PCA) was performed. Raman spectral data showed successful discrimination for about the source of the fats or oils. Temperature effect was also studied at six different temperatures (25, 30, 40, 50, 60 and 70 °C) in order to obtain the best spectral information. Raman spectra collected at higher temperatures were more intense. Obtained results showed that the performance of Raman spectroscopy with PCA was very promising and can be expected to provide a simple and quick way for the discrimination of foreign fats and oils in both milk cream and yogurt. Fermentation and yogurt processing affected clustering of fat samples by PCA, probably depending on some lipolysis or production of new products that can affect the Raman scattering. However, those changes did not affect differentiation of samples by Raman spectroscopy.

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1. Introduction

Adulteration is an illegal practice, but it may be used by some food producers and suppliers to increase profits. Adulteration of foods for economic gain is an important concern for consumers as it may affect consumer health, food safety, food quality, and loss of value in food products [1]. Food safety problem has become a serious problem all over the world and this situation makes special interest for determination of adulteration [2].

Dairy products are among the most adulterated food products. Especially, the milk fat can be replaced with different origin of fats or oils because of the high commercial value of itself. The full or partial substitution of milk fat is not allowed in dairy products unless indicated by labeling or addition of foreign fats or oils to dairy products is completely forbidden. However, expensive milk fats have often been adulterated, with proportional to increasing demand for dairy products in the markets in order to reduce production cost and it induces problems associated with certification and quality control [3–6].

Several methods have been reported in the literature for detection of milk and dairy products adulteration such as electrical admittance spectroscopy, single frequency conductance measurements, digital imaging, chromatography, matrix-assisted laser desorption/ionization-quadrupole time of flight mass spectroscopy, isotopic ratio mass spectroscopy, nuclear magnetic resonance, ultraviolet–visible light spectroscopy, enzyme-linked immunosorbent assays and thermal analysis [7–11]. Especially, gas chromatography (GC), gas chromatography–mass spectroscopy (GC–MS) and high performance liquid chromatography (HPLC) are the common conventional methods. Analysis of fatty acid composition and sterols are the main study for identifying the adulteration [3,4,6,8,12,13]. Stable isotope ratio analysis is another method for detecting the oil adulteration with the ratio of ^{13}C and ^{12}C [14]. These methods are considered to be time-consuming, not cost effective and labor-intensive due to sample pretreatment and the need of expensive equipment. Therefore, there is an increasing demand for rapid, simple and green methods for determination of the adulteration.

Vibrational spectroscopy that includes Raman spectroscopy based methods could offer alternative techniques to conventional methods. Raman spectroscopy is based on scattering and contains vibrational energy levels that mainly related to stretching or bending deformations of bonds [15,16]. Raman spectroscopy, with characteristic vibrational fingerprints of molecules, provides rapid, easy and non-destructive analysis without or simple sample preparation step. These features make Raman spectroscopy convenient for the quick authentication purposes. In addition, using chemometric methods with Raman spectroscopy enhances the determination performance of the method. The art of extracting chemically relevant data by means of mathematical or statistical tools is called chemometrics [17]. Application of chemometric strategy can yield useful information even complete separation is not achieved [18]. Principal component analysis (PCA) in chemometric, is a multivariate method of analysis whose main purpose is to reduce the dimension of the dataset with low information loss [19,20]. The information of each spectrum is defined by limited variables called principal components (PCs). PCA makes sense of seemingly minor differences in the spectra and provides classification by these minor differences [6,15].

In recent years, the evaluation of food quality and authenticity by vibrational spectroscopy has increased dramatically. There are some studies that use chemometric methods with Raman or NIR spectroscopies for the determination of adulteration. Some of them are; fat adulteration in bakery products [21], milk adulteration with small nitrogen-rich molecules and sucrose [22], butter adulteration with margarine [10], milk powder adulteration with maltodextrin [23], beef adulteration with horsemeat [24] and differentiating the olive oils from other edible oils [25–27]. Mid-infrared (MIR) technology has been also used to test butter adulteration [28].

In a study, dairy cream and its analogs with sunflower oil, coconut oil and palm oil in different milk fat/vegetable fat ratios were analyzed using Raman spectroscopy and classified by the linear discriminant analysis. It was stated that sunflower oil and milk fat samples were separated well on the contrary to the samples with coconut and palm oil, where the substantial overlapping occurred [29]. Beside detection of adulteration, it was indicated that vibrational spectroscopy can be used for process monitoring during production and compositional analysis for yogurt, cheese and other fermented dairy products because these methods are rapid, nondestructive and cost effective in comparison with traditional methods [30]. There are also chromatographic techniques to detect adulteration of milk fat. Kim et al. [8] used GC to detect adulteration of milk fat based on the fatty acids, triacylglycerol and cholesterol levels. All components should be evaluated separately in GC method. However, in vibrational methods like Raman spectroscopy, the effect of all components can be determined with a single spectrum.

According to our knowledge, there is no study about the determination of fat adulteration in yogurt as a dairy product by Raman spectroscopy. There has been only one study about the protein adulteration in yogurt [31] and the others were about the milk adulteration with small nitrogen-rich molecules [22,32]. Studies, intended to determine the fat adulteration, generally were performed with only one foreign fat or oil. But, in this study, we have discriminated six different foreign fats or oils at one time. Also, the classifications were performed for both cream and yogurt. In this way, our study can meet the deficit of the literature. Our research is mainly concerned on to develop a fast, simple and green method for the discrimination of non-milk based fats or oils in milk cream and yogurt as a dairy product. For this purpose, the milk cream was adulterated with commercially readily available oil (corn, sunflower) or vegetable fat blends having a similar melting point of milk fat which are the common adulterants for dairy products. Yogurt samples were manufactured in which fat was replaced partially or totally with these oils or vegetable fat blends. Discrimination assessment of these samples was carried out based on the developed method, which uses Raman spectroscopy coupled with PCA. Moreover, the

temperature effect on the Raman spectra was also evaluated to obtain the best spectral information.

2. Materials and methods

2.1. Fat or oil samples

Six non-milk based fats or oils and six milk cream were analyzed. In order to extend the heterogeneity of the milk cream population, six samples of milk cream were purchased from different local dairy industries in Turkey. Each cream sample was at different production parties of the companies. Also, six different non-milk based fat or oil samples (three of them were commercially available vegetable fat blends coded as T18, T30, ST which were selected according to their potential or current use in dairy industry and preferred according to personal communication with dairy products manufacturer, and the rests were sunflower oil, corn oil and margarine) were obtained from local markets. Binary mixtures of six different cream and six vegetable fat blends or oils were prepared by mixing them to obtain 1:1 (w/w) fat ratio in the final product and totally 36 binary mixtures were obtained. All samples were stored at +4 °C until analysis. These samples were also used in yogurt production.

The abbreviations of C1, C2, C3, C4, C5, C6 for six different cream (milk fat) samples, T18, T30 and ST for different vegetable fat blends, CO for corn oil, SF for Sunflower oil, MG for margarine were used in this text. The binary mixtures of these fats/oils were displayed as using both of the abbreviations that correspond to relating fat/oil.

2.2. Yogurt production

Ultra-high temperature (UHT) skim milk was obtained from a local manufacturer in Turkey. Milk creams, external fats or oils, and their binary mixtures which are mentioned above were added to skim milk to obtain 3% (w/w) fat content in milk. Each prepared milk samples was homogenized at 50 °C by using homogenizer (Heidolph SilentCrusher M, Germany) and pasteurized. After pasteurization, they were used for set type yogurt production. Briefly, each milk sample was put into a plastic container (100 mL) and starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) was added at 45 °C, and then well stirred. Samples were incubated at 42–45 °C until yogurt pH reaches to 4.5–4.6. After fermentation, yogurt samples were stored at 4 °C until analysis. In this way, 36 adulterated yogurt samples were produced with binary mixtures, 6 adulterated yogurt samples were produced with external fats or oils, and control yogurt samples were produced with 6 different milk fats. Totally, 48 yogurt samples were produced.

2.3. Sample preparation and gas chromatography analysis

Before analysis, a lipid extraction procedure was done to remove matrix interface. Lipid extractions from the samples were performed according to Folch method with some modifications [33]. Briefly, 10 g of fat or oil and 20 mL of chloroform:methanol (2:1, v/v) were mixed together vigorously for 5 min. Subsequently, 6 mL of NaCl solution (2%, w/v) was added to the mixture, vortexed and centrifuged (10 min, 4000 *g*). The lower chloroformic phase was filtered through anhydrous sodium sulfate and collected. The filtrate was heated in a water bath at 65 °C and chloroform was evaporated under a nitrogen stream.

The lipid extraction was also performed for yogurt samples. But, the amount of sample was increased 2 fold with proportion to extraction solutions, and then the extraction steps as mentioned above were applied identically. The extracted lipid samples were stored in amber glass vials under refrigerated conditions until analysis. All chemicals were analytical grade and purchased from Merck (Darmstadt, Germany) unless specified otherwise.

The fatty acid composition of the extracted lipid samples was determined as fatty acid methyl esters (FAMEs) by gas chromatography. FAMEs were prepared according to the International Dairy Federation Standard [34]. ThermoScientific Trace GC Ultra (Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a flame ionization detector and a capillary column (TR-WaxMS 60 m × 0.25 mm i.d. and 0.25 μm film thickness, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to separate and detect the FAMEs. The injection volume was 1 μL. The helium carrier gas flow rate was 1 mL/min with a split ratio of 1:40. The temperature of the GC oven was adjusted to 120 °C for 1 min, then increased by 6 °C/min to a final temperature of 240 °C, at which point the samples were held for 10 min at this temperature. The injector and detector temperatures were 250 °C. Fatty acid methyl esters (FAMEs) were identified by comparison of retention times with authentic standards (Supelco 37 comp. FAME mix). Results were expressed as a percentage (%) of total fatty acids detected.

2.4. Raman spectroscopy

Raman spectroscopy measurements were carried out by a DeltaNu Examiner Raman Microscopy system (DeltaNu Inc., Laramie, WY) with a 785 nm laser source and a cooled charge-coupled device (CCD, at 0 °C) detector. The extracted lipid samples were heated to 70 °C in a water-bath before Raman spectroscopy analysis. The liquid or molten sample of 200 μL was transferred into a sealed glass Raman spectroscopy cuvette and kept at 70 °C before measurements, then immediately entered into the system. Raman spectra were obtained in the range of 200–2000 cm^{-1} at a resolution of 2 cm^{-1} with the constant measurement parameters (10 s acquisition time, three measurements for every sample, 100 mW laser power). Raman measurements were conducted triplicate for each sample. RSD values of triplicate measurements were lower than 7% ($p > 0.05$). Therefore, the average results were used for data processing.

2.5. Temperature effect

Before sample data collection by Raman spectroscopy, the temperature effect was also studied in order to obtain the best spectral information. For this purpose, extracted lipid samples were put into the Raman cuvettes and were kept in a water

bath at six different temperatures (25, 30, 40, 50, 60 and 70 °C) for 1 h. Then, Raman spectra of samples were collected immediately as described above.

2.6. Chemometric data treatment

Chemometric method, PCA, was used to determine variations in the data matrix, and to form groups using (dis)similarity between the fat or oil types (milk fat, vegetable fat blends, sunflower oil, corn oil, binary mixtures). Raman spectra of each sample were collected and pre-processed before PCA. For pre-processing, first derivative (Savitzky–Golay, filter width: 15, polynomial order: 2) and mean centering operations were applied to the raw data in order to suppress or reduce any variations during measurements such as small temperature differences, the variability associated with the total amount of fat/oil sample used, and other sources of variance affecting the intensity of the peaks. Pre-processed spectral data gives much more detail information about the sample by resolving the previously overlapped bands in the raw spectra.

The pre-processed data were then analyzed with PCA by Stand-alone Chemometrics Software (Version Solo 6.5, Eigenvector Research Inc., Wenatchee, WA,). Through PCA, the huge number of data set (totally 1024 data) transformed into sufficient amount of data that can define the whole spectrum. The obtained scores of PCA were placed in a 3D coordinate. Certain distinctions were obtained between milk fat, external fats or oils and their binary mixtures with PCA analysis. Loadings obtained from PCA scores of pre-processed Raman spectra were also placed in a 3D coordinate to identify the wavelengths, which are the responsible for engendering the sample discrimination.

3. Results and discussion

3.1. Fatty acid composition of samples

The fatty acid composition of the milk fat and external fat or oil samples were determined by GC. The results were given in Table 1. Milk fat, margarine, T18, T30, and ST had higher saturated fatty acids (SFA) content than monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), while vegetable oil (corn oil and sunflower oil) samples had high PUFA content as expected. These results are coherent with the previous knowledge.

Milk fat was different from vegetable oil samples in terms of short and medium chain fatty acids such as butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0) and lauric (C12:0) acids (Table 1). The overall fatty acid composition of milk fat was in agreement with the literature [2,3,35]. In addition, the level of SFA, MUFA or PUFA in milk fat was clearly different from the vegetable fats or oils.

Sunflower and corn oil were rich in PUFA, which were calculated as 57.04% and 54.75%, respectively. The fatty acid composition of oils was consistent with the literature [3,35,36]. Margarine, T18, T30 and ST are commercially available vegetable fat blends and generally used by dairy industry for the replacement of milk fat. According to GC results, the fatty acid composition of the margarine, T18, and ST were pretty close to

Table 1 – Fatty acid composition of fat or oil samples determined by GC analysis (% total fatty acids).

Fatty acid	Milk fat[a]	T18	T30	ST	Margarine	Sunflower oil	Corn oil
C4:0 (Butryic)	2.85	nd	nd	nd	nd	nd	nd
C6:0 (Caproic)	1.55	nd	0.21	nd	nd	nd	nd
C8:0 (Caprylic)	0.94	0.05	2.91	0.35	0.58	nd	nd
C10:0 (Capric)	2.14	0.04	2.88	0.32	0.54	nd	nd
C12:0 (Lauric)	2.64	0.59	42.81	4.04	7.83	nd	nd
C13:0 (Tridecanoic)	0.09	nd	0.05	nd	nd	nd	nd
C14:0 (Myristic)	9.81	1.12	15.17	2.27	3.44	0.08	0.04
C14:1 (Myristoleic)	0.90	nd	nd	nd	nd	nd	nd
C15:0 (Pentadecanoic)	1.21	0.04	nd	0.04	0.03	nd	nd
C16:0 (Palmitic)	30.69	41.78	10.62	39.04	33.42	7.47	10.85
C16:1 (Palmitoleic)	1.63	0.17	nd	0.19	0.15	0.10	0.11
C17:0 (Heptadecanoic)	0.72	0.10	0.03	0.09	0.08	0.04	0.07
C18:0 (Stearic)	10.44	4.55	21.28	5.60	6.44	4.19	2.07
C18:1 c9 (Oleic)	22.57	36.82	2.10	28.65	28.54	28.34	30.04
C18:1 c11(Vaccenic)	2.47	0.77	0.02	0.64	0.69	0.76	0.59
C18:2 (Linoleic)	2.22	12.58	1.58	17.63	16.51	54.94	53.88
C18:3 (Linolenic)	0.23	0.54	0.02	0.27	0.58	0.10	0.87
C20:0 (Arachidic)	0.19	0.38	0.26	0.33	0.33	0.32	0.45
C20:1 (cis-11-Eicosenoic)	0.20	0.18	nd	0.13	0.17	0.28	0.41
C22:0 (Behenic)	0.08	0.09	0.04	0.09	0.13	0.86	0.14
SFA	63.34	48.74	96.26	52.20	52.85	12.96	13.65
MUFA	27.77	37.94	2.12	29.61	29.55	29.48	31.15
PUFA	2.45	13.12	1.60	17.90	17.09	57.04	54.75
TUFA	30.22	51.06	3.72	47.51	46.64	86.52	85.9

[a] Mean value of six different milk fat samples. All determinations were carried out in duplicate and mean values were reported, nd: none detected, MUFA: mono-unsaturated fatty acid, PUFA: poly-unsaturated fatty acid, SFA: saturated fatty acid, TUFA: total unsaturated fatty acid.

each other. Only slight differences such as the relatively low content of lauric acid (C12:0), myristic acid (C14:0), stearic acid (C18:0), linoleic acid (C18:2) were detected in T18. Especially, the ST and margarine were almost similar. The highest oleic acid (C18:1c9) content of 36.82% was determined in T18. In addition, palmitic acid (C16:0) level was high in T18 (41.78%), ST (39.04%), margarine (33.42%) and milk fat (30.69%) compared with other fat or oil samples.

T30 had the interesting result with the extremely high (96.26%) SFA content, which is comprised by the high level of lauric acid (42.81%, C12:0), myristic acid (15.17%, C14:0) and stearic acid (21.28%, C18:0). The extremely high content of lauric acid makes differ T30 from the other fat or oil samples. Also, the lowest levels of oleic acid (2.10%, C18:1c9), linoleic acid (1.58%, C18:2), MUFA (2.12%) and PUFA (1.60%) were determined in T30. Similar fatty acid profiles were determined from the lipid extracts of yogurt samples (data are not shown). The variation of fatty acid profile of fats or oils is important for discrimination. In a study, it has been showed that FT-Raman can classify edible oil and fats according to their degree of unsaturation which was found a correlation with GC results [35]. Similarly, in this study, obtained classification by Raman spectroscopy was found to be related with fatty acid composition of the samples (Fig. 4). The results show that Raman spectra discriminate the samples by not only the degree of unsaturation but also the balance between fatty acid profiles.

3.2. *Temperature effect*

Temperature effect was studied in order to obtain the best spectral information. For this purpose, Raman spectral data of the extraction applied milk fat samples were collected at six different temperatures (25, 30, 40, 50, 60 and 70 °C). Fig. 1 shows Raman spectra of milk fat at different temperature ranges. Raman spectra of milk fat were dependent on physical state of fat, which was highly correlated with temperature. At room temperature (25 °C), band broadening increased and a decrease in the spectral feature were observed (Fig. 1). Milk fat was completely liquid above 40 °C, and the intensity of Raman spectra increased between the region of 600–1450 cm^{-1} with the temperature elevation that improves the accuracy of the determination and discrimination (Fig. 1). Similar results were found in a study that discriminates the adulterated olive oil from pure olive oil. Authors showed that discrimination accuracy of olive and soybean oil was improved around 80–90 °C due to the enhanced spectral selectivity [37]. In another study, Raman spectra of anhydrous milk fat (AMF) collected from 0 to 50 °C had more intense peaks at higher temperatures [38]. The authors stated that Raman spectra of AMF samples are dependent both on composition and physical state which are in agreement with our findings. However, in the present study, oils (corn oil, sunflower oil) showed no distinct intensity difference according to temperature elevation (data are not shown). In addition, vegetable fat blends (T18, T30, ST) showed similar spectral variations as milk fat.

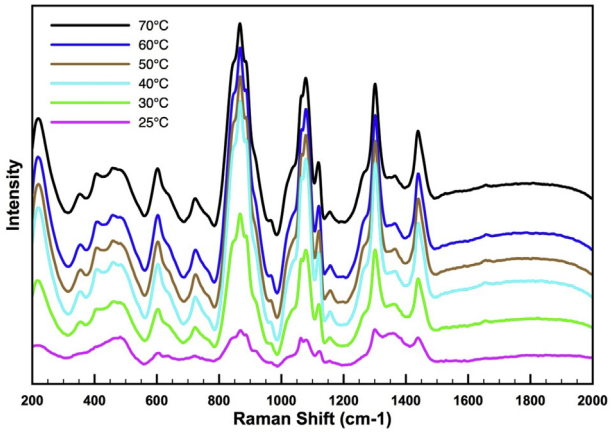

Fig. 1 – The raw Raman spectra of extraction applied milk fat sample collected at different temperatures.

To provide the unity and prevent any temperature effects, 70 °C was chosen as the standard measurement temperature in whole study because of the enhancement of spectral information.

3.3. *Raw Raman spectra of the samples*

Raman spectroscopy is designed to detect the scattered light from the sample and each of the scattered light generates a band in the Raman spectrum in the concerning wavelength shift. The main bands of fat or oil samples were at 483, 603, 670, 722, 846, 870, 876, 972, 1076, 1117, 1264, 1300, 1439 cm^{-1} (Fig. 2a). These bands are coherent with the results of the study of fat adulteration in bakery products [21]. Raman spectra with different intensities, could be assigned as follows; band at 1439 cm^{-1} to methylene scissoring deformation δ(CH2)sc [21,39], 1300 cm^{-1} to methylene twisting deformation δ(CH2)tw or to aliphatic ν (C–C)g stretch in gauche [39,40], 1264 cm^{-1} to deformation of in-plane *cis* double bond δ(=CH)ip [39], 1117 cm^{-1} to in-phase aliphatic C–C stretch all-*trans* ν (C–C)ip [10], 1076 cm^{-1} to aliphatic C–C stretch ν (C–C)g [39], 972 cm^{-1} to symmetric phosphate vibration [41], 876 cm^{-1} to C1 – C2 stretching vibration (the carbonyl carbon and the first carbon in the chain) ν (C1–C2), CH3 rocking and ν (C–O) [42]. Bands at between the range of 845–895 cm^{-1} may correspond to phospholipid group which contains 870-875 cm^{-1} choline band and 846 cm^{-1} inositol residue band [39]. The band at 603-607 cm^{-1} could be assigned to phospholipid group. The raw spectral variations on the region of 400–800 cm^{-1} of T30 were similar to milk fat, while margarine, T18, ST, corn and sunflower oil were considerably different (Fig. 2A). When we focus on the region at 1200-1400 cm^{-1}, the band at 1300 cm^{-1} was present in all samples, but in margarine, T18, ST, milk fat, corn and sunflower oil had an extra band at 1264 cm^{-1} probably correlated with unsaturation degree of samples. The band intensity at 1264 cm^{-1} increased with the degree of unsaturation of samples, that was in agreement with the GC results (Table 1). Decreasing order of samples within the context of total unsaturated fatty acid level (TUFA) was, sunflower oil, corn oil, T18, ST, margarine, milk fat and T30. The band at 1264 cm^{-1} was coherent with this order and in T30 the band disappeared because of the low TUFA content (3.72%) (Figs. 2A and 3A).

According to the similarity of specific Raman spectrum of the samples, we can visually distinguish these samples into three groups (Fig. 2A). The first group consisted of margarine, T18, and ST, the second group consisted of corn and sunflower

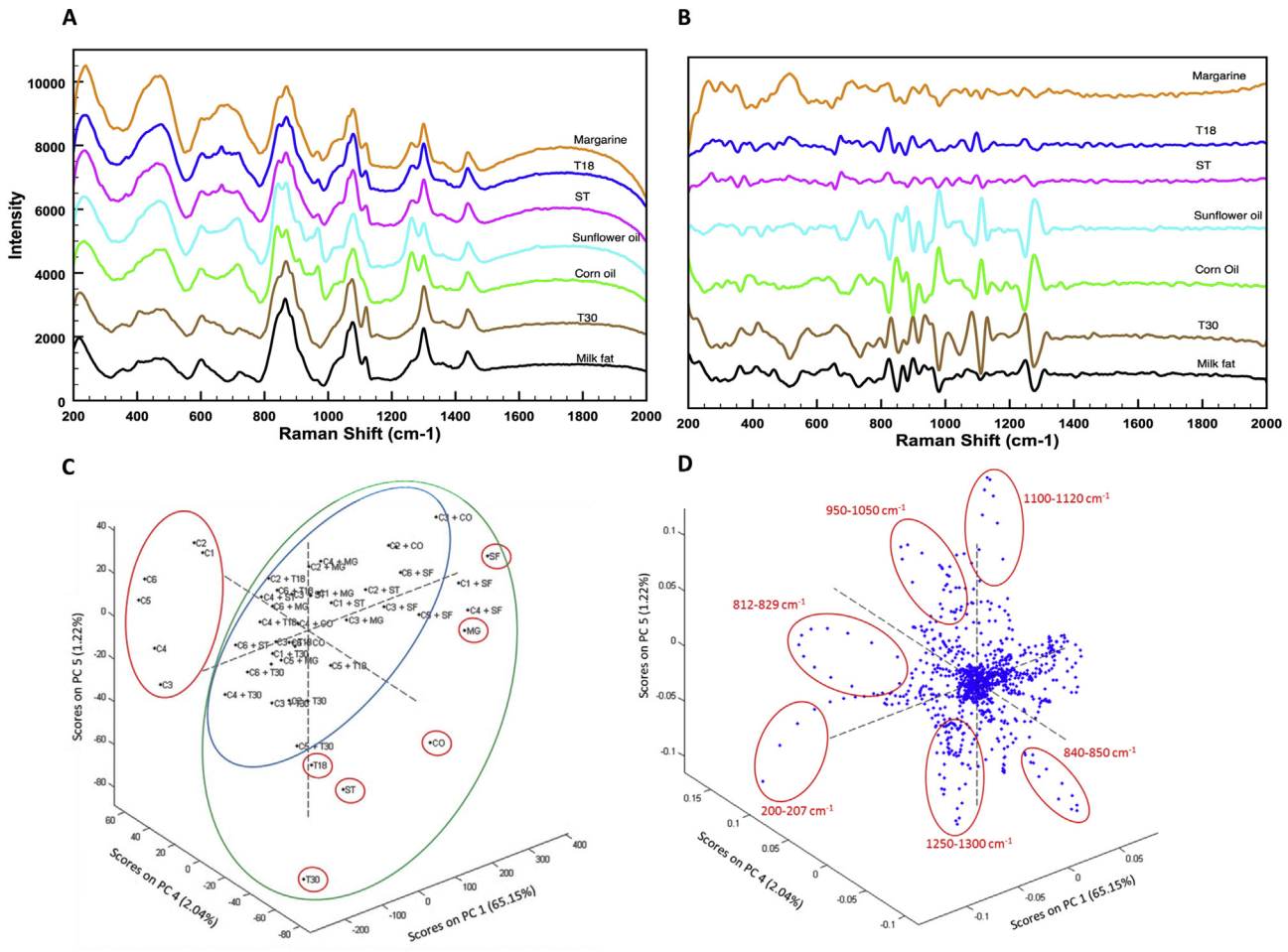

Fig. 2 – (A) Raw Raman spectra of samples, (B) pre-processed Raman spectra of samples (the first derivative, mean centering), (C) 3D PC scores of pure cream, other fat/oil samples and binary mixtures of them, (D) loading values of related PCs. C1, C2, C3, C4, C5, C6 corresponds to different milk fat samples, CO: Corn oil, MG: Margarine, SF: Sunflower oil, ST, T18, T30: Commercial vegetable fat blends.

oil and the third group consisted of T30 and milk fat. However, identification of differences in binary mixtures is difficult, so, an extra processing like PCA is needed to distinguish each fat samples.

To confirm the usability of this method in dairy products, 48 different yogurt samples were produced and lipid extraction were done for each yogurt sample. Raman measurements were performed for extracted lipids. The raw Raman spectra of extracted samples (milk fat, T18, T30, ST, margarine, corn oil and sunflower oil) from yogurt were given in Fig. 3A. Although yogurt is a fermented dairy product, this fermentation process did not influence the raw Raman spectra greatly. The main bands were similar to fat or oil samples that were given in Fig. 2A.

For sensitive discrimination of fats or oils and identification of adulteration from the raw spectrum, data processing and chemometric methods were needed.

3.4. *Chemometric data treatment of Raman spectra and classifying the samples*

As a chemometric data treatment, PCA that reduces multidimensional data down to a few dimensions is one of the most frequently used method. In this study, spectral changes of Raman bands were barely noticeable (Figs. 2A and 3A). Especially, discrimination of the binary mixtures of the adulterated samples was very difficult by the naked eye (Raman spectra are not given). So, to differentiate and visualize the minor differences, the entire raw Raman spectra were pre-processed before PCA analysis. All pre-processing techniques have the aim of reducing the un-modeled variability and enhancement of the interest spectra [43]. Before model was created, some examinations on pre-processing operations such as different derivative orders, baseline correction, smoothing, normalizing, auto scaling, mean centering were performed to find the best fitting model for clear discrimination of the samples. Then, first derivative and mean centering that resulted as the best fitting model was used for pre-processing of entire raw Raman spectra. The first derivative is generally used for the enhancement of spectral differences. The mean center is the average x and y coordinates and it is useful for tracking changes in the distribution or comparing the distributions of different types of features [17]. The pre-processed spectra of the extracted lipids from fats and oils and from yogurt samples were given in Figs. 2B and 3B, respectively. It can be clearly seen that minor differences in the raw data became

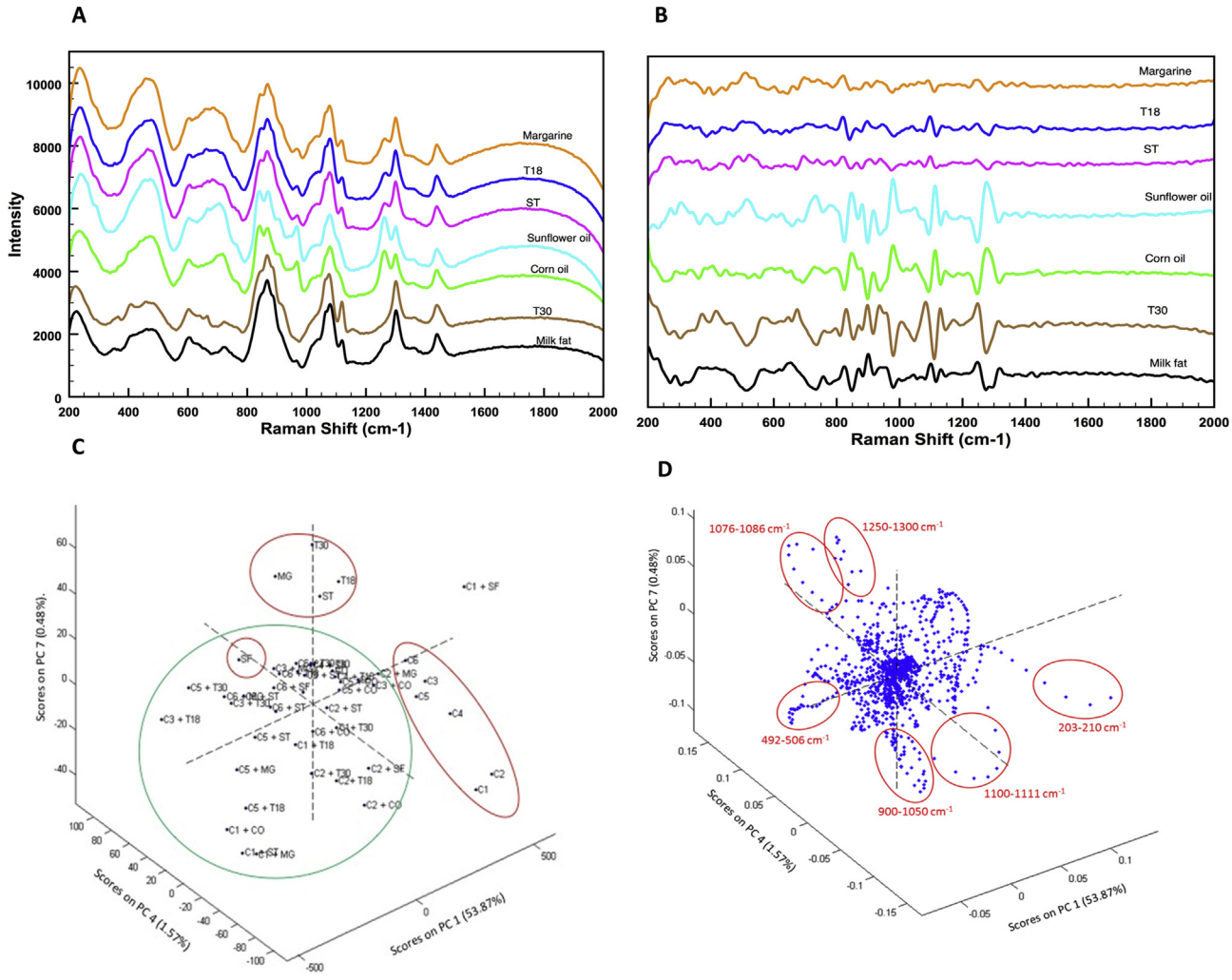

Fig. 3 – (A) Raw Raman spectra of extracted samples from yogurt, (B) pre-processed Raman spectra of extracted samples from yogurt (first derivative, mean centering), (C) 3D PC scores of extracted milk fat, external fat/oil samples and binary mixtures of them, (D) loading values of related PCs.

apparent. Especially, the spectral region of 800–1400 cm^{-1} became more diverse in all samples. The pre-processing operations improved the classification accuracy of the samples. After pre-processing, minor components such as carotenoids, sterols or other fat-soluble components could be visible and may help to detect adulteration or discrimination. For example, a band at around 1000 cm^{-1} which is probably related with carotenoids [39] became clearly visible after pre-processing (Figs. 2B and 3B).

The pre-processed spectral data were then analyzed with PCA that transforms the data set into a new set of uncorrelated attributes called principal components (PCs). Six different milk fat samples, six different external non-milk based samples and 36 binary mixtures of these samples were classified within a 3D graph of PCs (Fig. 2C). The axes of the graph were comprised of PC1 (65.15%), PC4 (2.04%) and PC5 (1.22%) which represented 68.41% of the total variance. Milk fat samples, external fat or oil samples and the binary mixtures of them exhibited well-separated groups allowing the discrimination according to the type of adulterant (Fig. 2C). Milk fat samples were clustered on the left side of the graph, while external non-milk based samples were on the right side and the binary mixtures of these samples were located between them.

In order to determine the responsible Raman shifts for the classification, loading values of related PCs' were plotted. The factor loadings from PCA of the 1024 data were shown in Fig. 2D with numbers referring to Raman shifts (from 200 to 2000 cm^{-1}). The loadings indicated that a cluster of six main band regions (200-207 cm^{-1}, 812-829 cm^{-1}, 840-850 cm^{-1}, 950-1050 cm^{-1}, 1100-1120 cm^{-1}, 1250-1300 cm^{-1}) explained the most of the variance differences for the discrimination of samples. Bands at those regions which belonged to phospholipid group, aliphatic C–C stretch all-*trans* ν (C–C)ip, and deformation of in-plane *cis* double bond contributed to the discrimination of the samples. The band at 1264 cm^{-1} corresponds to the deformation of in-plane *cis* double bond and is important for discrimination depending on unsaturation level of fat or oil in samples.

The raw spectra of extracted lipids from yogurt samples were pre-processed as mentioned above. The pre-processed Raman spectra and 3D graphs of PCs for yogurt samples

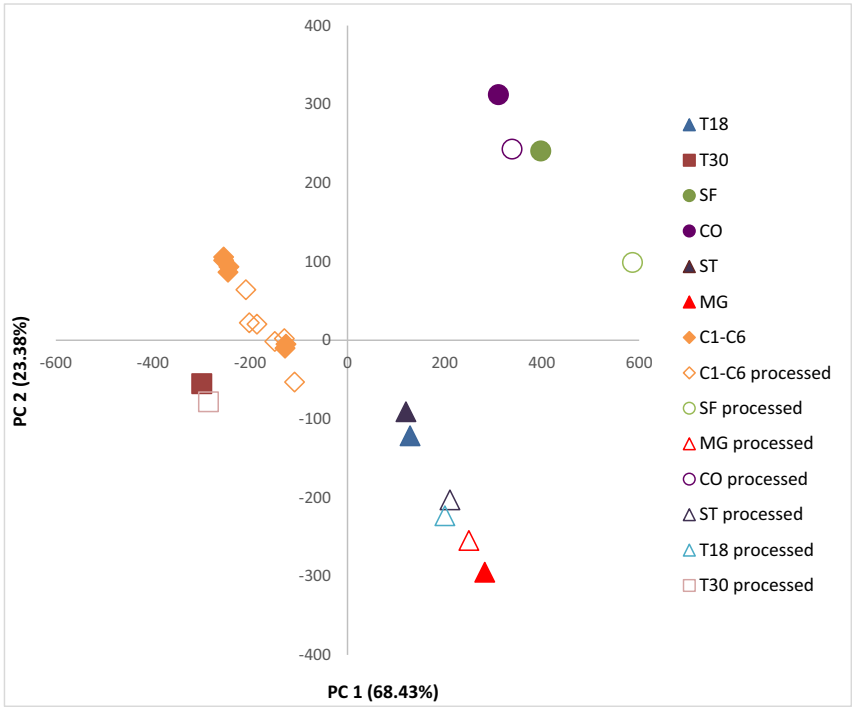

Fig. 4 – - PC scores of fat or oil samples before and after yogurt production. C1– C6 corresponds to different milk fat samples, CO: Corn oil, MG: Margarine, SF: Sunflower oil, ST, T18, T30: Commercial vegetable fat blends.

were given in Fig. 3B and C, respectively. Pre-processing operations made minor differences apparent for the detection of fat adulterant in yogurt samples. The axes of this 3D graph comprised of PC1 (53.87%), PC4 (1.57%) and PC7 (0.48%). This graph explained the 55.92% of the total variance. According to the PCA scores, extracted lipids were successfully classified into three main groups. External non-milk based fats were clustered in the upper part of the graph, while the milk fats were on the right side (Fig. 3C). Binary mixture of adulterated samples, corn, and sunflower oil samples were clustered together. The highest loadings of the three principal component axes of the PCA showed main differences in the discrimination of the samples were at regions around 203-210 cm^{-1}, 492-506 cm^{-1}, 900-1050 cm^{-1}, 1076-1086 cm^{-1}, 1100-1111 cm^{-1}, 1250-1300 cm^{-1} (Fig. 3D). Bands ~900 cm^{-1} correspond to ν (C–O–C) stretch and some other bands were mentioned as above. When Figs. 2C and 3C were compared, different discrimination by PCA was observed. It may be related with the fermentation process. Products such as free fatty acids or other fat-soluble metabolites, which were produced/consumed during fermentation or during storage, may cause this situation. However, discrimination of extracted lipid samples of both fat/oil and yogurt samples was successfully achieved by Raman spectroscopy coupled with PCA.

In order to visualize the yogurt processing effect on the extracted lipid samples, PCA was applied to the samples for both before and after yogurt production. First derivative and mean centering pre-processing techniques were implemented on raw Raman data before PCA processing as mentioned before. The scores of the PC1 (68.43%) and PC2 (23.38%) were placed in Fig. 4. The scores of the lipid samples before and after yogurt production was very close to each other. Therefore, it can be concluded that the effect of the yogurt processing to discrimination is minor. Clustering probably was depending on lipids' fatty acid compositions and it was independent from the processing. The obtained PCA results were consistent with the GC results. The samples having similar fatty acid compositions clustered closely (Fig. 4). These results are in agreement with Baeten et al. [35], which has been showed a high correlation between fatty acid profile and classification of fats and oils by FT-Raman spectroscopy.

There are some studies based on Raman and NIR spectroscopies combined with PCA for classification of lipid samples and detection of fat adulteration [10,21,35,37,44,45]. In some studies [10,21] detection of adulteration of milk fat by Raman was generally conducted just to discriminate milk fat and margarine. But, in this study, discrimination of seven different lipid samples (milk fat, T18, T30, ST, corn oil, sunflower oil, margarine) and adulteration of milk fat with six different sources either fat or oil was successfully determined within a single measurement.

4. Conclusions

The results represented in this study show that the application of Raman spectroscopy coupled with chemometric method offers a high potential for the rapid determination of milk fat adulteration with cheaper fats or oils. The proposed method of which has simple extraction and analysis steps,

provides the determination of fat adulteration within 30 min. When compared with chromatographic analyses such as GC, which takes about 1 h to detect fat adulteration, proposed method detects fat adulteration in a shorter time. In the proposed method, just extraction of lipid is needed for vibrational measurement without further sample pretreatments such as esterification.

The study also highlights the role of the measurement temperature of fats, on detection accuracy and intensity. The Raman spectra collected at higher temperatures were more intense and the discrimination performance of fat samples increased at elevated temperatures. However, oil measurements were not affected by temperature elevation when compared with fats.

According to results, discrimination of foreign fats or oils in milk cream and yogurt and clustering of the samples according to source of lipid using Raman spectroscopy were succeeded. The possible reason of this achievement could be related with sharp and intense spectrum of Raman bands that is mostly related to the saturation level of fats or oils. In addition, the overall differences between fats or oils in the samples have been analyzed at one time by vibrational spectroscopy which means discrimination of fats or oils is depending on not only fatty acid profiles but also other fat soluble components such as sterols, vitamins, carotenoids which may be helpful for discrimination.

Moreover, it should be emphasized that chemical properties of fats or oils may change on account of oxidation, polymerization, hydrolysis and lipolysis during the processing (heat treatment, fermentation, etc.) and storage of dairy products, which may affect the discrimination and detection capacity of adulterants in milk fat.

However, fat samples in yogurt successfully were distinguished by this technique with PCA, both before and after yogurt production. These data can be potentially useful in detecting foreign fats or oils in dairy products. The results of the study can be readily used for fast scanning of the multiplexed samples by the food control authorities and dairy industry for estimation of fat adulteration before analyzing them with conventional methods. Hence, results can be obtained in a short time with drastic reduction in consumption of chemical reagents required for sample preparation. Our method provides fast, non-destructive analysis without environmental side effects. Also, this method has potential to be used as a routine analysis by using portable Raman spectroscopy. However, further investigations are needed to determine milk fat authenticity for some other dairy products such as cheese which has long shelf life compared to yogurt.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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