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A rapid ATR-FTIR spectroscopic method for classification of gelatin gummy candies in relation to the gelatin source

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ABSTRACT

Gelatin is widely used in gummy candies because of its unique functional properties. Generally, porcine and bovine gelatins are used in the food industry. FTIR-ATR combined with chemometrics analysis such as hierarchical cluster analysis (HCA) (OPUS Version 7.2 software), principal component analysis (PCA) (OPUS Version 7.2 software) and partial least squares-discriminant analysis (PLS-DA) (Matlab R2017b) were used for classification and discrimination of gelatin gummy candies related to their gelatin source. The spectral region between 1734 and 1528 cm^{-1} was selected for chemometric analysis. The potential of FTIR spectroscopy for determination of bovine and porcine source in gummy candies was examined and validated by a real-time polymerase chain reaction (PCR) method. Twenty commercial samples were tested by developed ATR-FTIR methodology and RT-PCR technique, mutually confirming and supporting results were obtained. Gummy candies were classified and discriminated in relation to the bovine or porcine source of gelatin with 100% success without any sample preparation using FTIR-ATR technique.

1. Introduction

Gelatin is widely used in food industry due to its favorable and unique functional properties. Generally, gelatin is used as gelling, thickening and stabilizing agent (Baziwane & He, 2003). Other functional properties of gelatin can be listed as texturization, emulsification and adhesiveness. Food producers utilize gelatin in a wide variety of food stuffs such as gummy candies, jellies, milk desserts, marshmallows and more (Karim & Bhat, 2008). In general, gelatin is the thermal hydrolysis product of collagen from cattle bones, cattle hides and pork skins. Mainly porcine and bovine gelatins are produced and, exports and imports of them occur around the world in order to respond to the demands of especially confectionary industry. At this point, authentication of the gelatin comes into prominence since communities including Muslims and Hindus have sensitivity and selectivity in consumption of certain ingredients. For instance, Muslims are not allowed to consume any porcine derived food products according to the Islamic rules. However most of the commercial gelatin is extracted from skins of pig followed by bovine hides, bones and other sources (Karim & Bhat, 2009). When we consider the status in Turkey, gelatin production does not occur at a level that satisfies the demand of the industry especially confectionery factories therefore most of the gelatin is imported from other countries (Yetim, 2011). In this case, control mechanisms of government should determine the source of gelatin as a raw ingredient and once incorporated into the product (Yetim, 2011). Namely, there is an essential necessity for building reliable and strong methods in order to detect the source of commercial gelatin and detect the source of gelatin as an ingredient in food products.

Up to now, various studies were performed in order to detect the source of gelatin. Generally, polymerase chain reaction (PCR) based, electrophoretic, chromatographic and spectroscopic techniques were used (Eryılmaz et al., 2017). Previous studies have provided important information on determination of gelatin source both in the raw state and as an ingredient in the foodstuffs. Most of these studies included the determination of raw gelatins using various analytical techniques (Cebi, Durak, Toker, Sagdic, & Arici, 2016; Hashim et al., 2010; Jannat et al., 2017; Nemati, Oveisi, Abdollahi, & Sabzevari, 2004; Venien & Levieux, 2005; Zhang et al., 2009). Additionally, valuable studies were dedicated to the detection of gelatin source in processed foodstuffs. Yilmaz et al. (2013) successfully developed a ultra performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (nano UPLC-ESI-q-TOF-MS) method to determine the

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source of gelatin in yoghurt, cheese and ice cream. Raraswati, Triyana, and Rohman (2014) applied chemometrics based on amino acid compositions of soft candies in order to predict the gelatin source. In a technical note, Tan and Lock (2014) utilized liquid chromatography tandem mass spectroscopy (LC-MS/MS) method for determination of gelatin source in gummy bear soft candies. Demirhan, Ulca, and Senyuva (2012) presented a significant survey for determination of gelatin source in gumdrops, marshmallows and Turkish delight using real-time PCR technique. In another previous study, trace amounts of pork, beef, chicken, mutton and horseflesh were determined using a real time quantitative PCR method (Tanabe et al., 2007).

The mentioned methods have been known to be successful, accurate and precise. However disadvantages of these methods can be listed as being time consuming, destructive and arduous. Additionally, toxic chemicals are used and expert operator is needed to perform these studies. These challenges reveal a necessity to develop rapid, inexpensive and effective techniques for authenticity testing (source determination) of gelatin as a raw ingredient and once incorporated into the product. FTIR spectroscopy, in this respect, can be non-destructively and rapidly used to obtain biochemical fingerprints that would provide reliable information on molecular structure and composition (Alvarez-Ordonez & Prieto, 2012; Dufour, 2009; Sivakesava & Irudayaraj, 2001). Therefore, FTIR spectroscopic technique has been used as an effective and successful tool in a wide range of applications in food products (Gok, Severcan, Goormaghtigh, Kandemir, & Severcan, 2015; Ozen, Weiss, & Mauer, 2003; Wang, Kliks, Jun, Jackson, & Li, 2010). Chemometric techniques provide an opportunity for classification or discrimination of materials with respect to their similarities since these techniques have capability to extract distinctive properties from spectral data. Data from several studies suggest that application of spectroscopy combined chemometrics has great potential to solve adulteration and authentication problems (Cebi, Dogan, Develioglu, Yayla, & Sagdic, 2017; Gok et al., 2015; Ozen et al., 2003; Wang et al., 2010).

In this research, for the first time, a new, rapid, effective, non-destructive and cost-effective FTIR-ATR method was built with the aim of determination the source of gelatin in gummy candies. The chemometrics of hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed to process FTIR data. Additionally, partial least squares-discriminant analysis (PLS-DA) was performed for calibration and cross-validation using Matlab programming (Matlab R2017b – MathWorks Inc., Natick, MA). Eventually, results of test samples were validated using a real-time polymerase chain reaction (PCR) based method.

2. Materials and methods

2.1. Material and reagents

Perkin Elmer Frontier (Perkin Elmer Instruments, Waltham, Massachusetts, USA) FTIR/NIR with a potassium bromide (KBr) beamsplitter and a DTGS (deuterated triglycine sulfate) detector was used in this study. Three-bounce attenuated total reflectance (ATR) accessory (Perkin Elmer, Waltham, MA) equipped with a diamond crystal was used in all spectral acquisition. Standard bovine and porcine gelatins were used for the preparation of the gummy candy samples. These gelatins were obtained from Sigma Aldrich. The catalog numbers of porcine and bovine gelatins are G2500, G2625, G6144, G6144,

Table1
Ingredients of each gummy candy sample produced in laboratory.

Produced Gummy Candy (GC) Number	Ingredients of produced gummy candy samples (%)					
	0.72%	26%	40%	*Source and Name of standard gelatins 4.12%*	27%	2.16%
GC1	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma G2500)	Water	Citric acid
GC 2	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma G2500)	Water	Citric acid
GC 3	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma G2500)	Water	Citric acid
GC 4	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma 04055)	Water	Citric acid
GC 5	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma 04055)	Water	Citric acid
GC 6	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma 04055)	Water	Citric acid
GC 7	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma 48720)	Water	Citric acid
GC 8	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma 48720)	Water	Citric acid
GC 9	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma 48720)	Water	Citric acid
GC 10	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma 48724)	Water	Citric acid
GC 11	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma 48724)	Water	Citric acid
GC 12	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma 48724)	Water	Citric acid
GC 13	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma G6144)	Water	Citric acid
GC 14	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma G6144)	Water	Citric acid
GC 15	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma G6144)	Water	Citric acid
GC 16	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma 48722)	Water	Citric acid
GC 17	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma 48722)	Water	Citric acid
GC 18	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma 48722)	Water	Citric acid
GC 19	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma G2625)	Water	Citric acid
GC 20	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma G2625)	Water	Citric acid
GC 21	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma G2625)	Water	Citric acid
GC 22	Pectin	Sucrose	Fructose	Porcine gelatin (Sigma 39465)	Water	Citric acid
GC 23	Pectin	Sucrose	Glucose	Porcine gelatin (Sigma 39465)	Water	Citric acid
GC 24	Pectin	Sucrose	Maltose	Porcine gelatin (Sigma 39465)	Water	Citric acid
GC 25	Pectin	Sucrose	Fructose	Bovine gelatin (Sigma G9382)	Water	Citric acid
GC 26	Pectin	Sucrose	Glucose	Bovine gelatin (Sigma G9382)	Water	Citric acid
GC 27	Pectin	Sucrose	Maltose	Bovine gelatin (Sigma G9382)	Water	Citric acid
GC 28	Pectin	Sucrose	Fructose	Bovine gelatin (Sigma G9391)	Water	Citric acid
GC 29	Pectin	Sucrose	Glucose	Bovine gelatin (Sigma G9391)	Water	Citric acid
GC 30	Pectin	Sucrose	Maltose	Bovine gelatin (Sigma G9391)	Water	Citric acid

04055, 39465, 48720, 48722, 48724, G9382 and G9391. Sources and names of these standard gelatins are presented in Table 1 in detail. In addition, detailed information about produced gummy candies and their compositions are presented in Table 1. Produced gummy candies (GC) were codded as (GC1, GC2, GC3…) on the Table 1. Three different sugar compositions (sucrose + glucose, sucrose + maltose, sucrose + fructose) were formulated in order to enhance the sample number and diversity. Sugar composition for each gummy candy is presented in Table 1. Commercial products such as pectin, citric acid, glucose, fructose, sucrose, maltose and food colorants were obtained from Smart Kimya Tic. ve Dan. Ldt., Turkey. Ethanol and water were obtained from Sigma-Aldrich. Additionally, 20 samples were used as test materials. These gummy candies were purchased from markets in Turkey and abroad. Sure Food® Prep Advanced kit (CONGEN, R-Biopharm, Germany) and SureFood® Animal ID Pork SENS Plus (CONGEN, R-Biopharm, Germany) were used for DNA isolation and PCR amplification, respectively. In addition, RT-PCR studies were performed by using Applied Biosystems® 7500 Real-Time PCR.

2.2. Production of gummy candies

In this study, we produced totally 30 gummy candies in the laboratory on the basis of the recipe of Herbstreith and Fox (2004), which was chosen in order to obtain firm gummy candies. According to this recipe, pectin (7 g), sucrose (330 g), glucose syrup (480 g), water (320 g) and gelatin (50 g) and flavoring were used for production of gummy candy. Approximately 25 mL of citric acid (50%) was used to adjust the pH value to 3.2–3.4. At first, gelatin was swelled in some of the water. Then, pectin was mixed with approximately 100 g of sucrose. This mixture was stirred with water (200 g) and boiled while stirring till all pectin was completely dissolved. Remaining sucrose and glucose syrup were added and the mix was cooked. Swelled gelatin was stirred on a magnetic stirrer hot plate till completely dissolved in the water. Dissolved gelatin, color and flavor were added to previous pectin-sugar mixture. Lastly pH was adjusted to the 3.2–3.4 value. In this recipe, two types of sugar (sucrose and glucose syrup) are used. Sucrose is included in all lab-produced gummy candies and one of the sugars of glucose, fructose and maltose were used for each gelatin type with aim increasing sample diversity. Hence, three samples were prepared for each gelatin type. For instance, three gummy candies with gelatin G2500 were prepared. First one included sucrose + fructose, second one included sucrose + glucose and third one included sucrose + maltose as sugar ingredient in the composition. Detailed information about produced gummy candies and their compositions are presented in Table 1. Additionally, 20 samples were used as test materials. These gummy candies were purchased from markets in Turkey and abroad.

2.3. Spectral acquisition

FTIR-ATR spectra of all samples were obtained with a resolution of 8 cm^{-1}, accumulating 16 scans per spectra. Spectral measurements were performed within the mid-infrared range of 4000–650 cm^{-1}. All measurements were performed in triplicate with a diamond triple-bounce ATR accessory (Perkin Elmer, Waltham, MA). Average spectrum of triplicate analysis was obtained for each sample. Background air spectrum was collected before each sample measurement. All of the samples were directly compressed on attenuated total reflectance (ATR) crystal prior to analysis without any preliminary preparation. Ethanol and distilled water were used in order to clean the diamond ATR crystal.

2.4. Chemometrics

2.4.1. HCA and PCA

In this study, FTIR spectroscopy combined with chemometrics of HCA (hierarchical cluster analysis) and PCA (principal component analysis) were used for the classification of investigated materials (gummy candies) in relation with the source of gelatin.

Classification of gummy candy samples was performed by using OPUS Version 7.2 software. In the HCA chemometrics analysis, all spectra were preprocessed by obtaining first derivative spectra with 25 smoothing points through Ward's algorithm. In the developed methodology, the algorithm normal to the repro-level was selected in which spectral distances were separately calculated for each frequency range. 2D and 3D score plots were obtained through PCA (principal component analysis). The first derivatized and vector normalized (25 smoothing points) versions of all spectra were included in classification model and factorization algorithm was employed for calculating the spectral distances. The results from two and three-dimensional cluster analysis were obtained by using identity method of software. In addition, we performed experiments using other algorithms (single linkage, complete linkage, average linkage, weighted average linkage, median algorithm and centroid) for discrimination of gummy candies in relation to their gelatin source.

2.4.2. PLS-DA (partial least squares discriminate analysis)

In this study, Partial Least Squares (PLS) multivariate calibration methods were coded in Matlab programming (Matlab R2017b – MathWorks Inc., Natick, MA). Calibration was performed and method validation was performed with the option of "Leave-One-Out Cross Validation" mode.

2.5. Real-time polymerase chain reaction (RT-PCR) analysis for test samples

2.5.1. Isolation of DNA

Gummy candy has been known as highly processed food product with high amounts of gelatin in its composition. Gelatin is an already highly processed product. The main objective of PCR amplification is therefore obtaining sufficient DNA for analysis. Therefore, DNA isolation was performed by using a commercial kit which designed for maximal recovery of short DNA fragments. All steps of DNA extraction were performed following manufacturer's instructions with slight modifications. Applied kit instructions are presented in this section. DNA was extracted from gummy candies using the Sure Food® Prep Advanced kit (CONGEN, R-Biopharm, Germany). Lysis buffer (600 μL) and Proteinase K (40 μL) were added to 200 mg of sample and vortexed. The mixture was incubated at 65 °C for 60 min on a heating block under continuous shaking. The Lysate was removed by centrifuging in two steps. After centrifuging, a spin filter was placed in a receiver tube. 400 μL of the supernatant were transferred into the spin filter and centrifuged at 12,000 rpm for 1 min. Binding buffer (250 μL) was added to the filtrate and mixed. The filtrate was transferred to a new spin filter placed in a new receiver tube and centrifuged again at 12,000 rpm for 1 min after incubation at room temperature for 1 min. After the filtrate was discarded, 550 μL of pre-wash buffer was added to the spin filter and centrifuged at 12,000 rpm for 1 min. This step was repeated twice. After discarding the filtrate, receiver tube with spin filter was centrifuged for 2 min at 12,000 rpm to remove residual ethanol completely. A new spin filter was placed in a new 1.5 mL receiver tube; 50 μL of preheated elution buffer (65 °C) was transferred directly onto the spin filter and incubated at room temperature for 3 min at 65 °C. Finally, it was centrifuged for 1 min at 10,000 rpm and the purified DNA solution was stored at −20 °C until use.

2.5.2. PCR amplification

Amplification of DNA from gummy candies was performed in the same manner with the study of Demirhan et al. (2012). In this study, commercial kit (SureFood® Animal ID Pork SENS Plus) was used for porcine determination. This kit includes pork reaction mixture (specific primers) and Taq-Polymerase. Amplification was performed with a real-time PCR (Applied Biosystems® 7500). The thermal cycler program was

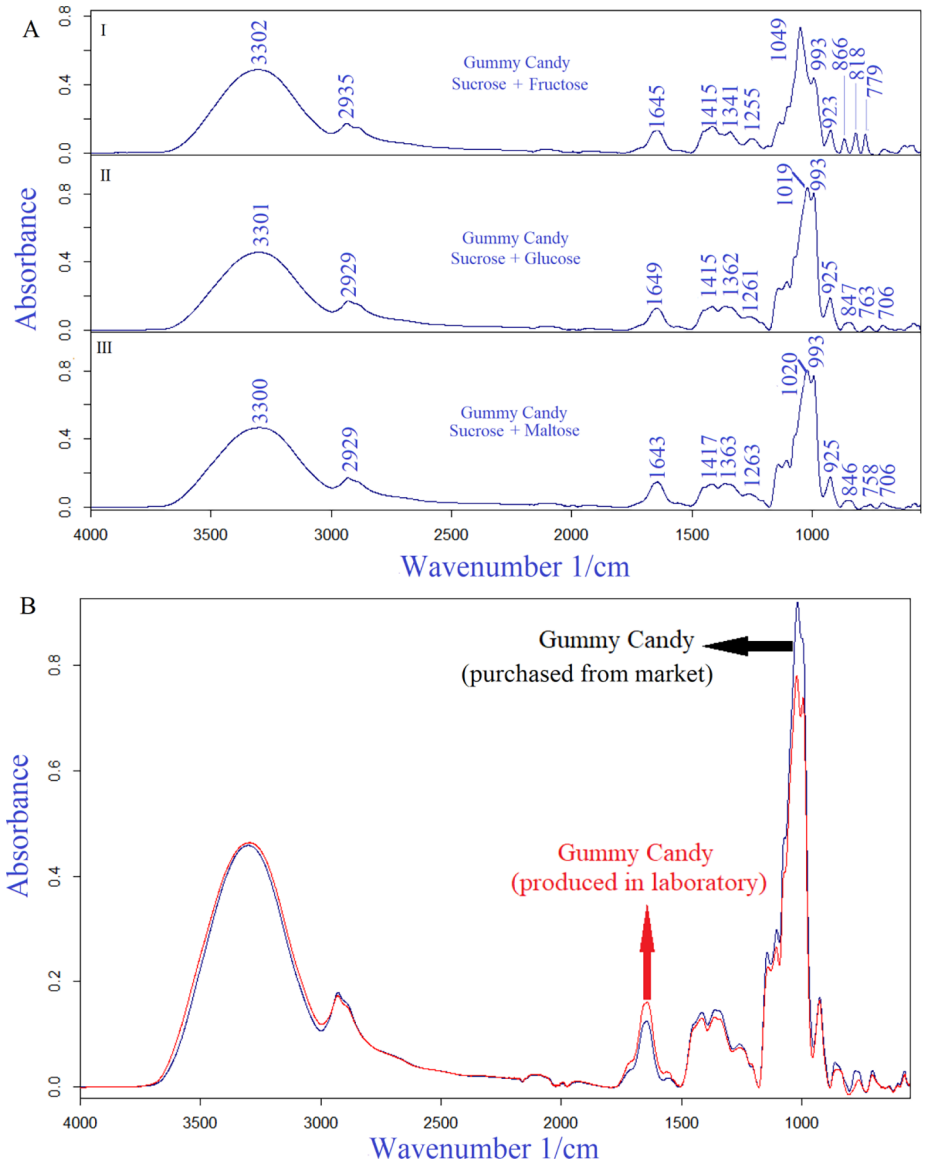

Fig. 1. (A) FTIR spectra of lab-produced gummy candy (Sucrose + Fructose) (I), FTIR spectra of lab-produced gummy candy (Sucrose + Glucose) (II) FTIR spectra of lab-produced gummy candy (Sucrose + Maltose)(III) (B) Representative FTIR spectra of lab-produced and purchased (commercial) gummy candies.

built according to the kit instructions. It includes initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C, annealing for 15 s and extension at 55 °C for 30 s. Fluorescent intensity was examined at 522 nm (FAM) and 553 nm (VIC).

3. Results

3.1. Interpretation of gummy candy spectrum

The FTIR spectrum of gummy candy (produced in laboratory) is presented in Fig. 1. Major components of gummy candy are water, sugar (glucose, fructose, maltose, and sucrose) and gelatin. FTIR spectra mainly include the vibrations arising from these major components. In other words, spectral bands are associated with the chemical groups of components in the structure of gummy candies. Similar spectral characteristics among samples were indicated in the Fig. 1. The band around 3300 cm^{-1} is mainly related to the OH stretching vibrations of water. The bands with peak point around 2930 cm^{-1} was due to the contributions from C—H stretching vibrations of carboxylic acids and NH_3^+ stretching vibrations of free amino acids (Gallardo-Velazquez, Osorio-Revilla, de Loa, & Rivera-Espinoza, 2009). Sugar-related bands are mainly observed in the spectral region between 750 and 1500 cm^{-1} (Tewarii & Irudayaraj, 2004). Spectral features arising from saccharides are observed at 900–750 cm^{-1} (Anjos, Campos, Ruiz, & Antunes, 2015). The band observed around 993 cm^{-1} has been known as the characteristic vibrational band of glycosidic links of sucrose. All gummy candies that we prepared included sucrose in their compositions. The band around 1049 cm^{-1} is due to the C—O and C—C stretching vibrations of carbohydrate structure in the fructose content (Gallardo-Velazquez et al., 2009; Wang et al., 2010). Spectral range between 1100 and 1000 cm^{-1} is resulted from the (C—O) and (C—O—C) absorptions of the carbohydrates (Cebi et al., 2016). The peaks observed around 925 cm^{-1}, 1255 cm^{-1} and 1415 cm^{-1} are arising from the C—H bending, C—O stretching and O—H stretching/bending vibrations of carbohydrates, respectively (Tewarii & Irudayaraj, 2004). Three major bands (1261, 1362, 1415 cm^{-1}) were observed between 1500 and 1200 cm^{-1} spectral range. The spectral range between 1500 and 1200 cm^{-1} is known for being a mixed region which is influenced by bending modes of $>CH_2$ and $-CH_3$ groups in proteins and C—H bending vibrations of carbohydrates (Alvarez-Ordonez & Prieto, 2012;

Dufour, 2009). Additionally, In addition, distinct spectral features were observed in the FTIR spectra of gummy candies which fructose was used in addition to the sucrose Fig. 1(A). The bands at 866, 818 and 779 cm^{-1} are due to the presence of fructose and correspond to the (δ CH + υ CC + δ CCH), δ CH, (δ CCO + CCH), respectively (Ibrahim, Alaam, El-Haes, Jalbout, & Leon, 2006). As mentioned previously, gelatin is one of the major components of gummy candies. In the FTIR spectra, we observe spectral features arising from the presence of gelatin. The most significant band (Amide I) related to the gelatin is observed in 1700–1600 cm^{-1} spectral range (Cebi et al., 2016). FTIR spectra of gummy candies which glucose and maltose were used in addition to the sucrose are presented in Fig. 1(A). In addition, representative FTIR spectra of lab-produced and purchased gummy candies are presented in Fig. 1(B). As it is seen in the Fig. 1(B), both lab-produced and commercial gummy candies have quite similar and comparable FTIR spectra. Findings from spectral comparison demonstrate that quite appropriate production recipe was applied in order to obtain most commercial-like gummy candies.

3.2. Discrimination and clusterization of gummy candies

The main objective of this research was to build a FTIR based methodology for discrimination or clusterization of the gummy candies based on the source of gelatin ingredient. FTIR-ATR technique combined chemometrics was used for this aim. At first, FTIR spectra of all gummy candies (produced in a laboratory) were obtained under same conditions. Afterwards, chemometrics was applied to all spectra in order to obtain significant classification according to the spectral resemblance or discrepancy of samples. Discrimination of gummy candies was accomplished utilizing from the spectral diversity among samples through multivariate chemometric techniques. In this study, the significant classification was observed for gummy candy samples by using normal to reprolevel and Ward's algorithm with first-derivative preprocessing of spectra. 1734–1528 cm^{-1} spectral region was selected for HCA and PCA analysis. The selected spectral region has contributions from Amide I (1700–1600 cm^{-1}) and Amide II (1565–1520 cm^{-1}) vibrational bands. In previous studies, Amide I band was mentioned as being responsible for the C=O stretching vibrations with a minor contribution from the C–N stretching vibration of the peptide linkages thus providing information about secondary structure of proteins. Amide I and Amide II spectral range that gives information about protein secondary structure and source of the gelatin (Cebi et al., 2016). Considering previous findings, same spectral region was selected for classification of gummy candies in relation with their gelatin source. HCA and PCA classification results for gummy candies are presented in Fig. 2. As it can been seen on the HCA dendrogram, mainly two well-separated clusters were observed. In other words, all gummy candy samples were clustered correctly related to their gelatin source. Gummy candy could be regarded as a quite complex matrix. In addition, no pretreatment was applied to the gummy candy samples. However, discrimination of samples was accomplished without any fault. This situation could be associated with the application of Ward's algorithm. In the scope of this research study, we performed experiments using other algorithms (single linkage, complete linkage, average linkage, weighted average linkage, median algorithm and centroid) but none of these algorithms provided significant discrimination of gummy candy

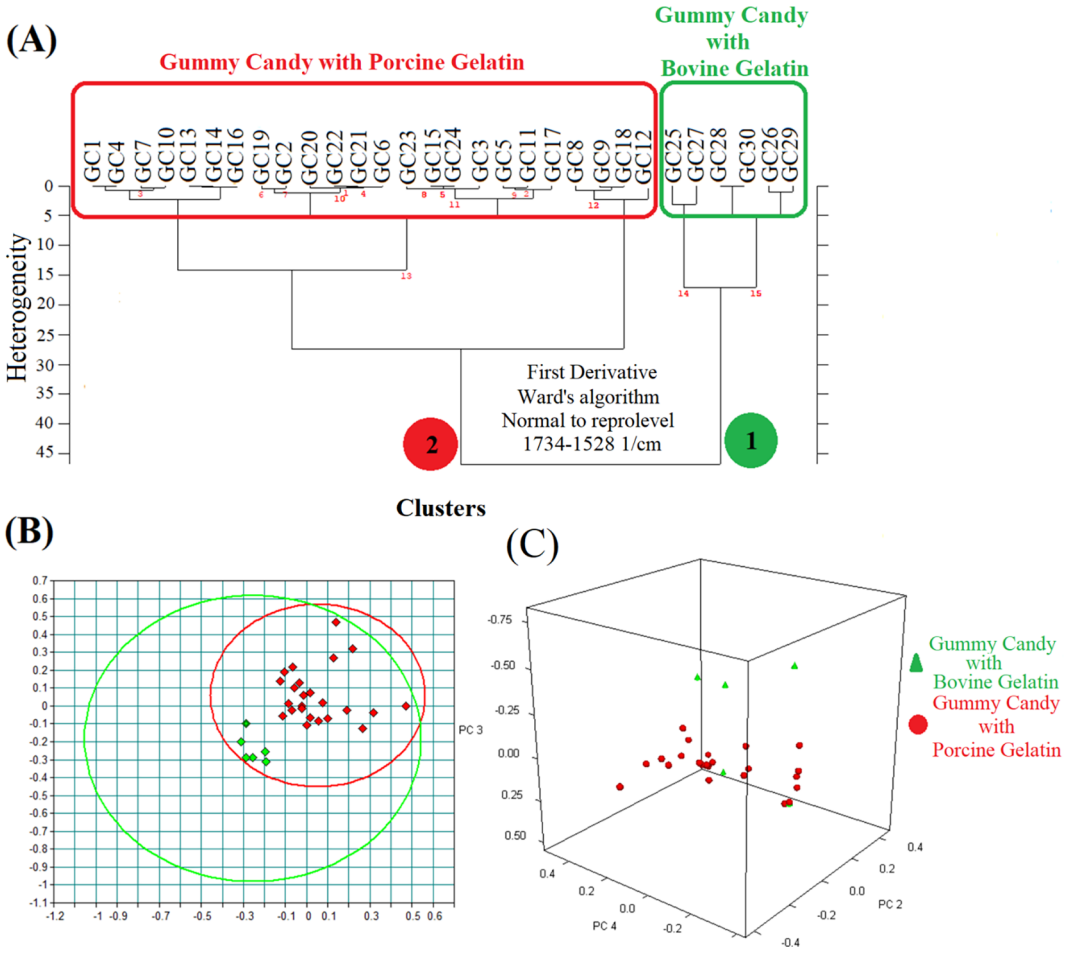

Fig. 2. Dendrogram of HCA analysis (Ward's algorithm) of FT-IR spectra from total 30 different samples of gummy candies (A), two dimensional PCA analysis maps for total 30 gummy candies (B) three dimensional PCA analysis maps for total 30 gummy candies (C).

samples. HCA results from chemometrics with different algorithms (single linkage, complete linkage, average linkage, weighted average linkage, median algorithm and centroid algorithm) are presented in Supplementary File. The right classification in which gummy candies clustered related to their gelatin source was only attained by using Ward's algorithm. Ward's method joins at each stage of the cluster pair whose merger minimizes the increase in the total within-group error sum of squares (Lorr, 1983). In addition, Ward's minimum variance criterion minimizes the total within-cluster variance. At each step, the pair of clusters with minimum cluster distance are merged (Murtagh & Legendre, 2011). Single linkage, complete linkage, average linkage, weighted average linkage, median and centroid algorithms merge the two groups which are most similar. Ward's Algorithm, however, tries to find as homogeneous groups as possible. This means that only two groups are merged which show the smallest growth in heterogeneity factor H. Instead of determining the spectral distance, the Ward's Algorithm determines the growth of heterogeneity H. In our study, high heterogeneity value of 45 was obtained in the hierarchical cluster analysis. All of the gummy candy samples that were produced using porcine gelatin were clustered together at the left side (numbered as 2) of HCA dendrogram in Fig. 2(A). Similarly, All of the gummy candy samples that were produced using bovine gelatin were clustered together at the right side (numbered as 1) of HCA dendrogram in Fig. 2(A). Eventually, the obtained HCA dendrogram showed that the gummy candy samples could be quickly and accurately distinguished from each other related to the source of the ingredient gelatin.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.10.125.

Two and three-dimensional PCA (principal component analysis) plots of cluster analysis are presented in Fig. 2(B) and (C), respectively. As it can been seen different shaped and colored symbols were assigned by the chemometric model for each different species on the basis of the spectral disparities. In other words, samples weren't defined as distinct groups in the software, all of the samples were loaded and classifications and assignments were performed by the software on the basis of selected algorithms and parameters. According to the PCA analysis, two distinct groups were observed. While one of the groups was marked with green triangles, other group was marked with red circles by cluster analysis software. It was observed that all of the green triangles were gummy candies with bovine gelatin and all of the red circles were gummy candies with porcine gelatin. Eventually, discrimination and classification of gummy candies could be accomplished using PCA analysis, too. When the results were evaluated, it was clearly seen that two different classes with right classifications were achieved by both two chemometric analysis (HCA and PCA). Furthermore diagnosis results of the chemometric analysis (HCA, PCA) are presented in Table 2. As it can been seen in Table 2, two diagnosis results were obtained; first one is obtained from the HCA and PCA analysis of totally 24 samples, which were gummy candy samples with porcine gelatin. Second diagnosis results arose from totally 6 samples that were gummy candy samples with bovine gelatin.

3.3. Evaluation of PLS-DA results

PLS regression is a widely used method in chemometrics, especially when constructing models for spectral data. This is because of the multicollinearity of the data that is the variables (i.e. absorbances at each wavelength) are correlated and the numbers of variables are significantly higher than the number of observations. In this type of cases, the ordinary regression methods fail to provide a generalized model which is expected to offer high predictive power on both calibration set and unseen data, PLS regression offers a remedy by constructing directions that maximizes covariance between dependent and independent variables (Gromski et al., 2015). Each direction is called loading and the projection of the data by using the direction yields a "component" or "latent variable (LV)". By omitting the directions of low covariance, modeling of noise and multicollinearity problem is avoided (Gromski et al., 2015). PLS algorithm can also be used for classification tasks in which case is called PLS-DA. To apply the PLS algorithm for classification, the binary encoding of class information takes place and resulting matrix is used as dependent variables (Hirri, Bassbasi, Platikanov, Tauler, & Oussama, 2016). The prediction, then, can be carried out by assigning classes based on which class prediction has the highest value. Predictions from PLS-DA have been reported with a bars graph. In a binary classification case, each sample has two bars that blue represents the 'gummy candy with porcine gelatin' class whereas orange representing 'gummy candy with bovine gelatin' class. For instance, if a sample's corresponding blue bar is higher than the orange bar, this sample can be predicted to be belonging to 'gummy candy with porcine gelatin' class. PLS-DA calibration study was performed for gummy candies, which were prepared in the laboratory (30 samples, Fig. 3). The signals within 1734–1528 cm^{-1} were used for

Table 2
Diagnosis results of chemometric analysis (Hierarchical cluster analysis and Principle component analysis).

Diagnosis Number	1. Class	2. Class
Diagnosis for gummy candy samples(produced in laboratory) Data preprocessing: First derivative Ward's algorithm, normal to reprolevel Frequency Ranges=1734-1528 cm^{-1} Standart (Euclidian Distance)	Gummy Candy 1 Gummy Candy 2 Gummy Candy 3 Gummy Candy 4 Gummy Candy 5 Gummy Candy 6 Gummy Candy 7 Gummy Candy 8 Gummy Candy 9 Gummy Candy 10 Gummy Candy 11 Gummy Candy 12 Gummy Candy 13 Gummy Candy 14 Gummy Candy 15 Gummy Candy 16 Gummy Candy 17 Gummy Candy 18 Gummy Candy 19 Gummy Candy 20 Gummy Candy 21 Gummy Candy 22 Gummy Candy 23 Gummy Candy 24 Gummy candy with porcine gelatin	Gummy Candy 25 Gummy Candy 26 Gummy Candy 27 Gummy Candy 28 Gummy Candy 29 Gummy Candy 30 Gummy candy with bovine gelatin

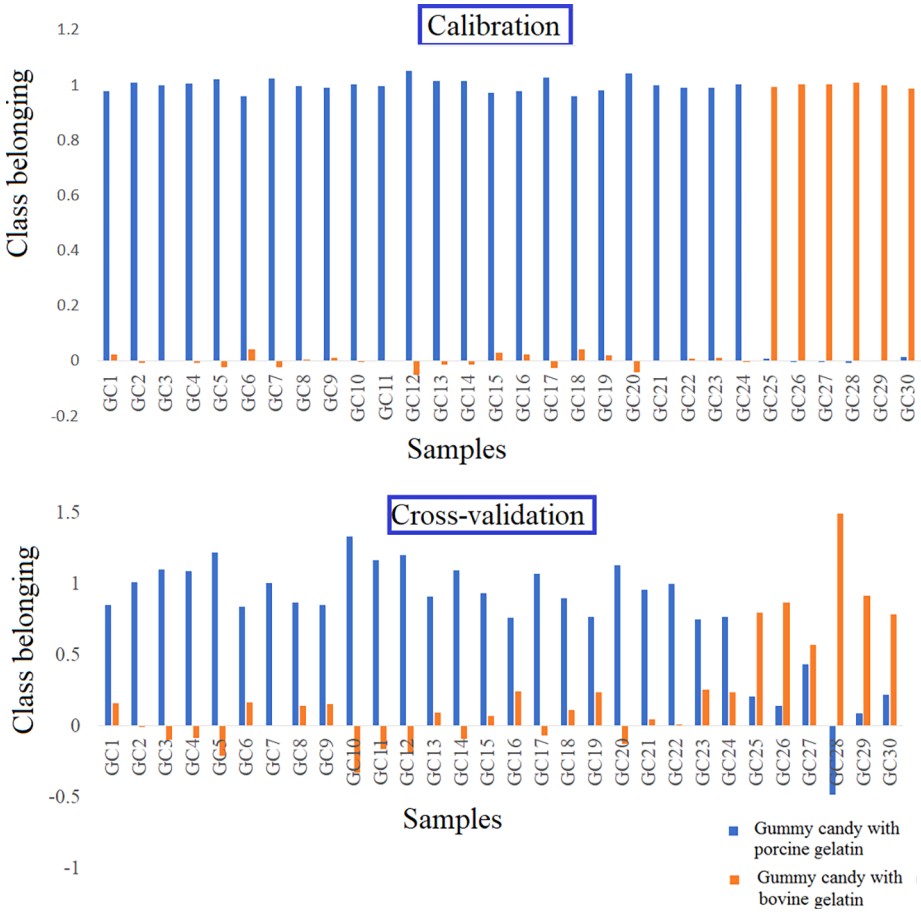

Fig. 3. PLS-DA class prediction (calibration and cross-validation) graphs for gummy candy samples.

PLS-DA analysis. Wavelength signals were used as X variables, while Y variables were related to with the two different gummy candy classes. As it can been seen in Fig. 3, two bar graphs were observed for each gummy candy sample. The highest one of these two bars was determined the belonging class of gummy candies. For example, in the calibration model, gelatin source was determined to be porcine for gummy candy 1 since the highest bar was blue (porcine gelatin). According to the PLS-DA results, 100% success was obtained in the calibration and cross-validation model without any error or fault. All of the porcine originated samples were predicted as porcine; also all of the bovine originated samples were predicted as bovine for gummy candies (gummy candies produced in laboratory).

Table 3
Comparison of ATR-FTIR results and PCR results for test samples.

Test Sample Number	FTIR results [a]	PCR results [b]
1	Bovine gelatin	Porcine negative
2	Bovine gelatin	Porcine negative
3	Bovine gelatin	Porcine negative
4	Bovine gelatin	Porcine negative
5	Bovine gelatin	Porcine negative
6	Bovine gelatin	Porcine negative
7	Bovine gelatin	Porcine negative
8	Porcine gelatin	Porcine positive
9	Porcine gelatin	Porcine positive
10	Porcine gelatin	Porcine positive
11	Bovine gelatin	Porcine negative
12	Bovine gelatin	Porcine negative
13	Bovine gelatin	Porcine negative
14	Bovine gelatin	Porcine negative
15	Bovine gelatin	Porcine negative
16	Bovine gelatin	Porcine negative
17	Bovine gelatin	Porcine negative
18	Bovine gelatin	Porcine negative
19	Bovine gelatin	Porcine negative
20	Bovine gelatin	Porcine negative

[a] HCA analysis test results of gummy candies. [b] RT-PCR porcine positive test samples.

3.4. Identification of test samples

In this research study, 20 gummy candy samples were tested using developed FTIR-ATR methodology. Commercial test samples (20 samples) were purchased from markets in Turkey and abroad. FTIR-ATR spectra of these 20 test samples were evaluated using HCA dendrogram of developed methodology. Each spectrum of test samples was tested using both identity test method and HCA analysis. Gelatin source of test samples was determined according to the location of the sample on the HCA dendrogram. When the sample was located in the porcine cluster among the porcine originated gummy candies, the gelatin source of the sample was determined to be porcine since the HCA dendrogram was built on the basis of spectral diversity of samples. In addition, real-time PCR analysis was performed for 20 samples. Applied PCR technique is the reference method for the determination of gelatin source in gummy candies. A negative control and positive control were analyzed in all measurements. Amplification of DNA was observed in FAM channel when the sample was detected to be pork positive. In other words, pork positive gummy candy samples were determined using sensitive and precise real time-PCR technique. In our study, a new method was not developed for PCR analysis of gummy candies. Confirmatory PCR analysis was applied in the same manner with the method of Demirhan et al. (2012). Comparison of HCA analysis results and real time-PCR analysis results of 20 test samples are presented in Table 3. As it can been seen in Table 3, gummy candies with porcine source were marked with a red rectangle. Three of the 20 commercial test samples were determined to include porcine gelatin in their compositions. All of the test samples which were predicted to include porcine gelatin by FTIR method were also determined to be pork positive in the real time-PCR analysis. Satisfactorily, identification of all test samples with respect to their gelatin source was confirmed using PCR technique with 100% accuracy without any disarrangements or false prediction. Eventually, one can conclude from these findings that the established FTIR-ATR based chemometric method has the capability to work perfectly for identification of gelatin source in gummy candies.

4. Discussion

Findings from this study prove that FTIR-ATR technique combined chemometrics (HCA and PCA) had significant capability to determine the source of gelatin in gummy candies. Results from HCA and PCA analysis were supported and confirmed each other. When we evaluate the outputs of cluster analysis (HCA and PCA), it is seen that more detailed information about arrangements of clusters was obtained by HCA dendrogram. In other words, a HCA dendrogram clearly illustrates the diversity or contiguity between clusters and each integral part in a dendrogram. In this research study, Ward's algorithm with first-derivative preprocessing of spectra (1734–1528 cm^{-1}) was implemented in HCA analysis. The most important superiority of HCA analysis arises from the application of Ward's algorithm. Ward (1963) expressed that hierarchical grouping procedure is based on greatest amount of information, thus not limited to classification problems and finds applications such as genetic background based taxonomy in the building of plants and animals, organizing of materials, storage of documents in libraries. In this study, for the first time, Ward's algorithm through FTIR and chemometrics was used in order to determine the source of gelatin in gummy candies. At this point, it is not possible to compare findings from this study with results from previous studies. However, the academic literature on food adulteration and authentication revealed the superiorities of Ward's algorithm in chemometric classification and discrimination issues. A great deal of previous research into authenticity problems has focused on application of FTIR-ATR technique combined chemometrics (Cebi et al., 2017; Cebi, Yilmaz, & Sagdic, 2017; Fabian et al., 2006; Gok et al., 2015; Ropodi, Pavlidis, Mohareb, Panagou, & Nychas, 2015). Another important superiority of the developed methodology arises from the use of 3-bounce diamond ATR crystal with enhanced signal and absorbance (Rodriguez-Saona & Allendorf, 2011). Multi-bounce ATR provides a higher quality spectrum than single-bounce ATR because a sample is probed multiple times, thereby increasing the signal-to-noise ratio.

5. Conclusions

In our study, to the best of our knowledge, for the first time, the capability of FTIR-ATR spectroscopy combined chemometrics was evaluated for determination of gelatin source in gummy candies. As chemometrics, HCA, PCA and PLS-DA were performed using triple-bounce FTIR data. Gummy candy samples could be perfectly distinguished in terms of their gelatin source by all of these applied techniques. The spectral range 1734–1528 cm^{-1} was used to generate the dendrogram through Ward's algorithm (HCA). In addition, PLS-DA analysis was performed using same spectral range (1734–1528 cm^{-1}) for calibration and cross-validation. Class predictions of gummy candies were performed with 100% correct classification. In our study, while HCA and PCA analysis were performed using OPUS software, PLS-DA analysis was performed using Matlab. Quite confirmative and coherent results were obtained for gummy candies in the all chemometric applications. Furthermore, gelatin sources of twenty commercial gummy candies were evaluated using a RT-PCR technique. Satisfactorily, identification of all test samples with respect to their gelatin source by ATR-FTIR technique was confirmed using PCR technique with 100% accuracy without any disarrangements or false prediction.

Obtained results show that, discrimination and classification of gummy candies on the basis of their gelatin source were successfully performed by FTIR-ATR technique without any sample preparation. Finally, it can be concluded that the developed FTIR-ATR technique is cost-effective, rapid, easy to operate, non-destructive and can be mentioned as "green analytical technique" since no solvents and reagents have been used during the study.

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Compliance with ethics requirements

All authors have declared that they don't have any conflict of interest for publishing the research. In addition, this article does not contain any studies with human or animal subjects.

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