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Identification and differentiation of goat and sheep milk based on diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) using cluster analysis

C.S. Pappas [a], P.A. Tarantilis [a,*], E. Moschopoulou [b], G. Moatsou [b], I. Kandarakis [b], M.G. Polissiou [a]

[a] *Laboratory of Chemistry, Agricultural University of Athens, 75 Iera Odos Street, 11855 Athens, Greece*
[b] *Laboratory of Dairy Technology, Agricultural University of Athens, 75 Iera Odos Street, 11855 Athens, Greece*

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Abstract

A new methodology for identification and differentiation of goat and sheep milk was developed based on FT-IR spectroscopy using hierarchical and discriminant analysis. Forty-nine goat and 38 sheep defatted and freeze-dried Greek milk samples were analyzed. FT-IR spectra were obtained in the diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) mode. The spectral region 1840–950 cm^{-1} was used to 'fingerprint' milk types. Main peak used for differentiation of goat/sheep milk is located at 1745 cm^{-1}, which is correlated to the degree of sugars carboxyl methyl esterification. Hierarchical and discriminant analyses were based on the absorptions of the above spectral region. These analyses showed that the samples of goat milk can be differentiated from the samples of sheep's.

Keywords: Goat milk; Sheep milk; DRIFTS; Differentiation; Hierarchical analysis; Discriminant analysis

1. Introduction

Many traditional dairy products (mainly cheeses) that are accepted by the consumers worldwide are made from sheep or goat milk or from their mixtures. As the composition of cheese milk affects the characteristics and therefore the acceptability of the final product, there is an increased demand for genuine and accurately labeled dairy products, which necessitates protection against adulteration of milk kinds. The substitution of sheep milk by goat milk in the dairy products is a frequent problem, because sheep milk has a higher price. In addition to that, there are mixed flocks of goats and sheep that results in accidental or fraudulent substitution of sheep milk by caprine and vice-versa.

The substitution of sheep or goat milk with cow milk is detected by the E.U. reference method (Commission Regulation, 1996) based on the isoelectric focusing of γ-caseins. However, the substitution of sheep milk with goat milk is not possible by this method. A limited number of studies have been reported on this subject, which are for the most part based on enzyme-linked immusorbent assays (ELISAs) (Moatsou & Anifantakis, 2003). Atomic absorption spectrophotometry has been used for the differentiation of some Spanish cheese varieties (Fresno, Pietro, Urdiales, Sarmiento, & Carballo, 1995). Cation-exchange chromatography (Mayer, Heidler, & Rockenbauer, 1997; Moatsou, Hatzinaki, Psathas, & Anifantakis, 2004) and capillary electrophoresis (Bonhomme, 1981; Recio et al., 2004) have been applied for the detection of caprine casein in ovine cheeses.

Many works have been published using Fourier transform infrared (FT-IR) spectroscopy to analyze milk samples (Agnet, 1998; Deng, Zhou, & Sun, 2006; Etzion, Linker,

* Corresponding author. Fax: +30 210 5294265.
E-mail address: ptara@aua.gr (P.A. Tarantilis).

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Cogan, & Shmulevich, 2004; Lefier, 1998; Navratilova et al., 2006; Qin et al., 2004; Van de Voort, 1992). The advantages of FT-IR spectroscopy are known. The technique is simple, rapid and non-destructive for the samples. The majority of researchers focus on the quantitative determination of milk components as fat, proteins and lactose (Agnet, 1998; Deng et al., 2006; Etzion et al., 2004; Lefier, 1998; Navratilova et al., 2006; Van de Voort, 1992). Qin et al. (2004) studied the stability of powdered milk based on the determination of fat. Inon, Garrigues, and Guardia (2004) grounded on the FT-IR using the attenuated total reflectance (ATR) technique and chemometric analyses to classify commercial milks. Hewavitharana and Van Brakel (1997) determined the casein in raw milk using FT-IR spectroscopy and chemometric analyses. Zaleska, Tomasik, and Lii (2002) used FT-IR in the study of formation of carboxymethyl cellulose–casein complexes. Lanher (1996) used the Aegys Mi 600 Fourier transform infrared milk analyzer for the determination of fat, protein, lactose, and nonfat solids.

This work describes the development of a new method for the identification and differentiation of goat and sheep milks. The method is based on DRIFTS using hierarchical and discriminant analysis.

2. Materials and methods

2.1. Materials

2.1.1. Preparation of milk samples

Forty-nine individual goats' milk samples and 38 individual ewes' milk samples were randomly collected after the complete morning milking and to all of them NaN_3 at a ratio 0.05% was added soon after the collection. The ewes' milk samples were collected from the stock-farm of the Agricultural University of Athens and goats' milk samples from farms in Greece in the region of Athens and Larissa. After pH determination, the fat was removed by centrifugation at 2000*g* for 30 min at 4 °C and finally each sample was freeze-dried.

2.1.2. Isolation of whole acid casein

Bulk goats' milk was centrifuged at 2000*g* for 30 min at 4 °C for fat removal. The skimmed milk was acidified to pH 4.5 with acetic acid (1 mol L^{-1}) under continuous stirring at 25 °C. After setting for 20 min casein was precipitated. Then, the mixture was filtered through Whatman No. 40 paper and casein was separated. The separated casein was washed with distilled water, dissolved with the addition of NaOH 10 g L^{-1} until it reached pH 7.0 and precipitated again. Four successive cycles of precipitation and washing were carried out. The final precipitate was dissolved in 20 mmol L^{-1} phosphate buffer, pH 7.0, heated at 80 °C for 30 min to inactivate plasmin, dialyzed (MWCO 12 kg mol^{-1}, Sigma-Aldrich Chemie Gmbh, Germany) against distilled water and lyophilized. The same procedure was followed for bulk ewes' milk.

Table 1
Differentiation of goat and sheep milk samples using Omnic TQ analyst

Sample	Actual class	Calculated class	Distance to goat	Distance to sheep
g1	Goat	Goat	0.408	0.840
g2	Goat	Goat	0.035	0.438
g3	Goat	Goat	0.116	0.562
g4	Goat	Goat	0.126	0.380
g5	Goat	Goat	0.074	0.485
g6	Goat	Goat	0.224	0.880
g7	Goat	Goat	0.036	0.556
g8	Goat	Goat	0.051	0.367
g9	Goat	Goat	0.124	0.591
g10	Goat	Goat	0.264	0.710
g11	Goat	Goat	0.133	0.702
g12	Goat	Goat	0.084	0.583
g13	Goat	Goat	0.082	0.511
g14	Goat	Goat	0.085	0.503
g15	Goat	Goat	0.044	0.410
g16	Goat	Goat	0.128	0.419
g17	Goat	Goat	0.140	0.708
g18	Goat	Goat	0.101	0.717
g19	Goat	Goat	0.226	1.000
g20	Goat	Goat	0.138	0.764
g21	Goat	Goat	0.253	0.766
g22	Goat	Goat	0.160	0.741
g23	Goat	Goat	0.205	0.782
g24	Goat	Goat	0.092	0.496
g25	Goat	Goat	0.119	0.728
g26	Goat	Goat	0.069	0.433
g27	Goat	Goat	0.076	0.422
g28	Goat	Goat	0.200	0.674
g29	Goat	Goat	0.072	0.460
g30	Goat	Goat	0.144	0.658
g31	Goat	Goat	0.110	0.420
g32	Goat	Goat	0.100	0.357
g33	Goat	Goat	0.205	0.613
g34	Goat	Goat	0.175	0.188
g35	Goat	Goat	0.119	0.499
g36	Goat	Goat	0.229	0.647
g37	Goat	Goat	0.052	0.320
g38	Goat	Goat	0.162	0.371
g39	Goat	Goat	0.064	0.404
g40	Goat	Goat	0.115	0.387
g41	Goat	Goat	0.017	0.435
g42	Goat	Goat	0.076	0.391
g43	Goat	Goat	0.117	0.553
g44	Goat	Goat	0.042	0.525
g45	Goat	Goat	0.098	0.287
g46	Goat	Goat	0.210	0.400
g47	Goat	Goat	0.050	0.365
g48	Goat	Goat	0.091	0.293
g49	Goat	Goat	0.122	0.570
s1	Sheep	Sheep	0.621	0.090
s2	Sheep	Sheep	0.421	0.077
s3	Sheep	Sheep	0.737	0.095
s4	Sheep	Sheep	0.507	0.079
s5	Sheep	Sheep	0.289	0.124
s6	Sheep	Sheep	0.463	0.094
s7	Sheep	Sheep	0.571	0.045
s8	Sheep	Sheep	0.293	0.081
s9	Sheep	Sheep	0.558	0.050
s10	Sheep	Sheep	0.482	0.084
s11	Sheep	Sheep	0.570	0.031
s12	Sheep	Sheep	0.679	0.101
s13	Sheep	Sheep	0.575	0.275
s14	Sheep	Sheep	0.495	0.144

Table 1 (*continued*)

Sample	Actual class	Calculated class	Distance to goat	Distance to sheep
s15	Sheep	Sheep	0.433	0.087
s16	Sheep	Sheep	0.536	0.077
s17	Sheep	Sheep	0.651	0.050
s18	Sheep	Sheep	0.466	0.086
s19	Sheep	Sheep	0.409	0.118
s20	Sheep	Sheep	0.584	0.124
s21	Sheep	Sheep	0.616	0.059
s22	Sheep	Sheep	0.448	0.053
s23	Sheep	Sheep	0.417	0.073
s24	Sheep	Sheep	0.448	0.097
s25	Sheep	Sheep	0.858	0.364
s26	Sheep	Sheep	0.472	0.117
s27	Sheep	Sheep	0.221	0.136
s28	Sheep	Sheep	0.421	0.040
s29	Sheep	Sheep	0.590	0.094
s30	Sheep	Sheep	0.608	0.074
s31	Sheep	Sheep	0.429	0.105
s32	Sheep	Sheep	0.321	0.079
s33	Sheep	Sheep	0.645	0.153
s34	Sheep	Sheep	0.328	0.082
s35	Sheep	Sheep	0.420	0.101
s36	Sheep	Sheep	0.692	0.119
s37	Sheep	Sheep	0.769	0.096
s38	Sheep	Sheep	0.425	0.086

2.2. Lactose was purchased from Aldrich Company and was lyophilized for 24 h

2.2.1. FT-IR spectroscopy

FT-IR spectra were obtained in diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) mode using the micro sampling cup of a Spectra-Tech diffuse reflectance accessory (2 mg of dried sample) against a KBr background on a Nicolet 750 FT-IR spectrometer (DTGS detector; Nichrome source; KBr beamsplitter), with a total of 100 scans (resolution, 4 cm^{-1}). Spectra were collected and manipulated using the OMNIC (ver. 3.1) software supplied from the manufacturer of the spectrometer.

All spectra were smoothed using the 'automatic smooth' function of the above software, which uses the Savitsky–Golay algorithm (95-point moving second-degree polynomial). After that, the baseline was corrected using the 'automatic baseline correct' and the spectra scale was normalized with the 'normalize scale' function. The spectroscopic region 1840–950 cm^{-1} was used for hierarchical cluster analysis.

2.3. Cluster analysis

2.3.1. Hierarchical cluster analysis

The spectroscopic region 1840–950 cm^{-1} of each spectrum was exported as an Excel file. So eighty-seven excel files were obtained. The absorptions values in these files were used for the differentiation of the milk samples using SPSS (ver. 12.0) software. The between-groups linkage method for the absorptions and the Pearson correlation were chosen for the measurement of the distances between clusters.

2.3.2. Discriminant analysis

The spectral region 1840–950 cm^{-1} of samples' spectra were used for discriminant analysis with Omnic TQ analyst (ver. 1.1.1.6) software. The samples were classified in two actual classes (goat and sheep). Then the software classified the samples in two classes (calculated classes) using Mehalonobis distance algorithm (Table 1).

3. Results and discussion

3.1. FT-IR study

The main components of lyophilized defatted milk samples are proteins, lactose, salts – mainly phosphate, citrate, lactate, carbonate (Salaün, Mietton, & Gaucheron, 2005) and non-removed water.

Our interest was focused on the 1840–950 cm^{-1} spectral region, because in this area carbonyl group absorbs and the 'fingerprint' region is included. Consequently, in this region any differences between the spectra can be detected.

Fig. 1 shows typical FT-IR spectra of goat and sheep whole acid caseins in the spectroscopic region 1840–950 cm^{-1}. Eleven peaks appear. The first peak is centered at 1745 cm^{-1}. It is due to the stretching of carbonyl ester group (Choudhurry, Mukherjee, & Adhikari, 2005). The second broad peak at 1683–1682 cm^{-1} has been assigned to the carbonyl (C=O) stretching (amide I) (Zaleska et al., 2002). In the same broad band, the non-removed water absorbs (bending mode) (Pappas, Tarantilis, Daliani, Mavromoustakos, & Polissiou, 2002) and the C=C of aromatic aminoacids (phenyl nucleus) (Nakanishi & Solomon, 1977). The absorption at 1549–1547 cm^{-1} corresponds to the N–H bending with the contribution from C–N stretching (amide II) (Hewavitharana & Van Brakel, 1997; Zaleska et al., 2002). Furthermore, in the same spectral region the phenyl nucleus (C=C) absorbs (Nakanishi & Solomon, 1977). The peaks at 1455–1452 cm^{-1} and 1397 cm^{-1} are associated with CH bending (Zaleska et al., 2002). The weak absorptions at 1346–1344 cm^{-1} and 1314–1323 cm^{-1} have been assigned to CH bending and CH_2 wagging (Lewis & Mc, 1998; Nakanishi & Solomon, 1977). The peak at 1245–1243 cm^{-1} corresponds to CH bending and C–N stretching with the contribution from N–H bending (amide III) (Hewavitharana & Van Brakel, 1997; Zaleska et al., 2002). In this spectral region also absorb the O–P–O (asymmetric stretching vibration) (Lewis & Mc, 1998) the C–O (stretching vibration) bonds (Socrates, 1997). The band at 1168 cm^{-1} is due to $-NH_2$ deformation (Socrates, 1997). The last two peaks at 1099–1096 cm^{-1} and 973–971 cm^{-1} have been assigned to COH bending, and C–C stretching with the contribution from OH bending (Zaleska et al., 2002).

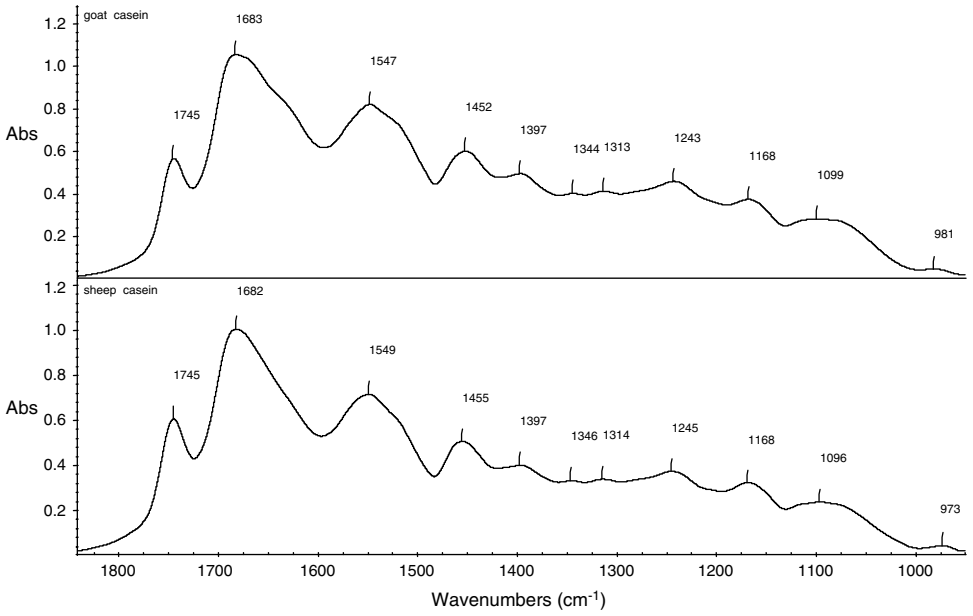

Fig. 1. FT-IR spectra in 1840–950 cm^{-1} spectral region of sheep and goat caseins.

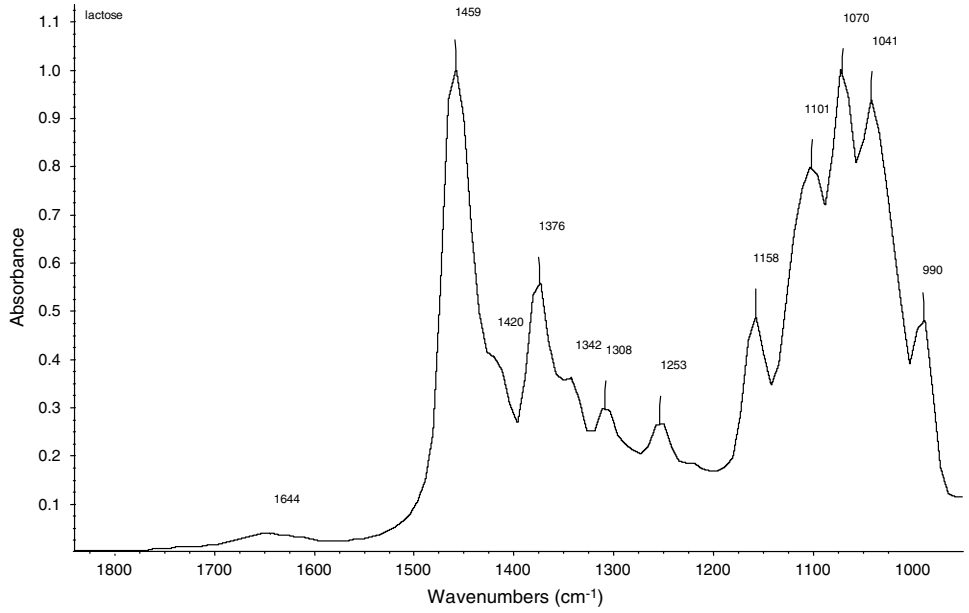

Fig. 2. FT-IR spectrum in 1840–950 cm^{-1} spectral region of lactose.

Fig. 2 shows the FT-IR spectrum of lactose in the spectroscopic region 1840–950 cm^{-1}. Twelve peaks are distinguished. The first broad and weak peak at 1644 cm^{-1} is correlated with the non-removed water (bending vibration) (Pappas et al., 2002). The very strong absorbance at 1459 cm^{-1} has been assigned to –OH bending (Druliolle, Kokoh, Hahn, Lamy, & Beden, 1997) and CH_2 scissoring (Nakanishi & Solomon, 1977). The third weak peak at 1420 cm^{-1} has been attributed to CH_2 bending and O–H in-plane bending (Nakanishi & Solomon, 1977). The 1376 cm^{-1} strong band corresponds to CH_2 bending (Druliolle et al., 1997). The weak peak at 1342 cm^{-1} is correlated with CH bending (Nakanishi & Solomon, 1977) and C–C, C–O skeletal vibrations (Pappas et al., 2002). The sixth medium in intensity absorption at 1306 cm^{-1} is due to O–H deformation (Druliolle et al., 1997). The band at 1253 cm^{-1} is attributed to CH bending (Druliolle et al., 1997). The next peak has been correlated with the C–O stretching from CHOH (Druliolle et al., 1997). T he following three absorptions have been assigned to the C–O stretching from CH_2OH. The final peak at 990 cm^{-1} is due to the stretching and twisting of C–H ((Harwood, Moody, & Percy, 1999).

In Fig. 3 appears the FT-IR spectra of goat and sheep defatted milk in spectral region 1845–1190 cm^{-1}. Twelve bands are shown at 1745–1744, 1683–1672, 1557–1553,

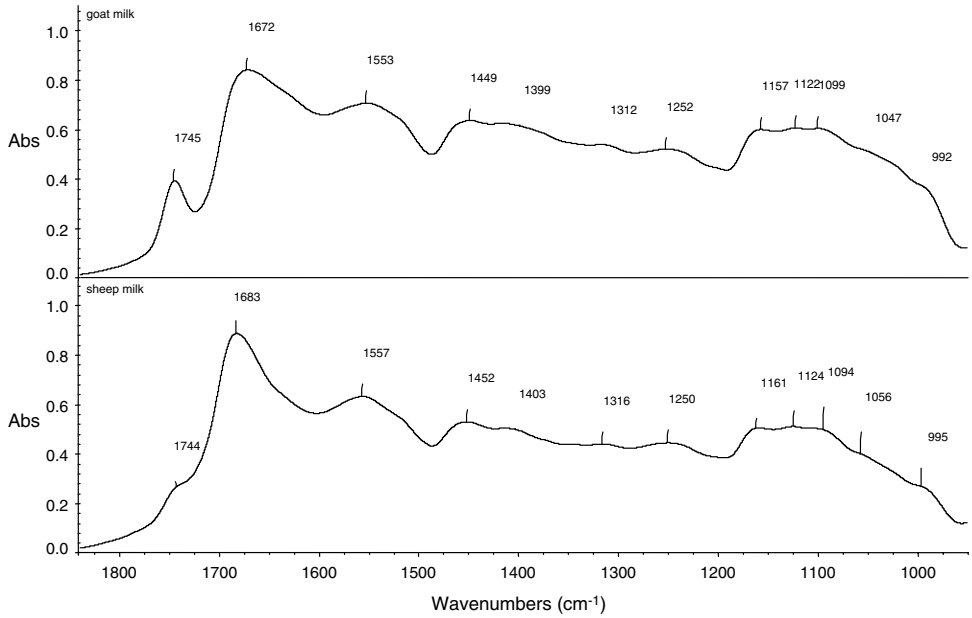

Fig. 3. FT-IR spectra in 1840–950 cm^{-1} spectral region of goat and sheep milk.

1452–1449, 1403–1399, 1316–1312, 1252–1250, 1161–1157, 1124–1122, 1099–1094, 1056–1047 and 995–992 cm^{-1}. The spectra texture is very similar to corresponding caseins spectra, but they are broader and present a significant shift.

The above absorptions are combination of corresponding absorptions of caseins, lactose, salts, sodium azide (whish was added to all milk samples) and non-removed water. The $-COO^-$ of salts absorb mainly at about 1610 cm^{-1} (antisymmetric vibration of C–O) (Dzwolak, Kato, & Taniguchi, 2002; Nakanishi & Solomon, 1977) and 1400 cm^{-1} (symmetric vibration) (Nakanishi & Solomon, 1977), while the carbonate (CO_3^{2-}) of salts at 1450–1410 cm^{-1} (Nakanishi & Solomon, 1977). The main peaks of sodium azide in this region are at 1555, 1510, 1458 and 1362 cm^{-1}. So, the peaks of milk components are overlapped. As we can see the peaks of casein cover these of the other milk components. So, the texture of milk samples spectra resembles the corresponding spectra of caseins. On the other hand, the interaction of caseins and the remaining milk components causes shift of the spectra milk peaks concerning those of caseins. For the above reasons the peaks of the milk samples appear broader than those of caseins.

There are minor differences between the milk samples spectra. The main difference is located at 1745 cm^{-1} of carbonyl vibration (ester group). This peak is shown as a shoulder in the sheep milk spectrum, but is very clear in the goat milk spectrum and has been correlated to the degree of sugars, carboxyl methyl esterification (Chatjigakis et al., 1998). The degree of esterification of the goat milk is higher than that of sheep milk (Fig. 3).

In our study, the discrimination between goat and sheep milk samples is based on small differences between spectra. An alteration was observed between goat and sheep milk samples in the carbonyl spectroscopic region. Furthermore, the spectroscopic region 1500–950 cm^{-1} is the 'fingerprint' area. So the 1840–950 cm^{-1} spectral region has been chosen.

3.2. Cluster analysis

In hierarchical cluster analysis, the similarity between samples is calculated using the distance concept, based on a mathematical relationship of numerical properties of the samples. In a successive procedure, each sample is linked to the closest sample or group of samples and a characteristic distance is used to describe this union. This distance between groups of samples can be evaluated in different ways and is the main difference among common linkage methods. The results are represented in a dendrogram, which shows at which normalized or rescaled distance a group of samples is differentiated from others, when it is read from bottom to top.

Fig. 4 shows the dendrogram of milk samples using between-groups linkage method and Pearson correlation. SPSS software was used. As can be seen, the clustering is successful. The distance fluctuated between 2 and 15 for the goat milk cluster and 2–10 for the sheep. The distance between goat and sheep milk cluster was 25. So, we remark a clear differentiation between the cluster of goat and sheep milk samples.

The differentiation between the goat and sheep milk samples was confirmed using discriminant analysis. The same spectroscopic region and Omnic TQ analyst software were used. This software uses the Mehalonobis algorithm for the calculation of the distances. Initially the samples were separated in two classes, goat and sheep actual class. Then the software calculated the distances and created two new classes, goat and sheep calculated class. Afterwards,

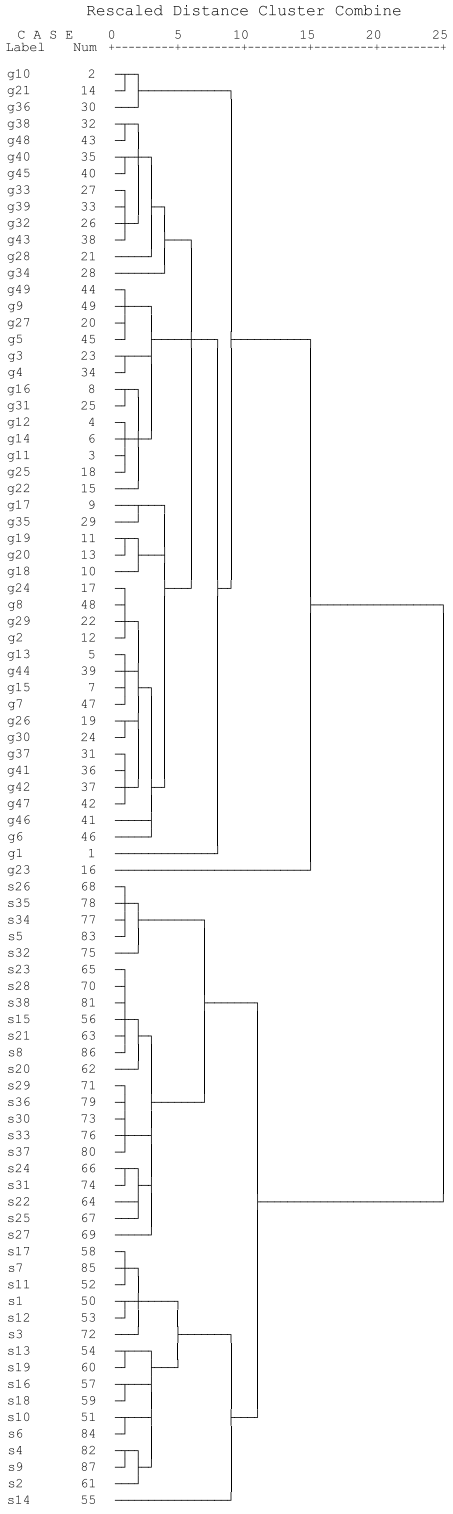

Fig. 4. Dendrogram of milk samples using between-groups linkage method and Pearson correlation.

the software inserted each sample in a calculated class (Table 1, Fig. 5). Every sample was classified correct (Table 1). The distances between goat milk samples fluctuated from 0.017 to 0.408 in calculated goat class and 0.031–0.364 for the sheep milk samples in the corresponding sheep class (Table 1, Fig. 5).

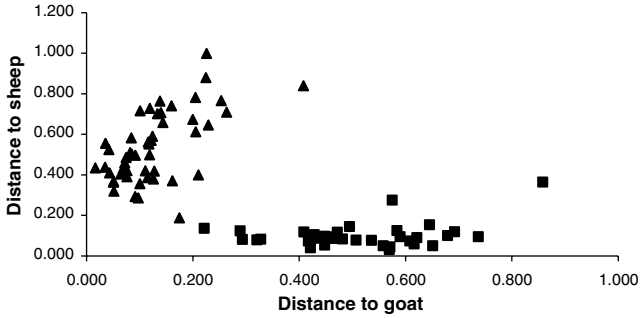

Fig. 5. Scatter plot of the distances between goat and sheep milk samples (▲ = goat sample, ■ = sheep milk).

4. Conclusions

According to the findings of the present study, Fourier transform infrared spectroscopy offers fast and accurate identification of the defatted milk kind and in combination with cluster analysis can differentiate the goat and sheep defatted milk samples. The method developed, using FT-IR spectra as a data bank, can detect minor differences between milk kinds, being rapid, accurate and non-destructive for the samples.

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