

# Discrimination of Cheddar and Kefalotyri Cheese Samples: Analysis by Chemometrics of Proton-NMR and FTIR Spectra

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**Abstract:** Cheddar and Kefalotyri cheese belong to the category of hard cheeses. Cheddar has an English origin, while Kefalotyri is a traditional cheese in Greece and a well-consumed dairy product in Cyprus. Discrimination of dairy products can be determined through several chemical methods. The aim of this study was to discriminate the samples of Cheddar and Kefalotyri cheese by analyzing various samples, from different brands. Two spectroscopic techniques namely proton nuclear magnetic resonance ( $^1\text{H-NMR}$ ) and Fourier-transformed infrared (FTIR) spectroscopy were chosen in order to chemically characterise the samples. The first step of the methodology was the freeze-drying process for lyophilisation of the samples. The number of samples reached 28, including 14 Cheddar samples and 14 samples of Kefalotyri cheese. After that, measurements for each sample have been obtained by FTIR (% transmittance-wavenumber in  $\text{cm}^{-1}$ ) and  $^1\text{H-NMR}$  (signal intensity-chemical shift in ppm) techniques. The data were analysed using SIMCA software. The proposed techniques along with chemometrics allow the discrimination of those two types of cheese. Both techniques employed are of significant importance, since they provide information about good classification of the samples when they are combined together. Interpretation of results and classification by using chemometric methods confirmed the different recipe of the two types of cheese. This study is the initial step of the future work. Future research will focus on discrimination based on the species' origin of milk of these and other cheese samples.

**Key words:** Cheddar cheese, Kefalotyri cheese, discrimination, chemometrics,  $^1\text{H-NMR}$ , FTIR.

## 1. Introduction

Adulteration of dairy products is a well-known phenomenon, and there are numerous published studies about the authenticity of cheese specifically [1-4]. Moreover, there are several analytical techniques which can indicate food authenticity [5-7].

The word Kefalotyri comes from the Greek term "head-shaped" which portrays how a whole Kefalotyri cheese looks like [8]. It is a famous Greek product, mainly made of goat's or sheep's milk or a mixture of these. Kefalotyri is a hard rind, heavily salted cheese [9, 10]. It has a strong flavor, white-yellow colour with small holes all over the mass of cheese [9].

Cheddar cheese also belongs to the category of hard cheese [11]. The cheese took its name from the village Cheddar in the English county called Somerset [12]. Cheddar cheese is made using cow's milk. Its production includes an additional step compared to other cheeses named the "cheddaring" process, which relates to curd draining. The pH is reduced between 1-2 h in length, resulting in tight packaging of the curds and slow draining off the whey [13].

Cheddar cheese has been studied enough regarding chemical characterization and authenticity in contrast with Kefalotyri cheese. For instance, previous studies in relation to Cheddar cheese have focused on textural analysis (fat, protein and moisture) [14, 15], flavor and chemical composition (moisture, pH, salt, fat content) [16], flavor and sensory analysis during ripening [17, 18], aroma (volatile components) for sensory analysis

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during ripening [19], composition, sensory and rheological parameters [2], following different experimental procedures compared to this study. As far as it can be determined from existing literature this is the first study on authenticity of Kefalotyri cheese with chemical analytical techniques.

Various studies have mentioned that spectroscopic measurements and chemometrics can be a successful combination in the field of food and beverage classification [2, 20-22]. Dairy products are among those foodstuffs which are often adulterated. Substitution of a portion of fat and/or proteins, adulteration with milk of other species' origin and mislabeling of ingredients are some of the food authenticity issues [23]. There are not any adulterations published related to Cheddar and Kefalotyri cheese, however it was considered important to investigate it since adulteration in cheese products is very common [24-27].

In this study, analytical methods were used, which are normally employed by this group in solid state chemistry-related research, such as Fourier-transformed infrared (FTIR) and nuclear magnetic resonance (NMR) to characterize and classify traditional Cypriot products in combination with chemometrics [28-30]. Regarding cheese, FTIR with chemometrics has been recently used for studying Pecorino cheese [31], Cantal-type cheese [32], Edamer, Gouda, Emmentaler and Feta [33], as well as proton nuclear magnetic resonance ( $^1\text{H-NMR}$ ) with chemometrics for Parmigiano Reggiano [34], Mozzarella [35], etc. Subsequently, there is a research gap related to these two types of cheese studied here, and an emerging interest to combine them in this study.

The methodology used here is fast, simple and using less reagents than others. The aim of the work was to differentiate the two different types of hard cheese, to confirm that they are made following their respective traditional recipes, by using the combination of the analytical techniques already mentioned and chemometrics. At first, principal

component analysis (PCA) was used followed by orthogonal projections like orthogonal projections to latent structures discriminant analysis (OPLS-DA) and O2PLS-DA. This present work was the first report highlighting the use of spectroscopic techniques modeled with OPLS-DA for the particular two types of dairy products. It is the first time when a study combines FTIR and  $^1\text{H-NMR}$  with chemometrics for the discrimination of Cheddar and Kefalotyri cheese samples.

## **2. Materials and Methods**

### *2.1 Cheese Sampling*

A total of 28 samples (14 commercial samples of Kefalotyri cheese and 14 commercial samples of Cheddar cheese) were purchased from local supermarkets in Cyprus. Table 1 shows details of the samples regarding the animal origin of milk and the country of provenance.

### *2.2 Preparation (Lyophilization) of the Samples*

Freeze drying of the samples was necessary to avoid the presence of water from the samples. Lucas *et al.* [36] reported a freeze-drying method and conditions for preparing some cheese samples and based on that the parameters of freeze-drying method were optimized. More particularly the period of freeze drying for the samples was reduced to 5 h. Due to that, the mass of each sample was decreased, too. The samples of the present study were grated and 5 g of each sample was subjected to freeze-drying using a Christ, Alpha 1-2 freeze-drier. The condenser temperature was 233 K and the final pressure in the drying chamber was 3 mPa. After the freeze-drying procedure, the residue was homogenised and used for FTIR and  $^1\text{H-NMR}$  measurements.

### *2.3 FTIR*

The FTIR spectra were measured in duplicate on a Shimadzu Fourier Transform-8900 Spectrometer instrument employing a KBr beam splitter. Twenty

**Table 1** Details of samples.

S/n	Label	Grouping	Type	Animal origin of milk	Country of provenance
1	S1	1	Cheddar	Cow	New Zealand
2	S2	1	Cheddar, mild	Cow	Somerset, England
3	S3	1	Cheddar	Cow	Somerset, England
4	S4	1	Cheddar, mature	Cow	Davidstow, United Kingdom
5	S5	1	Cheddar, mild	Cow	England
6	S6	1	Cheddar	Cow	Ireland
7	S7	1	Cheddar, mild	Cow	England
8	S8	1	Cheddar	Cow	Ireland
9	S9	1	Cheddar, mild	Cow	Ireland
10	S10	1	Cheddar	Cow	Ireland
11	S11	1	Cheddar	Cow	England
12	S12	1	Cheddar, medium	Cow	United Kingdom
13	S13	1	Cheddar	Cow	The Netherlands
14	S14	1	Cheddar, mild	Cow	Somerset, England
-	Removed	-	Cheddar, vegan	Plant	Europe
15	S16	2	Kefalotyri	Cow	Denmark
16	S17	2	Kefalotyri	Sheep	Cyprus
17	S18	2	Kefalotyri	Cow	Greece
18	S19	2	Kefalotyri	Sheep & goat	Greece
19	S20	2	Kefalotyri	Sheep & goat	Greece
20	S21	2	Kefalotyri	Cow	Greece
21	S22	2	Kefalotyri	Sheep & goat	Cyprus
22	S23	2	Kefalotyri	Mix	Mytilene
23	S24	2	Kefalotyri	Sheep & goat	Cyprus
24	S25	2	Kefalotyri	Sheep & goat	Cyprus
25	S26	2	Kefalotyri	Cow	Greece
26	S27	2	Kefalotyri	Cow	Cyprus
27	S28	2	Kefalotyri	Sheep & goat	Greece
28	S29	2	Kefalotyri	Sheep	Greece

S/n = sample number.

scans were co-added at a normal resolution of 8 cm<sup>-1</sup> in the 400-4,000 cm<sup>-1</sup> region. The samples were recorded against a background of air to minimize the interference due to carbon dioxide and water vapor in the atmosphere. Samples were recorded as pressed KBr pellets.

#### 2.4 <sup>1</sup>H-NMR

The samples to acquire <sup>1</sup>H-NMR spectra were prepared by dissolving 80 mg of cheese sample in 600 μL of deuterium oxide (D<sub>2</sub>O). After filtration, only 500 μL of each sample was transferred in the NMR tube for proceeding to measurement. The <sup>1</sup>H-NMR spectra were measured on a Bruker Avance 300

Ultrasield spectrometer (Bruker BioSpin, Rheinstetten, Germany). Sixteen scans were acquired with a spectral width of 12.0 ppm. All NMR spectra were baseline-corrected.

#### 2.5 Chemometrics

All the data acquired from the spectra were analyzed by SIMCA (version 14.1 & 15.0.2; Umetrics; Sweden, Switzerland). The variables (spectroscopic data) are correlated and summarized by other new variables usually the t<sub>0</sub>, t<sub>1</sub> and t<sub>2</sub> scores whose values explain the variation represented by each principal component. Those scores are orthogonal, meaning to be completely independent between them. There are

as many scores as components in the model. The score  $t_1$  (first component) explains the largest variation of the X space, followed by  $t_2$ , etc. A score plot shows the possible presence of outliers, groups, similarities and other patterns in the data, thus a score plot can be characterized as a map of the observations [37, 38]. Two-dimensional score plots have the tolerance ellipse based on Hotelling's  $T^2$  (95% tolerance was set for all the data analysis during this study). Observations (samples) situated outside the ellipse are outliers. Pattern recognition tool shown in this work was as follows.

### 2.6 OPLS-DA

It is a method usually used for group separation. It fits an OPLS/O2PLS model using SIMCA created dummy Y variables, one for each class, and it is available after grouping observations in two or more classes. This technique is a powerful tool which can handle the variation in X that is orthogonal to Y. The particular model provides high transparency and interpretability. Later extensions of OPLS gave rise to OPLS-DA in 2005 thus making it appropriate for use for discriminant analysis along with prediction purposes. The latest can separate predictive from non-predictive (orthogonal) variation [38].

## 3. Results and Discussion

One of the main ways to study the data structure is to search for natural groupings in the samples [39-41]. The initial goal was to achieve a good separation of the two groups of cheese presented in Table 1. Sample S15 of Table 1 was the only cheese having plant origin as it was a vegan cheese, and due to that reason, it was hampering the results of this study. S15 was finally removed from the observations during chemometric analysis to achieve a more balanced soft sensor model. Thus, the two groups were as follows:

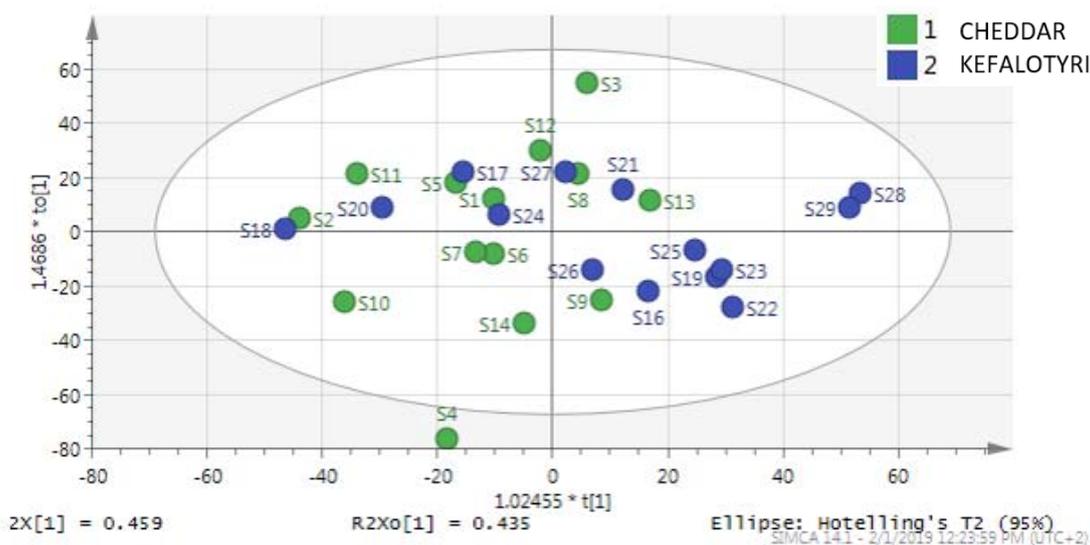
Group 1: Cheddar cheese samples (#14: S1-S14);

Group 2: Kefalotyri cheese samples (#14: S16-S29).

Chemometric results obtained with PCA and O2PLS-DA are not presented here, as PCA was not producing significant results, however O2PLS-DA was giving the same results with OPLS-DA for all the results presented below.

### 3.1 Chemometric Results of $^1\text{H-NMR}$ and FTIR Data

Regarding the type of cheese, Fig. 1 shows the classification of samples from OPLS-DA modeling and all the data (excluding the region 2,800-1,700  $\text{cm}^{-1}$  which might interfere with the extraction of



**Fig. 1** Score scatter plot ( $t_0/t_1$ ) from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from  $^1\text{H-NMR}$  and FTIR spectra,  $R^2X$  (cum) = 0.89,  $Q^2$  (cum) = 0.19.

useful information) obtained from <sup>1</sup>H-NMR and FTIR spectra were used. Although the Fischer value is lower than 0.05 (Table 2), the percentage of successful classification was only 71.43%, since 8 of 28 samples were classified incorrectly indicating that the combination of all the data obtained from <sup>1</sup>H-NMR and FTIR data disturbs the success of the chemometric model.

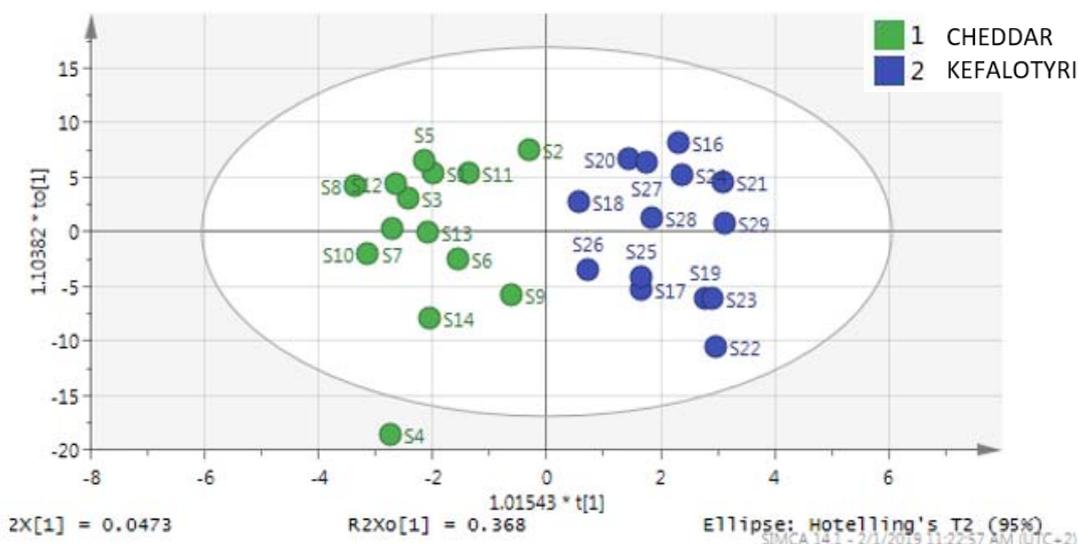
A second chemometric analysis, was carried out by using all the data from <sup>1</sup>H-NMR and only the region 1,000-400 cm<sup>-1</sup> from FTIR spectra. These results are displayed in Fig. 2. Most of the Cheddar cheese samples mapped at the left side of the 95% Hotelling

T2 ellipse and Kefalotyri cheese samples mapped at the right side. Table 3 shows a 100% correct prediction for all samples, according to the type of cheese, since all the samples classify correctly, while the low Fischer value of  $p < 0.05$  emphasizes the statistical importance of the model. However, the values  $R^2X$  (cum) = 0.6 and  $Q^2$  (cum) = 0.4 (Fig. 2) are not satisfactory. The samples do not seem to differentiate in their groups according to their provenance or type of milk, therefore chemometric analysis continued by changing the region used from FTIR spectra.

Chemometric analysis was carried out by using all

**Table 2 Misclassification from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from <sup>1</sup>H-NMR and FTIR spectra.**

Group	Members	Correct	1-Cheddar	2-Kefalotyri
1	14	71.43%	10	4
2	14	71.43%	4	10
Total	28	71.43%	14	14
Fisher's prob.	0.028			



**Fig. 2 Score scatter plot (to/t1) from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from <sup>1</sup>H-NMR and only the region 1,000-400 cm<sup>-1</sup> from FTIR spectra,  $R^2X$  (cum) = 0.6,  $Q^2$  (cum) = 0.4.**

**Table 3 Misclassification from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from <sup>1</sup>H-NMR and only the region 1,000-400 cm<sup>-1</sup> from FTIR spectra.**

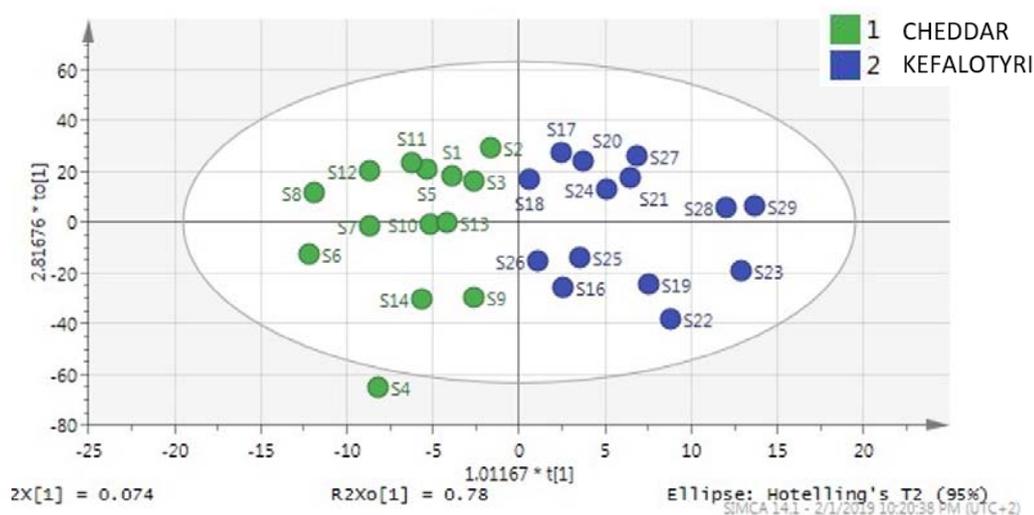
	Members	Correct	1-Cheddar	2-Kefalotyri
1	14	100%	14	0
2	14	100%	0	14
Total	28	100%	14	14
Fisher's prob.	2.5e-008			

the data obtained from  $^1\text{H-NMR}$  and only the region  $1,900\text{-}400\text{ cm}^{-1}$  from FTIR spectra, producing Fig. 3. This was a satisfactory outcome and it is also confirmed by the Table 4. In addition, goodness fit of the model is confirmed by the value 0.94 for  $R^2X$  (cum) which is higher than 0.5, and  $Q^2$  (cum) is equal to 0.5. The difference between these two values  $R^2X$  (cum) and  $Q^2$  (cum) is 0.44 which is relatively low. The region  $1,500\text{-}900\text{ cm}^{-1}$ , called the fingerprint region, is included in this range. In addition,  $\text{-C=O}$  of acids and esters ( $1,750\text{-}1,650\text{ cm}^{-1}$ ), amide I and amide II of proteins ( $1,650\text{-}1,450\text{ cm}^{-1}$ ), esters and aliphatic chains of fatty acids ( $1,460\text{-}1,150\text{ cm}^{-1}$ ),  $\text{C=O}$  and  $\text{C-C}$  stretching of acids ( $1,200\text{-}800\text{ cm}^{-1}$ ) and absorbance from acidic amino acids, such as glutamic acid, and the aliphatic chains of fatty acids ( $1,450\text{ cm}^{-1}$  and  $1,410\text{ cm}^{-1}$ ) in FTIR spectra contribute significantly to the discrimination of the samples [31, 42-44].

Pillonel *et al.* [1] suggested that the region  $4,000\text{-}3,050\text{ cm}^{-1}$  contained no useful chemical information. However, Paradkar and Irudayaraj [45] indicated that  $3,200\text{-}2,800\text{ cm}^{-1}$  is important for cholesterol characterization. Subsequently, the next chemometric step was the use of data corresponding to the region  $4,000\text{-}2,700\text{ cm}^{-1}$  from FTIR data as well as

all the  $^1\text{H-NMR}$  data. The results are presented in Fig. 4, which are not as satisfactory as those in Figs. 2 and 3. Also, the prediction percentages as shown in Table 5 are an indication of the correctness of the distribution of samples in the groups. Despite the fact that this fell to 71.43%, the Fisher's value was less 0.05, indicating that this model as presented is valid. Subsequently,  $\text{-O-H}$  stretching in hydroxyl groups ( $3,700\text{-}3,000\text{ cm}^{-1}$ ) and  $\text{-C-H}$  stretching of methyl groups of cholesterol structure and fatty acids ( $3,000\text{-}2,800\text{ cm}^{-1}$ ) are similar for both types of cheese and there is not significant contribution to discrimination [31, 42, 43, 45].

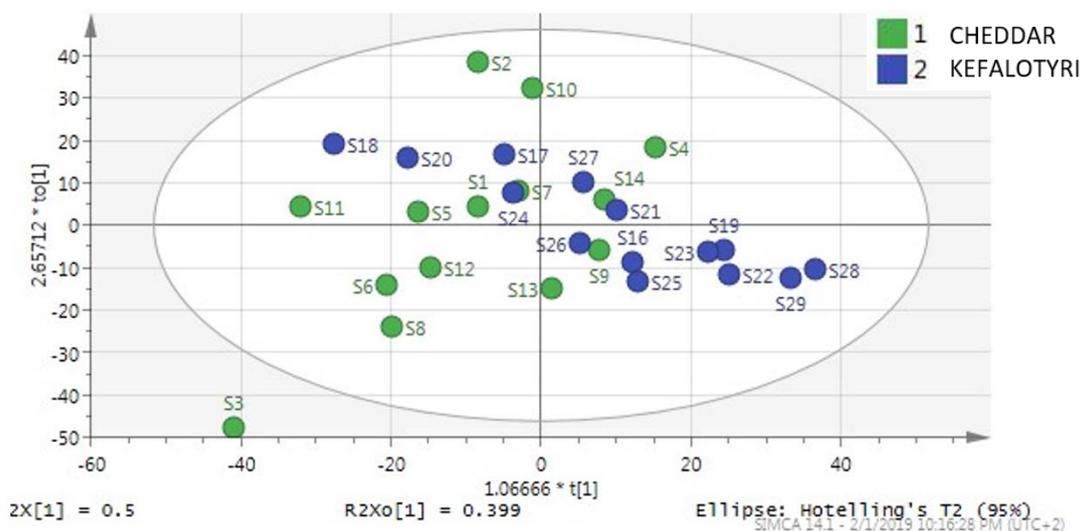
These last results suggest that analysis based on a combination of  $^1\text{H-NMR}$  data with some of the data from FTIR spectra specifically the region  $1,900\text{-}400\text{ cm}^{-1}$  gives the best results with chemometric analysis. In the case of Cheddar cheese this region includes the above mentioned functional groups contained in organic acids, alcohols, short chain fatty acids and their esters, amino acids and small water-soluble peptides, all of which are important for its unique flavor [16]. Since Cheddar and Kefalotyri cheese have different flavor it was expected that the particular region would be important for their discrimination.



**Fig. 3** Score scatter plot (to/t1) from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from  $^1\text{H-NMR}$  and only the region  $1,900\text{-}400\text{ cm}^{-1}$  from FTIR spectra,  $R^2X$  (cum) = 0.94,  $Q^2$  (cum) = 0.5.

**Table 4** Misclassification from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from <sup>1</sup>H-NMR and only the region 1,900-400 cm<sup>-1</sup> from FTIR spectra.

	Members	Correct	1-Cheddar	2-Kefalotyri
1	14	100%	14	0
2	14	100%	0	14
Total	28	100%	14	14
Fisher's prob.		2.5e-008		



**Fig. 4** Score scatter plot (to/t1) from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from <sup>1</sup>H-NMR and only the region 4,000-2,700 cm<sup>-1</sup> from FTIR spectra, R<sup>2</sup>X (cum) = 0.9, Q<sup>2</sup> (cum) = 0.015.

**Table 5** Misclassification from OPLS-DA modeling of the samples regarding the type of cheese for all the data obtained from <sup>1</sup>H-NMR and only the region 4,000-2,700 cm<sup>-1</sup> from FTIR spectra.

	Members	Correct	1-Cheddar	2-Kefalotyri
1	14	71.43%	10	4
2	14	71.43%	4	10
Total	28	71.43%	14	14
Fisher's prob.		0.028		

#### 4. Conclusions

<sup>1</sup>H-NMR and FTIR spectroscopy in combination with OPLS-DA were able to differentiate the two types of cheese (Cheddar and Kefalotyri) and additionally they seem to be a useful combination for classifications. When the <sup>1</sup>H-NMR and FTIR data (especially the region 1,900-400 cm<sup>-1</sup>) are used together, excellent results are produced regarding classifications based on the type of cheese. With regard to the chemometric procedures, other chemometric methods than OPLS-DA and O2PLS-DA can be tested for the same samples in the future, e.g., canonical discriminant analysis (CDA)

and classification and regression trees (CARTs). The results gained here compromise the initial step of setting up a method able to differentiate cheese samples regarding the type of milk (species' origin) which has been used for their production. This will lighten up the field of food authenticity by determining the adulterations in milk taking place among dairy products.

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