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1 **Major and trace elements in milk and Halloumi cheese as markers for**
2 **authentication of goat feeding regimes and geographical origin**

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1 **ABSTRACT**

2 Sixty samples of milk (n=24), Halloumi cheese (n=23) and local grazing plants (i.e.
3 shrubs) (n=13) were collected over a year from dairy farms located on three different
4 locations of the island of Cyprus. Major and trace elements (Ag, Al, B, Ba, Co, Fe, Li,
5 Mn, Se, Sr, Y, Ca, K, Mg, Na and P) were quantified using Inductively Coupled
6 Plasma-Atomic Emission Spectroscopy (ICP-AES). Milk and Halloumi cheese
7 produced in different geographical locations of Cyprus presented significant
8 differences in the concentration of some of the elements analysed. Principal
9 component analysis showed grouping of samples according to region of production
10 for both, milk and cheese samples. By applying a stepwise canonical discriminant
11 analysis, a good discrimination of milk (88.5% of the samples correctly assigned to
12 the area of production) and cheese (100% of the samples correctly classified)
13 according to geographical origin and feeding regimes was obtained. These findings
14 show that the assay of element compounds can provide useful fingerprints for the
15 characterisation of dairy products according to geographical location and production
16 system.

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18 **Keywords:** authentication, elemental markers, geographical origin, feeding regime,
19 goat milk, Halloumi cheese

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1 **1. INTRODUCTION**

2 The differentiation of dairy products obtained from different regions in Cyprus
3 (highland areas i.e. Paphos vs lowland areas i.e. Kofinou) is of high importance since
4 milk and cheese produced in highland areas could carry a 'quality label' i.e.
5 "mountain product" (EC 1151/2012). It has to be proved, however, that they are
6 distinctively different from milk and cheese produced in lowland areas. Products
7 endorsed with a quality label have an added value and benefit for the producers and
8 consumers alike. The production of goat and sheep milk in Cyprus is about 38 million
9 L year⁻¹ with approx. 21 million L (55 %) used for the production of Halloumi cheese
10 (Statistical Service Cyprus, 2014). Halloumi is the traditional goat and sheep cheese
11 produced in Cyprus that has gained international recognition with exports of 10,530t
12 currently valued at 70 million € annually (Statistical Service Cyprus, 2014). It is a
13 semi-hard, elastic, easily sliceable cheese that can be grilled, fried, or cooked whilst
14 retaining its texture. It has a pleasant milky flavour while salt adds to its taste. It is
15 made primarily of a mixture of goat and sheep milk (usually at 70:30 ratio), while
16 during the last 20 years cow's milk has also been introduced to the milk mixture
17 (Papademas, 2006). Although Halloumi cheese has not been yet assigned to any EU
18 quality schemes (such as Protected Designation of Origin –PDO– or Protected
19 Geographical Indication –PGI–), in order to protect its authenticity, some parameters
20 such as area of origin and processing of the milk must be established. These
21 characteristics depend on the original features of the milk and hence on the conditions
22 under which the milk has been produced such as feeding regime of the animals or
23 geographical location. The composition of the goat/sheep milk depends on production
24 factors constituting the farming system such as genotype, agro-climatic conditions
25 and feeding and milking of animals (Morand-Fehr, Fedele, Decandia, & Le Frileux,

1 2007). Due to the regime under which the goats and sheep are raised (usually goats
2 are kept outdoors several months per year), the feeding factor has been proven to be
3 one of the main factors influencing the composition of the milk (Morand-Fehr et al.,
4 2007) and it is linked inevitably to geographical origin where the feed ingested by the
5 animals is growing. Consequently, geography of the farming area might affect the
6 kind of plants that are available to the animals. For instance, highlands farm suitable
7 for extensive livestock production where no other agricultural practices are possible,
8 have a wider botanical diversity than lowlands where agricultural practices are
9 suitable (Mariaca et al., 1997).

10 Previous studies have established a relationship between some aspects of the chain;
11 plant-animal-milk-cheese and have proposed different compounds as markers of that
12 chain for tracing cheeses. For instance, quantification of specific compounds or
13 groups of compounds in milk and cheese that can act as dietary and geographical sites
14 markers has been reported; these include fatty acids (Collomb, Bütikofer, Sieber,
15 Jeangros, & Bosseta, 2002), volatile compounds –mainly terpenes (Pillonel, Ampuero,
16 Tabacchi, & Bosset, 2003; Zeppa, Giordano, Lombardi, Gerbi, 2003) and vitamin A
17 and E (Agabriel et al., 2007).

18 The use of multi-element analysis has been proved to be a good tool for origin
19 authentication purposes of different foods such as onions (Ariyama et al., 2007;
20 Chope & Terry, 2009; Furia, Naccarato, Sindona, Stabile, & Tagarelli, 2011), wine
21 (Taylor, Longerich & Greenough, 2003; Coetzee et al., 2005), tea (Moreda-Piñeiro,
22 Fisher & Hill, 2003) and tomatoes (Lo Feudo, Naccarato, Sindona, & Tagarelli, 2010),
23 milk i.e. cow vs buffalo (Benincasa, Lewis, Sindona, & Tagarelli, 2008) The
24 aforementioned technique has also been applied for origin authentication purposes to
25 cheeses (Suhaj & Koreňovská, 2008). The latter authors revealed significant

1 correlations between the concentration of elemental markers in soil and their content
2 in grass, milk and cheese. However, there is no study on the utility of mineral and
3 trace element analysis as a tool for discrimination of milk according to geographical
4 regions and/or feeding regimes. For the latter purpose, multi-element analysis has
5 been used in combination with multi-element isotope analysis in milk (Sacco et al.,
6 2009) and cheese (Pillonel et al., 2010; Bontempo et al., 2011). Factors influencing
7 the mineral and trace element concentrations in dairy products were studied by
8 Herrera et al. (2006). The concentration of minor and trace elements differed amongst
9 varieties of dairy products, i.e. raw milk vs whey vs fresh cheese vs semi-hard cheese;
10 these differences were also revealed by Coni et al. (1996). However, the region of
11 production was found to have less influence on the mineral concentration of dairy
12 products than the season of production (Herrera et al., 2006).

13 This study is based on the existing goat/sheep production systems from three different
14 areas of Cyprus well known for Halloumi cheese production. Available information
15 from the geochemical atlas of soils in Cyprus revealed substantial differences between
16 the three points of interest where the farms are located. Consequently, this paper
17 aimed at (a) investigating the differences of the metallic and trace elements in grazing
18 plants, goat/sheep milk and Halloumi cheese originating from those three areas and (b)
19 establishing a relationship between these elements as potential markers and the region
20 of production. Potential elemental markers could be used to assess authentication of
21 dairy products in Cyprus.

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1 2. MATERIAL AND METHODS

2 2.1. Experimental design

3 Three goat/sheep farms from different locations in Cyprus were studied. Group A
4 animals were bred in Anogyra area, a region in the SW of Cyprus where all animals
5 were 100% Damascus goat breed and grazed for 6h/d on a land mainly characterised
6 by the following plants: *Pistacia lentiscus* and *Ceratonia siliqua* and 18h/d kept in
7 indoor confinement. Indoor animals were offered a diet based on concentrate and
8 forage (mean daily DM intake per animal of 1 kg concentrate and *ad libitum* forage i.e.
9 straw). A typical concentrate composition is: 615 g kg⁻¹ barley, 230 g kg⁻¹ soya, 130 g
10 kg⁻¹ corn grains, 25 g kg⁻¹ mineral/vitamin premix containing calcium, phosphorus,
11 sodium and magnesium. All animals were milked twice daily. Group B animals were
12 bred in Kofinou area where all animals were 100% Damascus goat breed with a
13 feeding regime consisting of 2 h free-grazing and 22 h indoor feeding. Grazing in the
14 land where group B animals were raised was minimal and mainly characterized by the
15 plant species *Malva sylvestris*. Indoor animals were fed concentrate and straw (mean
16 daily DM intake per animal of 3 kg concentrate and 0.8-1.0 kg forage i.e. straw). The
17 composition of the concentrate was similar to the one mentioned above. Animals from
18 group C were bred in the region of Paphos in the west part of Cyprus. In this area,
19 goats and sheep after morning milking were led to the Paphos highlands in the spring
20 period (from March to June) and to the lowlands in the winter period (from September
21 –February) for approximately 8-10 h/d. Grazing during the very hot summer months
22 (July, August) is generally limited. The highland flora is characterised by the
23 following plants: *Thymus capitatus*, *Calicotome villosa*, *Myrtus communis*, *Olea*
24 *europaea*, *Quercus coccinea*, *Inula viscosa*, *Cistus creticus*, *Sarcopoterium spinosum*,
25 *Crataegus azarolus*, *Helichrysum italicum*, and a mixture of plants growing together

1 such as *Plantago lanceolata*, *Medicago truncatula* and *Poaceae*, whereas the lowland
2 flora is characterized by the straw remained after cereal harvest. When animals are
3 indoors they are offered *ad libitum* straw and barley in addition to feed concentrates.

4 **2.2. Sampling**

5 A total of 24 bulk-tank milk samples (approx. 1000 mL) were collected from March
6 2011 to February 2012 from the three dairy farms in Cyprus and 23 samples of
7 Halloumi cheese made from the same batch of milk were also collected. Halloumi
8 cheese was elaborated in each farm following the standard manufacturing procedure
9 excluding a dry mint coating of the cheeses to avoid possible external sources rather
10 than milk. Additionally, 13 samples of the predominant grazing plants were collected
11 (March - June 2011) from the three sampling regions and submitted to multi-element
12 analysis. All samples were stored at -80 °C for subsequent analysis.

13 **2.3. Chemicals**

14 The reagents used for mineralization, HNO₃ (65%) and H₂O₂ (30%) were provided
15 by Merck (Darmstadt, Germany). A multi-element solution of Ag, Al, Ba, Be, Bi, Cd,
16 Co, Cr, Cs, Cu, Ga, In, Li, Mg, Mn, Mo, Ni, Pb, Rb, Sr, Tl, V and Zn (10 mg L⁻¹) and
17 Ca, Fe, K and Na (100 mg L⁻¹) (Fluka, Buchs, Switzerland) as well as 5 individual
18 standards, As, Se and Y (Merck, Germany), B and P (Fluka, Switzerland) were used
19 to prepare the calibration standards. The accuracy of the method was assessed by
20 analysing the certified reference material BCR-063R (Skim milk power, IRMM -
21 European Commission-Joint Research Centre, Geel, Belgium).

22 **2.4. Analytical procedure**

23 Milk and Halloumi cheese samples were defrosted overnight. Milk samples were
24 thoroughly mixed prior to digestion. Aliquots of 3 mL of milk were weighed directly
25 into the digestion vessels and 9 mL of HNO₃ (65%) was added to each vessel.

1 Halloumi samples were cut into small cubes and ground using a manual grater.
2 Aliquots of 0.65 g of Halloumi cheese were weighed directly into the digestion
3 vessels and 4 mL of HNO₃ (65%) and 2 mL H₂O₂ (30%) were added to each vessel
4 as mentioned in the study of Bontempo et al. (2011). Fresh plants were dried in an air-
5 oven (Memmert, Germany) at 75°C for 48 h and dry material was ground and
6 homogenised using an electric mixer (Taurus, Spain). About 0.5 g of each dry plant
7 was weighed into the digestion vessels. Digestion was performed by adding 7 mL of
8 HNO₃ (65%) and 2 mL H₂O₂ (30%) to each vessel.

9 All samples were digested in close vessels using a Milestone Ethos 1 microwave oven
10 (Milestone Srl., Milan, Italy). The operating conditions used for the microwave
11 digestion system are shown in Table 1. All samples were digested once and analysis
12 was carried out in triplicates. Additionally, two milk samples and two Halloumi
13 samples were digested in triplicate and duplicate respectively, in order to check the
14 reproducibility of the digestion procedure and analysed in triplicate. After digestion
15 and cooling down at room temperature, all the digested liquors were quantitatively
16 transferred into volumetric flasks and made up to volume 12 mL for milk digested
17 liquors, 25 mL for Halloumi cheese digested liquors and 15 mL for plant digested
18 liquors with ultrapure water obtained from a Elix Milli-Q system (Millipore, USA). 1
19 mL of each digested liquors was transferred a second time to a test plastic tube and
20 made up to a volume 20 mL using ultrapure water in order to quantify those elements
21 present in high concentration in the samples (Ca, K, Mg, Na, P and Zn).

22 Analysis of Ag, Al, Ba, Be, Bi, Cd, Co, Cr, Cs, Cu, Ga, In, Li, Mg, Mn, Mo, Ni, Pb,
23 Rb, Sr, Tl, V, Zn, Ca, Fe, K and Na were determined by Inductively Coupled Plasma-
24 Atomic Emission Spectrometry (ICP-AES) (ICPE-9000 Shimadzu, Japan). Instrument

1 operating conditions were: radiofrequency power, 1200 W; plasma gas flow, 10.0
2 L/min; auxiliary gas flow, 0.6 L/min; carrier gas flow 0.7 L/min.

3 To assess the accuracy of the whole process, a certified reference material, BCR-063R
4 (Skim milk powder, IRMM - European Commission-Joint Research Centre, Geel,
5 Belgium) was subjected to the same analytical process as other samples and analysed
6 in triplicate (Table 2).

7 A minimum of two spiked samples with five elements: P (20 ppm or 20 µg/g), Na (20
8 µg/g), Ca (20 µg/g), K (10 µg/g) and Mg (10 µg/g) were analysed at the end of each
9 of the three analytical runs (cheese, milk and plants) for quality control and reliability
10 purposes.

11 Blanks were run at the beginning of each analytical batch to calculate the limit of
12 detection and quantification. Limit of detection (LOD) and limit of quantification
13 (LOQ) were defined as 3 and 10 times respectively, the standard deviation of the
14 signal from reagent blanks after correction for sample weight and dilution. Elements
15 below the LOQ value were not accepted.

16 ***2.5. Calibration procedure***

17 Calibration standards were prepared by diluting a multi-element solution containing
18 10 mg L⁻¹ of each of the following elements, Ag, Al, Ba, Be, Bi, Cd, Co, Cr, Cs, Cu,
19 Ga, In, Li, Mg, Mn, Mo, Ni, Pb, Rb, Sr, Tl, V and Zn and 100 mg L⁻¹ of Ca, Fe, K, Na
20 as well as five individual standards not included in the multielement solution. These
21 five standards were As, B, P, Se and Y in a solution of 0.08% HNO₃. The
22 concentration range for the elements Ag, Al, Ba, Be, Bi, Cd, Co, Cr, Cs, Cu, Ga, In,
23 Li, Mg, Mn, Mo, Ni, Pb, Rb, Sr, Tl, V and Zn was 0.001 – 0.2 whereas the
24 concentration range for the elements Ca, Fe, K and Na was 0.01 – 2 µg/g. The set of
25 calibration standards were analysed at the beginning of the sample runs and a seven-

1 point calibration curve was used for quantitative analysis. Six of those elements (Ca,
2 K, Mg, Na, P, and Zn) were present in high concentration in the dairy samples;
3 therefore a new set of calibration standards were prepared by diluting these six
4 elements in a solution of 0.08% HNO₃. The concentration range for Zn was 0.1 – 2.5
5 ppm whereas for Ca, K, Mg, Na, P and Zn was 2 – 50 µg/g. For the quantitative
6 analysis of those six elements, a five-point calibration curve was built.

7 ***2.6. Statistical analysis***

8 An exploratory one-way analysis of variance (ANOVA) for each measured variable
9 followed by a Tukey post hoc test was performed to assess the significance of the
10 differences among groups of samples of different production regimes using SPSS 20.0
11 (IBM SPSS Statistics, Inc., Chicago, IL). The data were also subjected to multivariate
12 statistical analysis to evaluate the possibility of differentiating milk and cheese
13 according to production regimen. Principal component analysis (PCA) was performed
14 to obtain a better perception of differences between elements of milk and Halloumi
15 cheese from different locations within Cyprus.

16 Canonical discriminant analysis (CDA) was performed to evaluate whether
17 discrimination of samples on the basis of the production regimen could be based on
18 the determined multi-elemental profile and to verify which elements contribute
19 toward classification. A stepwise method was used to select the most significant
20 variables and to exclude the redundant ones from the model. The procedure generates
21 a set of canonical discriminant functions based on the selected variables that provide
22 the best discrimination between the groups. Those functions can be applied to new
23 samples that have measurements for the determined elements but come from
24 unknown dietary groups. The statistical significance of each discriminant function
25 was evaluated on the basis of the Wilks' λ factor after the function was removed. To

1 verify the stability of the model, a “leave-one-out” cross validation was performed.
2 The success of the discrimination was measured by the proportion of cases correctly
3 classified using this cross-validation.

4
5 **3. RESULTS AND DISCUSSION**

6 **3.1. Milk**

7 Mineral and trace elements concentrations of cheese produced in three different areas
8 of Cyprus as well as the milk used to elaborate those cheeses are shown in Table 3.
9 Five elements (Li, Ca, Mn, Zn, Sr) showed significant differences according to the
10 producing areas of the milks. Milk samples from the highland area (Paphos) presented
11 higher concentrations of Li and Ca ($P<0.05$) than those from the other two producing
12 areas. In contrast to the findings of Garcia et al. (2006) who found that Ca
13 concentrations of goats’ dairy products were affected by seasonal variation rather than
14 by the region of production, Ca levels were found to be affected by the region of
15 production in the current study. While milks from lowland areas (Anogyra and
16 Kofinou) differed significantly in the concentration of Mn ($P<0.05$), milks from
17 Kofinou and Paphos differed significantly ($P<0.05$) in the concentration of Zn. The
18 differences in the mean concentration of Zn in milk from Kofinou and Paphos could
19 be attributed to the type of goats’ diet since Zn content in milk was found to be
20 dependent on this (Garcia et al., 2006). Additionally, the concentration of Sr was
21 significantly higher ($P<0.05$) in milk from Kofinou than in milk from Anogyra and
22 Paphos. Although a high variability in the concentration of Sr in the milk samples
23 from Kofinou was observed (Figure 1), it is clear that no overlap between Kofinou
24 samples and those from the other two regions occurred.

1 The higher concentrations of some of the minerals could be explained by the different
2 animals' diets (i.e. different type of grazing plants). Goats from Paphos are grazing
3 for prolonged periods of the year outdoors. As a consequence, higher levels of Zn, Ca
4 and Li occur in the milks from Paphos compared to the other areas, which are
5 reflected in the higher content of those minerals in the plants the animals grazed.
6 Element concentrations of plants collected from the three regions of production are
7 presented in Table 4. In terms of geographical location, wide plant diversity exists in
8 the highlands of Paphos where animals grazed *ad libitum*. Plants are known to
9 accumulate minerals and metals essential for their growth from the environment.

10 Principal component analysis (PCA) was applied to a data set composed of five
11 variables and 24 dairy samples. The variables were the elements that showed
12 significant differences between areas of production in the ANOVA, Li, Ca, Mn, Zn
13 and Sr. The score plot on the first two principal components (PCs) is showed in
14 Figure 2. The 100% original data variance was summarized by five uncorrelated
15 principal components. Only the first two PCs accounted for a significant piece of
16 information with eigenvalues ≥ 1 and they accounted for 61% of the total variance.

17 Samples from Kofinou and Paphos are clearly separated on the first principal
18 component, while samples from Anogyra and the other two regions (Kofinou and
19 Paphos) are mainly separated on the second principal component. Li, Sr and Zn have
20 the highest loading coefficients on the PC1, being negative for Sr and positive for Li
21 and Zn. On the other hand, Mn and Ca have the highest loading coefficients on the
22 PC2, both of them being positive. Samples from Kofinou were all situated the bottom
23 left of the score plot due to their high Sr content. Anogyra showed an almost-
24 complete separation from the other two groups with the exception of two samples,

1 mainly due to their high Mn and Ca content. Paphos samples were mainly situated at
2 the bottom right of the score plot due to their high content of Zn and Li.

3 ***3.1.1. Canonical discriminant analysis***

4 A canonical discriminant analysis (CDA) was performed to classify milk according to
5 region of origin on the basis of minor and trace elements. CDA was applied to five
6 variables (same as above, Li, Mn, Sr, Ca and Zn) considering all the milk samples
7 from three different regions of origin (Anogyra, Kofinou and Paphos). A stepwise
8 method was used in order to identify redundant variables and exclude them from the
9 model. In this case, three elements were selected due to their significant contribution
10 to the discrimination of milk samples: Ca, Sr and Zn. The results showed two
11 discriminant functions. Both discriminant functions were statistically significant for
12 the discrimination (Wilk's $\lambda < 0.2$), with the first being the most significant ($\lambda < 0.002$).
13 The first function explained 98% of the variance and it was mainly correlated with Ca.
14 The second discriminant function accounted for 2% of the variation and it was mainly
15 correlated with Sr and Zn. In the classification results (Table 3), 88.5% of the original
16 grouped cases were correctly classified. A slightly higher correct classification
17 (92.3%) was obtained when cross-validation analysis was performed. In the cross-
18 validation, all samples from Anogyra and Paphos were correctly classified, whereas
19 two samples from Kofinou were misclassified. This suggests that Ca, Sr and Zn
20 markers could be used as a tool in identifying milk originated from the three Cypriot
21 regions.

22 The separation between the three regions of origin in the discriminant space
23 was checked by plotting the first and second discriminant functions shown in Figure 3.
24 Results showed a good separation between the three groups with the exception of two
25 samples from Paphos that were clustered with samples from Anogyra.

1 **3.2. Halloumi cheese**

2 As expected, cheeses presented higher mean concentrations of all elements analysed
3 than the milk used for their production. This is due to the technological process for
4 elaboration of cheeses and the fact that cheeses are more concentrated (less moisture
5 content) product. Eight elements showed significant differences according to the
6 region of origin of the Halloumi cheeses, Ag, Ba, Ca, K, Mg, Mn, P and Sr.
7 Additionally, significant differences were observed in the concentration of Na. Since
8 salt (NaCl) is added as an ingredient in the cheese making process, Na (sodium)
9 should not be considered a possible marker of region of origin, thus it was excluded
10 from the models. However, cheeses from Paphos presented higher content of Na
11 followed by cheeses from Anogyra and Kofinou, respectively. Organoleptic
12 characterisation (i.e. sensory analysis) of Halloumi cheese clearly indicated that
13 cheeses from Paphos were saltier than those from Anogyra and Kofinou (data not
14 shown). Moreover, milk samples from the three areas of production had a similar Na
15 content, suggesting the addition of NaCl during cheese making affects the final
16 concentration. The concentrations of Li and B were below the detection limits of the
17 determination. Comparing the cheeses from the three regions, Halloumi from
18 Anogyra had higher ($P<0.01$) Ag, K and Mn mean concentration than the
19 corresponding mean values in Halloumi from Kofinou and Paphos. The high levels of
20 Ag could be explained by the fact that the surface soil of the Anogyra region presents
21 higher levels of Ag than the soil of the other two regions. Comparing with fresh and
22 semi-hard cheeses from Tenerife (Spain) (Garcia et al., 2006), Halloumi cheese from
23 the three regions had similar concentrations of K. Cheese samples from Kofinou had
24 higher levels ($P<0.01$) of Ba, P and Sr than Halloumi from the other two regions. The
25 concentration of Ba in both the surface soil and the subsoil was higher in the region of

1 Kofinou than that found in the other two regions (Cohen, Rutherford, Morisseau, &
2 Zissimos, 2011). Higher amount of Sr observed in the soil could also contribute to the
3 mean concentration of Sr present in Halloumi cheese samples from Kofinou.

4 PCA on cheese samples (n=23) was performed using five (Ag, Ba, K, Mn, Sr) of the
5 eight elements that showed significant differences between areas of production in the
6 ANOVA. Ca, Mg and P showed significant differences between the areas of
7 production, and were excluded from the PCA. The reason is that these minerals are
8 rather abundant in milk and their levels are not production depended. It is not
9 expected goat's milk to have different quantities of major elements even from
10 different areas since goat milk is from the same or similar breeds. These minerals,
11 therefore, they cannot serve as biomarkers.

12 Data processing with PCA showed that the three different production areas are well
13 separated in the score plot of the first two principal components (Figure 4), which
14 accounted for 73% of the total variance. 'Lowland' cheeses, however, are better
15 correlated with discriminant elements, i.e. have highest loading coefficients on one of
16 the PCs, than 'highland' cheeses. More specifically, 'lowland' Anogyra and Kofinou
17 samples are defined by their distinct Ag, Mn and K content and Ba and Sr content,
18 respectively. No particular element was found predominant from the highland
19 (Paphos) cheeses that made this area of production be sufficiently different from
20 lowland cheeses in terms of the elemental composition.

21 ***3.2.1. Canonical discriminant analysis***

22 CDA was applied to the concentration of the five elements of each sample mainly
23 contributing to separation of the groups in the PCA. These elements were Ag, Ba, K,
24 Mn and Sr. Following the stepwise procedure, three elements (K, Mn and Sr) were
25 selected due to their significant contribution to the discrimination of Halloumi

1 samples. The two first canonical discriminant functions were used in the analysis.
2 Both discriminant functions were statistically significant for the discrimination
3 (Wilk's $\lambda < 0.3$), with the first being the most significant ($\lambda < 0.05$). The first function
4 explained 65% of the variance and it was mainly correlated with Sr. The second
5 discriminant function accounted for 35% of the variation and it was mainly correlated
6 with K and Mn. The scores of the two canonical discriminant functions (Figure 5)
7 showed a clear separation between the three regions of cheese production. This good
8 separation of groups was confirmed by the classification results (Table 4); 100% of
9 the samples were correctly classified with 95.7% cross-validated correctly classified
10 samples. In the cross-validation, all samples from Anogyra and Paphos were correctly
11 classified and only one sample from Kofinou was misclassified. In a similar study,
12 different elemental markers of origin (Ba, Cu, Cr, Hg, Mg, Mn, Ni and V) were
13 successfully (>90% accuracy) able to classify Slovakian sheep cheeses from different
14 producing agricultural areas (Suhaj & Koreňovská, 2008) which highlighted the
15 potential of element content as markers for geographical identification of area of
16 cheese production. In the current study, K, Mn and Sr could be considered as markers
17 of origin for the identification of Halloumi cheese originating from different locations
18 of the island of Cyprus. From these, Sr is the only elemental marker of origin that
19 contributed to the successful identification of both milk and cheeses.

20

21 **4. CONCLUSIONS**

22 The vertical investigation of geochemical information (soil data *leading to* grazing
23 plants *leading to* milk *leading to* cheese, - here Halloumi cheese) is an essential
24 approach for authenticating the geographical origin of milk/cheese production that has
25 been attempted for some other products in the past. Its implementation, however, in

1 traditional dairy products can sometimes prove to be challenging. In the current study,
2 trace elements (Mn and Sr), are not present in high concentration in milk and cheese,
3 do not originate from cheesemaking equipment, nor are present in high quantities
4 naturally in milk or in feeding concentrates. These two trace elements have
5 demonstrated the capacity to provide a traceability record. Particularly strontium (Sr)
6 could be used as a potential biomarker for distinguishing at least the samples from one
7 area of production (i.e. Kofinou area) since the results could be well correlated for soil
8 data, milk and cheese as a final product. It is true that in order to build a more robust
9 model, more sample types (i.e. water from the animal farms) should be added and
10 overall sample numbers should be increased. On the other hand, if elemental data is
11 used in conjunction to other characteristic chemical indexes (i.e. fatty acid profiles,
12 plant terpenes, isotope analysis), a more holistic and accurate picture of halloumi
13 cheese authenticity could be created.

14

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20

1 **FIGURES / TABLES**

2

3 **Table 1.** Microwave digestion settings for milk, Halloumi cheese and plant samples

4

5 (a) Microwave digestion program for Halloumi cheese samples

Step	Time (min)	Temperature (°C)	Power (W)
1	7.00	90	1000
2	5.00	170	1000
3	5.00	210	1000
4	20.00	210	1000

6 (b) Microwave digestion program for milk samples

Step	Time (min)	Temperature (°C)	Power (W)
1	3.00	25-140	1000
2	4.00	140-150	1000
3	7.00	150	1000
4	10.00	150-180	1000
5	20.00	180	1000

7 (c) Microwave digestion program for plant samples

Step	Time (min)	Temperature (°C)	Power (W)
1	10.00	180	1000
2	10.00	180	1000

8

9

10 **Table 2.** Quality assurance material performance data - accuracy referred to skim

11 milk powder BCR-063R certified reference material (average from three analytical

12 runs).

Element	Certified value	Found value (mg/g)	Accuracy (%)
Ca	13.49 (mg/g) ± 0.10	13.56 ± 0.09	100.5
Fe	2.32 (µg/g) ± 0.23	3.48 ± 2.64	150
K	17.68 (mg/g) ± 0.19	16.56 ± 0.04	93.7
Mg	1.263 (mg/g) ± 0.024	1.23 ± 0.00	97.4
Na	4.37 (mg/g) ± 0.031	5.52 ± 0.05	126.3
P	11.10 (mg/g) ± 0.13	10.32 ± 0.31	92.9
Pb	18.50 (ng/g) ± 2.7	18.10 ± 1.27	95.1
Zn	49.00 (µg/g) ± 0.6	39.38±4.59	80.4

Table 3. Element content (mean $\mu\text{g/g} \pm \text{SD}$) in milk and Halloumi cheese from the three producing areas (Anogyra, Kofinou and Paphos).

Region Element content ($\mu\text{g/g}$)	Anogyra		Kofinou		Paphos	
	Milk (n=7)	Halloumi cheese (n=7)	Milk (n=7)	Halloumi cheese (n=7)	Milk (n=10)	Halloumi cheese (n=7)
Ag	n.d.	0.43 ^a ±0.25	n.d.	0.09 ^b ±0.06	n.d.	0.12 ^b ±0.10
Al	n.d.	1.47 ^a ±0.60	n.d.	1.24 ^a ±0.35	n.d.	1.33 ^a ±0.36
B	0.12 ^a ±0.06	n.d.	0.10 ^a ±0.04	n.d.	0.10 ^a ±0.03	n.d.
Ba	0.04 ^a ±0.03	0.60 ^b ±0.28	0.11 ^a ±0.06	1.23 ^a ±0.43	0.12 ^a ±0.12	0.48 ^b ±0.47
Co	0.14 ^a ±0.01	1.17 ^a ±0.08	0.14 ^a ±0.01	1.24 ^a ±0.06	0.16 ^a ±0.03	1.19 ^a ±0.09
Fe	0.14 ^a ±0.05	2.57 ^a ±2.15	0.16 ^a ±0.07	1.37 ^a ±0.82	0.32 ^a ±0.21	1.79 ^a ±0.93
Li	0.03 ^a ±0.00	n.d.	0.03 ^a ±0.00	n.d.	0.07 ^b ±0.03	n.d.
Mn	0.09 ^a ±0.04	1.01 ^a ±0.56	0.04 ^b ±0.01	0.37 ^b ±0.09	0.05 ^{a,b} ±0.02	0.32 ^b ±0.06
Se	0.31 ^a ±0.05	2.51 ^a ±0.21	0.25 ^a ±0.17	2.48 ^a ±0.40	0.32 ^a ±0.05	2.73 ^a ±0.41
Sr	0.89 ^b ±0.15	7.26 ^b ±1.31	3.34 ^a ±1.64	25.15 ^a ±11.22	1.03 ^b ±0.35	6.74 ^b ±1.36
Y	0.005 ^a ±0.00	0.05 ^a ±0.02	0.005 ^a ±0.00	0.04 ^a ±0.002	0.006 ^a ±0.001	0.04 ^a ±0.001
Ca	929.3 ^b ±52.9	6976.5 ^{a,b} ±684.8	785.9 ^b ±127.6	7568.4 ^a ±776.3	1228.5 ^a ±333.3	6583.4 ^b ±745.6
K	1195.6 ^a ±120.2	2038.8 ^a ±178.9	1151.8 ^a ±204.0	1729.1 ^b ±201	1415.1 ^a ±782.1	1516.7 ^b ±217.7
Mg	102.8 ^a ±29.0	288.6 ^a ±36.6	78.73 ^a ±18.14	349.6 ^b ±125.2	121.4 ^a ±60.2	236 ^a ±51.8
Na	389.3 ^a ±32.31	10774 ^{a,b} ±3557	356.2 ^a ±42.8	7952.1 ^b ±2966	372.4 ^a ±73.2	16193 ^a ±5822

P	720.5 ^a ±66,9	4786.4 ^b ±348,6	593.4 ^a ±78,3	5282.7 ^a ±541,8	815.0 ^a ±278,5	4686.6 ^b ±388,8
Zn	1.77 ^{a,b} ±0.45	26 ^a ±8,6	1.24 ^b ±0,22	25.2 ^a ±11,7	1.85 ^a ±0,71	31.7 ^a ±9.1

n: number of samples

n.d.: not detected

^{a,b} Different letters within a row indicate significant differences (P<0.05) between groups

Table 4. Average element content ($\mu\text{g/g}$ or ppm) in plants samples, mainly forage, from the three producing areas.

Plant species	Ag	Al	B	Ba	Co	Fe	Li	Mn	Ni	Sr	Y	Ca	K	Mg	Na	P	Zn
<i>Pistacia lentiscus</i> (A)	0.3	28.9	44.2	19.0	1.8	187.5	0.3	18.1	0.6	79.3	0.11	7235.4	5776.9	1471.8	2519.7	1188.1	10.0
<i>Ceratonia siliqua</i> (A)	0.3	48.9	2019.8	45.2	3.0	1492.8	0.4	628.7	1.9	2309.6	0.19	22628.0	4396.6	1837.0	4267.6	910.1	10.3
<i>Malva sylvestris</i> (K)	0.7	17.3	40.9	21.0	2.9	120.6	1.2	51.2	0.5	149.6	0.11	19888.1	26883.9	1319.2	4377.4	2568.5	27.7
<i>Myrtus communis</i> (P)	0.8	37.8	74.8	0.5	1.8	151.0	0.3	25.1	0.7	7.0	0.16	7295.4	4446.9	2254.9	2278.2	717.9	9.4
<i>Olea europaea</i> (P)	1.7	16.6	8.0	0.2	1.7	80.4	0.2	19.7	0.6	6.7	0.12	8228.5	4427.1	1155.1	2266.8	766.6	8.6
<i>Thymus capitatus</i> (P)	2.2	52.4	19.3	4.3	2.4	176.8	0.5	34.3	0.6	11.4	0.18	13972.1	8303.4	2315.4	2904.2	1147.7	24.2
<i>Inula viscosa</i> (P)	1.2	45.7	59.8	1.8	2.1	187.1	1.0	165.8	1.6	19.9	0.16	8503.5	15679.2	1089.2	5767.9	2241.8	18.6
<i>Cistus creticus</i> (P)	1.2	74.5	31.8	2.4	2.5	244.3	0.5	47.5	1.0	13.2	0.19	12215.4	6884.4	1536.9	2081.3	1483.8	42.4
<i>Sarcopoterium spinosum</i> (P)	0.2	70.4	31.8	2.0	2.4	252.5	0.5	38.9	0.8	14.9	0.22	13125.5	8108.5	3078.2	2208.7	1335.9	13.2
<i>Crataegus azarolus</i> (P)	1.0	8.2	32.5	0.6	1.8	66.7	0.2	26.2	0.4	5.8	0.11	6161.0	12308.7	1723.2	2371.9	1500.3	15.5
<i>Quercus coccinea</i> (P)	0.5	27.1	46.4	0.6	1.5	109.6	0.7	326.8	0.7	5.8	0.11	6122.9	2655.1	1017.4	2195.4	821.2	12.4
<i>Helichrysum italicum</i> (P)	0.3	1028 3.9	31.7	3.0	2.7	26507. 7	1.7	1333. 9	3.3	12.6	0.59	8711.5	8529.1	2624.1	4889.9	967.4	13.3
Mixture (<i>Plantago lanceolata</i>).	0.5	72.6	5.5	0.5	1.7	303.6	0.3	37.1	1.2	7.7	0.14	3947.6	18622.2	1389.2	2844.9	1625.7	16.9

<i>Medicago truncatula</i> (P)																	
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(A): Anogyra; (K): Kofinou; (P): Paphos

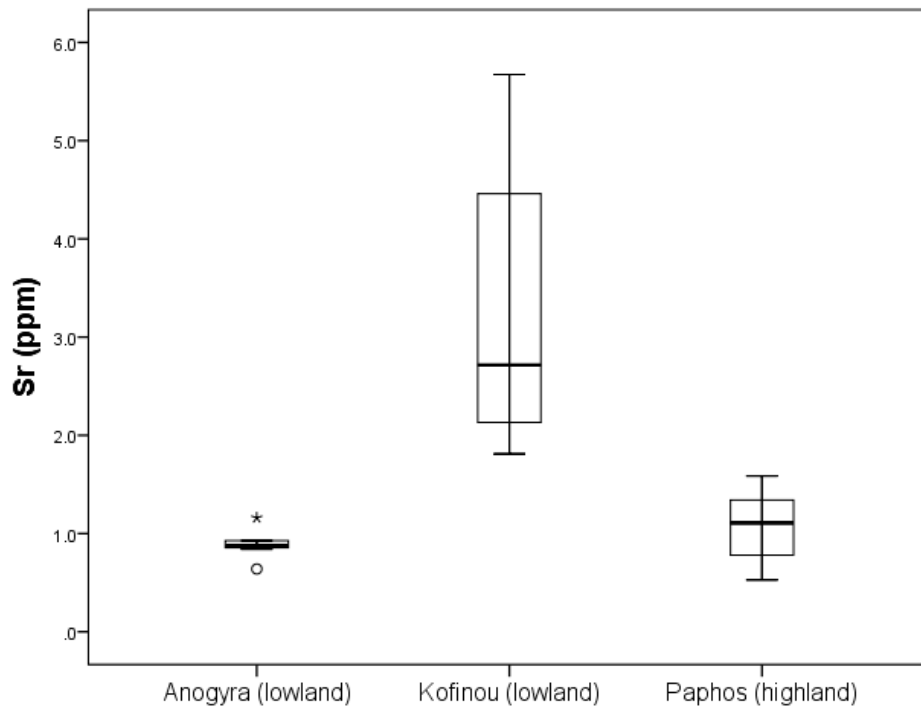


Figure 1. Box plot showing Sr concentration values from milk produced in different regions and under different production systems. (Line in the centre, median; box, 25-75th percentile; whisker, minimum non-outlier - maximum non-outlier. Symbols out of the boxplots: outliers, circles; extreme outliers, asterisks).

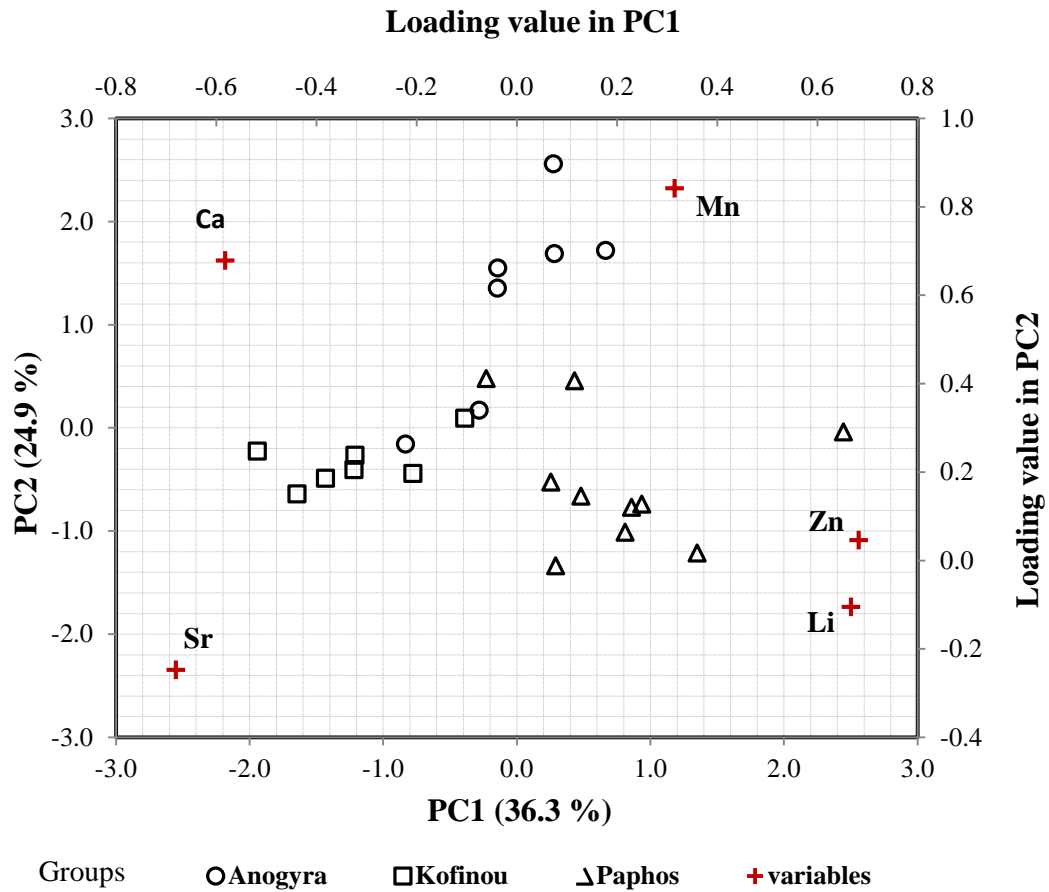


Figure 2. Principal component analysis on milk samples (n = 24). Biplot of principal component scores and loadings.

Table 3. Results of the classification of milk samples from different production regions of Cyprus on the basis of canonical discriminant analysis by means of Ca, Sr, and Zn^a.

		Origin	Predicted Group Membership			Total
			Anogyra	Kofinou	Paphos	
Original	Count	Anogyra	7	0	0	7
		Kofinou	1	6	0	7
		Paphos	2	0	10	12
	%	Anogyra	100.0	0.0	0.0	100.0
		Kofinou	14.3	85.7	0.0	100.0
		Paphos	16.7	0.0	83.3	100.0
Cross-validated	Count	Anogyra	7	0	0	7
		Kofinou	0	5	2	7
		Paphos	0	0	12	12
	%	Anogyra	100.0	0.0	0.0	100.0
		Kofinou	0.0	71.4	28.6	100.0
		Paphos	0.0	0.0	100.0	100.0

^a 88.5% of original grouped cases correctly classified. 92.3% of cross-validated grouped cases correctly classified.

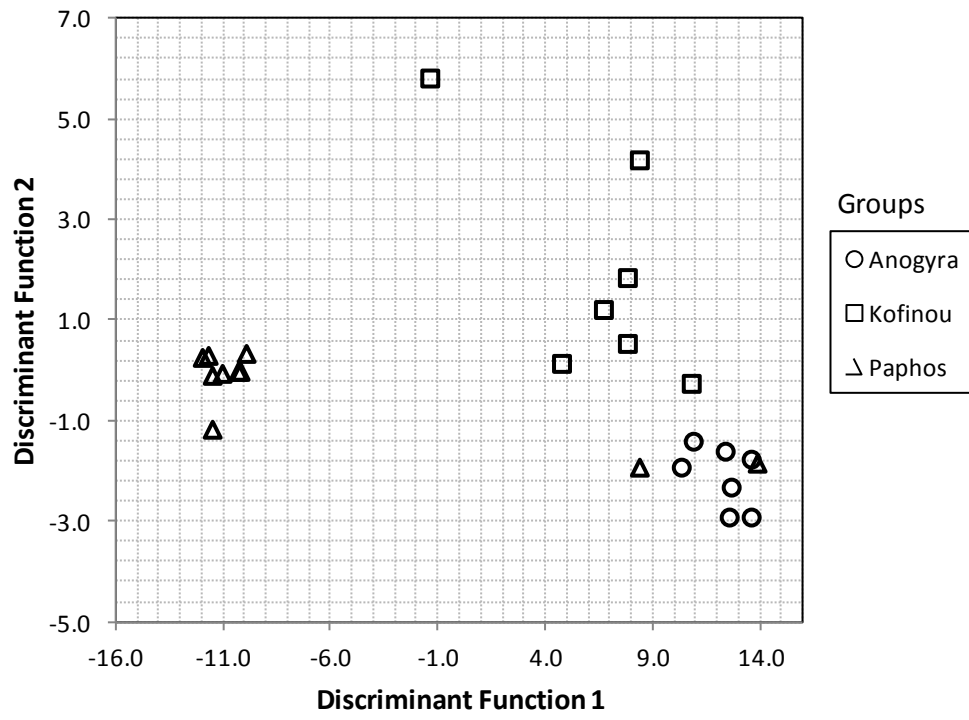


Figure 3. Plot showing the first two discriminant functions derived from milk samples produced in three different regions of Cyprus after canonical discriminant analysis.

Table 4. Results of the classification of Halloumi cheese samples from different production regions of Cyprus on the basis of canonical discriminant analysis by means of Sr, K and Mn^a.

		Origin	Predicted group membership			Total
			Anogyra	Kofinou	Paphos	
Original	Count	Anogyra	7	0	0	7
		Kofinou	0	7	0	7
		Paphos	0	0	9	9
	%	Anogyra	100.0	0.0	0.0	100.0
		Kofinou	0.0	100.0	0.0	100.0
		Paphos	0.0	0.0	100.0	100.0
Cross-validated	Count	Anogyra	7	0	0	7
		Kofinou	0	6	1	7
		Paphos	0	0	9	9
	%	Anogyra	100.0	0.0	0.0	100.0
		Kofinou	0.0	85.7	14.3	100.0
		Paphos	0.0	0.0	100.0	100.0

^a 100% of original grouped cases correctly classified. 95.7% of cross-validated grouped cases correctly classified.

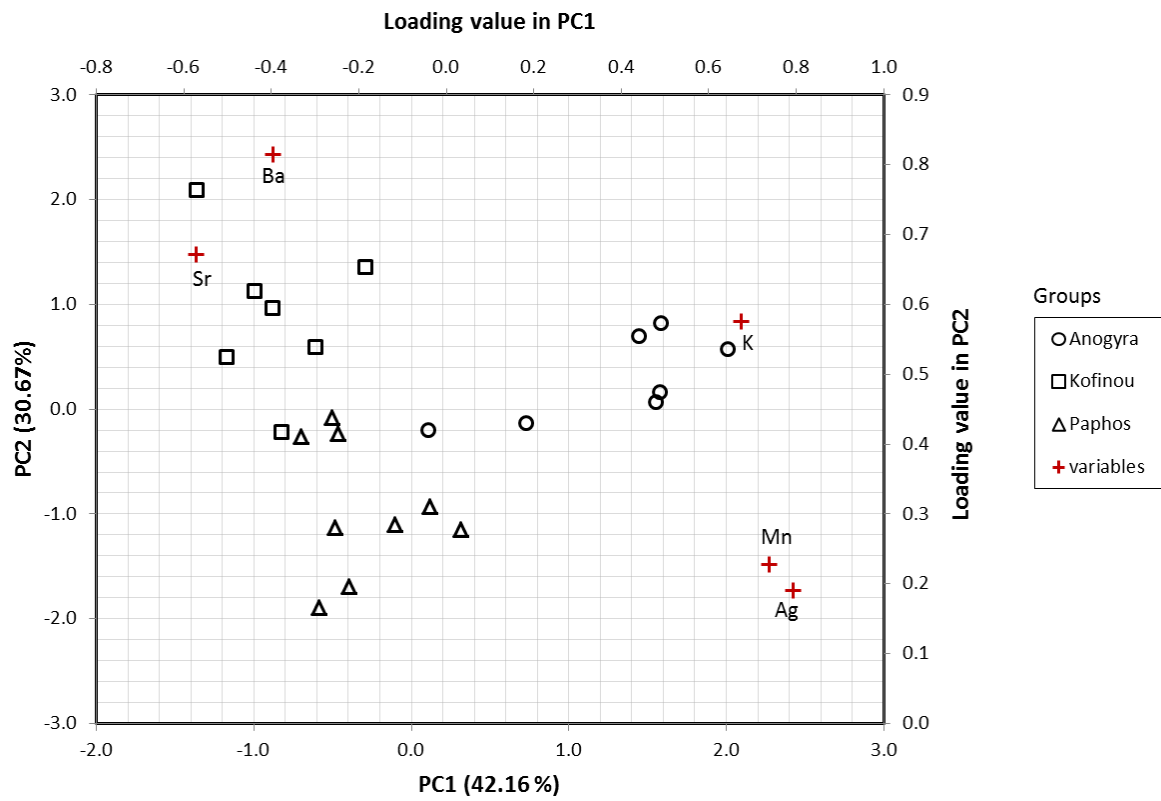


Figure 4. Principal component analysis on Halloumi cheese samples (n=23). Biplot of principal component scores and loadings.

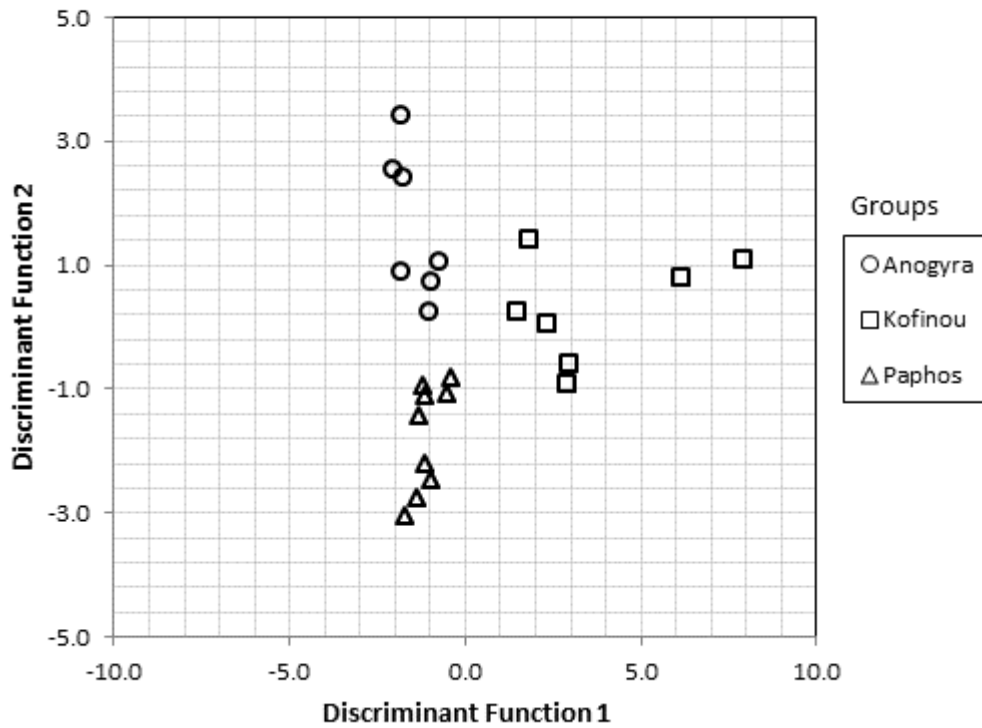


Figure 5. Canonical discriminant analysis plot showing the first two discriminant functions derived from Halloumi cheese samples produced in three different areas of the island.

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