



**HAL**  
open science

## Classification of the geographical origin of Italian donkey milk based on differences in inorganic anions

Giuseppa Di Bella, Vincenzo Lo Turco, Angela Giorgia Potortì, Rosario Rocco Luppino, Vincenzo Fotia, Francesca Conte, Giacomo Dugo

► **To cite this version:**

Giuseppa Di Bella, Vincenzo Lo Turco, Angela Giorgia Potortì, Rosario Rocco Luppino, Vincenzo Fotia, et al.. Classification of the geographical origin of Italian donkey milk based on differences in inorganic anions. Food Additives and Contaminants, 2012, pp.1. 10.1080/19440049.2012.674979 . hal-00812882

**HAL Id: hal-00812882**

**<https://hal.science/hal-00812882>**

Submitted on 13 Apr 2013

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**Classification of the geographical origin of Italian donkey milk based on differences in inorganic anions**

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2011-501.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	05-Mar-2012
Complete List of Authors:	Di Bella, Giuseppa; University of Messina, Department of Food and Environment Sciences Lo Turco, Vincenzo; University of Messina, Department of Food and Environment Sciences Potorti, Angela Giorgia; Experimental Zooprophyllactic Institute of Sicily, Luppino, Rosario Rocco; University of Messina, Department of Food and Environment Sciences Fotia, Vincenzo; University of Messina, Department of Food and Environment Sciences Conte, Francesca; University of Messina, Department of Veterinary Health Public Dugo, Giacomo; University of Messina, Department of Food and Environment Sciences
Methods/Techniques:	Statistical analysis, Traceability
Additives/Contaminants:	Nitrate, Nitrite
Food Types:	Baby food, Milk
Abstract:	In this work the content of chlorides, nitrites, nitrates, phosphates and sulphates were used to classify 45 donkey milk samples collected from different Italian regions. An ion exchange chromatography with conductivity detector and chemical suppression method was used. The quantitative results indicate phosphates (569.39-1304.40 mg kg <sup>-1</sup> ) and chlorides (545.93-1757.89 mg kg <sup>-1</sup> ) that as the most abundant anions, followed by and sulphates (109.52-200.69 mg kg <sup>-1</sup> ). The concentrations of nitrites and nitrates are found to be lower at 5.60 mg kg <sup>-1</sup> and 5.50 mg kg <sup>-1</sup> . The data set was subdivided into three groups according to the region of origin of milk, was statistically evaluated by analysis of variance (ANOVA). Chlorides and nitrates concentrations showed a significant difference among farms (p<0.001). In a first discriminant analysis procedure, functions based on linear combinations of the loge-transformed

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

	element concentrations of anions were generated to classify donkey milk samples from different regions. In an alternative approach, a three-step discriminant analysis procedure to classify a milk sample was tested. The obtained results lead to a correct classification of donkey milk samples based on their anions content so that 91.1 through 97.8% of the samples were correctly classified. The procedure proved to be very simple so it could be used as an evaluation method for traceability of donkey milk thus preserving this peculiar product against frauds or commercial disputes.

SCHOLARONE™  
Manuscripts

For Peer Review Only

1 **Classification of the geographical origin of Italian donkey milk**  
2 **based on differences in inorganic anions**

3  
4  
5 Giuseppa Di Bella<sup>1</sup>, Vincenzo Lo Turco<sup>1</sup>, Angela Giorgia Potortì<sup>2\*</sup>,  
6 Rosario Rocco Luppino<sup>1</sup>, Vincenzo Fotia<sup>1</sup>, Francesca Conte<sup>3</sup> and Giacomo Dugo<sup>1</sup>.

7  
8 <sup>1</sup>Department of Food and Environment Sciences “Prof. G. Stagno d’Alcontres”,  
9 University of Messina (Italy), V.le F. Stagno d’Alcontres 31, 98166 Messina,  
10 Italy.

11 <sup>2</sup>Experimental Zooprophyllactic Institute of Sicily “A. Mirri”, Via G. Marinuzzi 3,  
12 90129 Palermo, Italy.

13 <sup>3</sup>Department of Veterinary Health Public, University of Messina (Italy), V.le  
14 Annunziata, 98168 Messina, Italy.

15  
16 \* *Corresponding author:* Angela Giorgia Potortì, Experimental Zooprophyllactic  
17 Institute of Sicily “A. Mirri”, Via G. Marinuzzi 3, 90129 Palermo, Italy.

18 *Phone:* +39 090 676 5181; *fax:* +39 090 676 5436; *e-mail address:*

19 [agpotorti@unime.it](mailto:agpotorti@unime.it)

20

**Abstract:**

22 The content of chlorides, nitrites, nitrates, phosphates and sulphates were used to classify  
23 45 donkey milk samples collected from different Italian regions. A method was used  
24 employing ion exchange chromatography with conductivity detector and chemical  
25 suppression. The quantitative results indicated phosphates (569.4-1304.4 mg·kg<sup>-1</sup>) and  
26 chlorides (545.9-1757.9 mg·kg<sup>-1</sup>) as being the most abundant anions, followed by  
27 sulphates (109.5-200.7 mg·kg<sup>-1</sup>). The concentrations of nitrites and nitrates were found to  
28 be lower at 5.6 mg·kg<sup>-1</sup> and 5.5 mg·kg<sup>-1</sup> respectively. The data set was sub-divided into  
29 three groups according to the region of origin of milk, and was statistically evaluated by  
30 analysis of variance (ANOVA). Concentrations of chlorides and nitrites showed a  
31 significant difference among farms (p<0.001). In a first discriminant analysis procedure,  
32 functions based on linear combinations of the  $\log_e$ -transformed element concentrations of  
33 anions were generated to classify donkey milk samples from different regions. In an  
34 alternative approach, a three-step discriminant analysis procedure to classify a milk  
35 sample was tested. The results, which were obtained, led to a correct classification of  
36 donkey milk samples based on their anions content with 91 - 98% of the samples being  
37 correctly classified. The procedure proved to be very simple, so it could be used as an  
38 evaluation method for traceability of donkey milk thus defending this unique product  
39 against fraud or commercial disputes.

40

**Keywords:** milk; donkey; inorganic anions; discriminant analysis; traceability.

## Introduction

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

2 The added value of donkey milk is related to its being similar to human milk, more than bovine milk (Chiavari et al. 2005; Salimei et al. 2004). Today it has been re-discovered for its characteristics such as high digestibility, elevated nutrient value and physiological properties. It is the food of choice for infants affected by allergy to cow's milk protein (CMPA) (Monti et al. 2007), the most common food allergy, affecting about 3% of children in the first three years of life (Sampson 2004). Donkey's milk is very pleasant and well accepted by children because of its high lactose content (Schaafsma 2003). Some authors have also suggested using donkey milk for probiotic purposes, since it has several beneficial qualities, such as low microbial activity and high amount of lysozyme (Chiavari et al. 2005; Coppola et al. 2002). Donkey's milk contains bioactive compounds such as polyamines e.g. putrescine, spermidine and spermine. (La Torre et al. 2010). The good intestinal absorption of calcium makes it suitable for bone mineralization in children and for to prevent osteoporosis in the elderly (Tafaro et al. 2007).

31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

16 Considering the unique nutrient profile of donkey's milk it should be valued as a nutraceutical food to meet the high quality and nutritional requirements of consumers. Unfortunately, the daily production of donkey's milk is lower than cow's milk (1.4-2.0 kg/day vs. 28-63 kg/day) (Polidori et al. 2009; Norring et al. 2012), and in Italy it costs 15-20 euro per liter, whereas cow's milk costs around 1 euro per liter. Increasing attention has been paid to donkey's milk, but little is known of the mineral composition. The presence of inorganic elements in milk depends on factors such as the dietary intake of minerals and the time of lactation; furthermore, even technological processes such as thermal treatment, acidification and membrane separation can modify the main anion concentrations in the aqueous phase of the milk (Gaucheron et al. 1996).

51  
52  
53  
54  
55  
56  
57  
58  
59  
60

26 Generally, the analysis of inorganic anions in foods is very important from a toxicological point of view. Chloride it is one of the most common inorganic anions in food and as sodium chloride is employed as a preservative; therefore, its determination in food is essential to fulfill legal regulations and to meet quality control requirements.

1  
2  
3 Among all the studied inorganic anions, nitrites and nitrates are the most important in  
4  
5 2 terms of contamination due to their potential toxicity. Nitrites and nitrates are ubiquitous in  
6  
7 biological, food and environmental samples since they derive from livestock and human  
8  
9 4 excrement, from domestic and industrial organic wastes, and from the use of nitrogenous  
10  
11 fertilizers and herbicides in agriculture (Cemek et al. 2007).  
12

13 6 Sulphates and phosphates, because their low toxicity, are extensively employed in  
14  
15 agriculture; the former as pesticides and the latter as fertilizers (Dugo et.al. 2007).  
16  
17 8 Particularly in milk, phosphate is present as inorganic phosphate (soluble phosphate and  
18  
19 micellar calcium phosphate (MCP)), organic phosphate as small molecules, covalently  
20  
21 10 bound to the peptide chains of caseins.  
22

23  
24 The determination of the geographical origin of foodstuffs is becoming of increasing  
25  
26 12 interest to consumers and producers, since it may be used as a criterion to certify quality  
27  
28 and typicality. Generally, measurement of multi-element stable isotope ratios by isotope  
29  
30 14 ratio mass spectrometry (IRMS) has been used to determine the geographical origin of milk  
31  
32 and milk ingredients (Kornexl et al. 1997; Renou et al. 2004; Manca et al. 2006; Crittenden  
33  
34 16 et al.2007). Also, classical techniques, i.e. high performance ion chromatography (HPIC),  
35  
36 liquid chromatography–mass spectrometry (LC-MS), inductively coupled plasma emission  
37  
38 18 spectroscopy (ICP-AES) and nuclear magnetic resonance spectroscopy (NMR), were used  
39  
40 to determine different compounds in combination with chemometric methods to  
41  
42 20 discriminate the geographical origin of milk (Brescia et al. 2005; Calabrese et al. 2009;  
43  
44 Sacco et al, 2009).

45  
46 22 This research deals with the classification of donkey milk samples from different  
47  
48 Italian regions according to their anion content. The anion concentrations were easily  
49  
50 24 determined by Suppressed Ion Chromatography (SIC) following fast milk pre-treatment.  
51  
52 The data set which was obtained was sub-divided into three groups and subject to analysis  
53  
54 26 of variance (ANOVA) and Canonical Discriminant Analysis (CDA) to achieve some  
55  
56 statistical classification of donkey milk from different Italian areas according to the  
57  
58 28 presence of anions.  
59  
60

Using these tools, it was possible to elicit the geographical provenance of donkey milk. Assessing the geographic origin is of paramount importance to gain Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) trademarks from the European Union. In order to obtain these designations, the materials must have been produced and processed in the specific region from which the product gets its name.

## Materials and methods

### *Samples*

45 donkey milk samples were obtained from conventional Italian donkey farms, in Sicily (Farm A), Calabria (Farm B) and Emilia-Romagna (Farm C). The farms were selected in areas where the soil and weather conditions were similar, and where human activity on the environment was not high. Three bulk milk samples were collected from each farm in the months of September and November 2009, and in January, March and May 2010. All donkeys, mean age 3 to 8 years, were healthy and had no illness during pregnancy. The animals were fed on grass resulting from harvesting of poliphita meadow and fodder without any other added substances. Milk samples were collected using hand milking by standardized procedures at the same time of day (in the morning between 08.00 and 10.00 a.m.), and were preserved in PET containers; immediately refrigerated on ice, they were frozen at -20°C, and as they arrived in the laboratory, they were stored until analysis.

### *Chemicals*

All chemicals were of HPLC grade and all reagents, eluent, standard and sample solutions used for the determination were prepared by ultrapure water with a specific conductivity value less than  $18 \mu\text{S cm}^{-1}$ , purchased from Romil (Milan, Italy). The analytes, i.e., chloride, nitrite and nitrate ions as sodium salts, sulphate ion as dibasic sodium salt and phosphate ion as monobasic potassium salt were purchased from Dionex (Sunnyvale, CA, USA).



In this study 3% acetic acid solution was used, prepared from ultrapure acetic acid (99.9%) purchased from J. T. Baker (Deventer, Holland). Mobile phase (9mM Na<sub>2</sub>CO<sub>3</sub>) was prepared from 0.5M sodium carbonate obtained from Dionex (Sunnyvale, CA, USA).

#### *Standard solution and sample preparation*

Standard mixture concentrations ranged from 30 to 150 mg·L<sup>-1</sup> in ultrapure water (99.9%). Standard solutions were daily prepared by serial dilution of the standard mixture prior to use. After homogenization and agitation with a vortex, 10mL of every milk sample were transferred into a 20mL volumetric flask, spiked with 2mL of 3% acetic acid and brought to volume by ultrapure water. 1mL of this solution was diluted with ultrapure water again up to 50mL.

#### *Equipment*

Analysis were performed by an ICS 1000 ion chromatography system (Dionex, Sunnyvale, CA, USA) equipped with isocratic pump, conductivity detector, guard column (Dionex Ion Pac AG9-HC, 4 x 50mm) to prevent potential fouling of the analytical column, high capacity anion exchange analytical column (Dionex Ion Pack AS9-HC, 4 x 250mm, 9µm), 25µL sample loop and anion self-regenerating suppressor (ASRS 300, 4mm). Data acquisition and instrument control were performed using the Chromeleon software.

#### *Ion exchange chromatography analysis*

All experiments were performed at room temperature, with flow rate of 1.0 mL·min<sup>-1</sup> and 35°C flow cell temperature. Suppressor current was fixed at 45mA. The isocratic elution was carried out using a 9mM sodium carbonate solution. The standard and sample solutions were filtered through 0.22µm glass-microfiber GMF Whatman chromatographic filter before entering the IEC system. Data collection was performed in triplicate. Ultrapure water was injected before the unknown samples.

### *Statistical method*

The multivariate analysis was carried out using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL). Statistical methods were conducted on starting multivariate matrix where the cases were the 45 analyzed donkey milk samples and the concentrations of chlorides, nitrites, nitrates, phosphates and sulphates were the variables.

The data were sub-divided into three groups (each one formed of 15 samples) according to the milk origin, and were statistically evaluated by analysis of variance (ANOVA). Discriminant Analysis was performed in order to classify different donkey milks.

## **Results and discussion**

### *Anions determination*

The data showed that  $\text{Na}_2\text{CO}_3$  concentration of 9mM permitted a fast separation of the five anions (about 20 min) . The chromatographic peaks were well resolved and consequently the quantifications steps were easy. Identification of analytes was carried out by comparing the retention times in the sample with those of the standard mixture. For quantification, a calibration curve was obtained for each analyte by plotting peak areas versus their concentrations.

### *Validation of IEC analysis*

The analytical characteristics of the method are presented in Tables 1–3. The relative standard deviations (RSD%) on retention times and on peak area were determined considering a mixture of standard anions at the concentration level of 0.75 (chloride), 2.5 (nitrite and nitrate) and  $3.7 \text{ mg}\cdot\text{L}^{-1}$  (sulfate and phosphate). The measurements were performed, in the conditions reported in sections 2.4 and 2.5, within the same day ( $n=6$ ) and over a period of 10 days ( $n=10$ ). In the latter case, the 10 measured values represented the average of three determinations per day. The highest RSD values were 1.4 and 2.2% for  $T_R$  and 2.4 and 3.7% for areas for intra-day and inter-day repeatability, respectively (Table

1  
2  
3 1). Table 2 summarizes the sensitivity and linearity parameters for the analysis of anions  
4  
5 2 with the method used. The linearity of the method was assessed by analyzing seven  
6  
7 standard solutions obtained from the standard mixture. Three replicate analyses were  
8  
9 4 performed at each concentration level. Good linearity was observed in each concentration  
10  
11 range, with linear correlation coefficients ( $R^2$ ) better than 0.9873. As per Pharmacopoeia  
12  
13 6 (1999), the limits of detection (LODs) and the limits of quantification (LOQs) were  
14  
15 experimentally calculated as a signal-to-noise ratio of 3 and 10, respectively. Among all  
16  
17 8 anions, phosphates had the highest detection limit of  $20.16 \mu\text{g}\cdot\text{L}^{-1}$ .

18  
19  
20  
21 10 The accuracy of the method described for the determination of anions in milk samples was  
22  
23 evaluated at three spiking levels, with three replicates for each level. For the recovery test,  
24  
25 12 a sample of donkey milk was previously analyzed and then fortified with note amount of  
26  
27 standard anions. The results obtained for each anion are given in Table 3, where it can be  
28  
29 14 noted that the recoveries for each anion were almost constants. From this table, it is also  
30  
31 evident that the highest recoveries were determined for chlorides (96.6%) and phosphates  
32  
33 16 (95.1%). For the other three anions, the recoveries were around 88%. All the RSD values  
34  
35 were less than 2.8%, which shows the excellent precision of the method used for the  
36  
37 18 determination of anions in milk.

#### 40 41 20 *Anion contents in donkey milk*

42  
43 The quantitative results of anions found are show in Table 4. All milk samples were  
44  
45 22 analyzed in triplicate. The obtained data indicate that phosphates ( $569.4\text{-}1304.4 \text{ mg}\cdot\text{kg}^{-1}$ )  
46  
47 and chlorides ( $545.9\text{-}1757.9 \text{ mg}\cdot\text{kg}^{-1}$ ) are the most abundant anions, followed by sulphates  
48  
49 24 ( $109.5\text{-}200.7 \text{ mg}\cdot\text{kg}^{-1}$ ). The concentrations of nitrites and nitrates are found lower than  $5.6$   
50  
51  $\text{mg}\cdot\text{kg}^{-1}$  and  $5.5 \text{ mg}\cdot\text{kg}^{-1}$  (maximum values observed).  
52  
53

54  
55 26  
56  
57 The chloride concentrations found in these samples are similar to those determined in cow  
58  
59 28 milk (Gaucheron 2005) and twice higher than those determined for human breast milk  
60

(Wack et al. 1997). The values of phosphates are in accordance with the values reported in literature for cow milk (Gaucheron et al. 1996). There is no available literature for the sulfate content in milk. The nitrate values are comparable to those for cow milk (Gapper et al. 2004; Reis Lima et al. 2006) and lower than those for infant formula (Gapper et al. 2004).

The concentrations of nitrite resulted from 10 to 35 times higher than cow milk and infant formula (Gapper et al. 2004). According to the European Directives, there is no indication on allowed nitrite and nitrate concentration in milk. There is legislation on maximum limits for drinking water (Directive 2003/40/CE of 16 May 2003) and, therefore, it is possible refer to it. It establishes that the amount of nitrates for children cannot exceed 10 mg·L<sup>-1</sup>, while for nitrites the limit is set at 0.1 mg·L<sup>-1</sup>. In all milk analyzed samples concentration of nitrate were lower than fixed limit; conversely, all samples showed nitrites at concentration higher than 0.1 mg·L<sup>-1</sup>. This last data could be attributed to many factors including intake of water, feed or forages which could contain high levels of nitrites, due to nitrogenous fertilizer and herbicides, and industrial and domestic wastes in the environment. So the research on the presence of nitrites in donkey milk should be deeper, in order to reduce their contents at levels comparable to that of other milks, for human health and product quality.

#### *Statistical analysis*

In order to improve the robustness of the model given by discriminant analysis, anion concentrations were  $\log_e$ -transformed to reduce the effect of outliers on skewing the data distribution and to bring the concentrations of anions within the same range. To statistically define the seasonal variation in anions in the donkey milk, Kruskal Wallis tests were performed to try to link anions concentrations to the timing of sampling of the donkey milk. A plot of anion concentration according to timing of sampling was showed in

Figure 1. Within each farm, no significant seasonal variability, at p-level <0.01, has been observed in the anions levels (Table 5).

The ANOVA and the Wilks' Lambda test indicated that the variables that contributed most to the discriminant model were chlorides and nitrates, at p-level < 0.001, whereas nitrites, phosphate and sulfate were the variables that contributed the least to the statistical model (Table 6). It might be thought that the difference in concentration of chlorides and nitrates in donkey milk from three different farms could be due to various factors such as feeding regime, forage preservation, geographical of origin.

In the first explorative statistical approach we chose to enter all independents variables together. The following standardized canonical discriminant functions were derived by discriminant analysis:

$$F_1 = 0.976\ln(Cl^-) - 0.446\ln(PO_4^{3-}) - 0.158\ln(SO_4^{2-}) - 0.400\ln(NO_2^-) + 0.678\ln(NO_3^-) \text{ (eq.1)}$$

$$F_2 = -0.730\ln(Cl^-) - 0.054\ln(PO_4^{3-}) - 0.188\ln(SO_4^{2-}) + 0.555\ln(NO_2^-) + 0.763\ln(NO_3^-) \text{ (eq.2)}$$

A Scatterplot (Fig. 2) related to these discriminant functions shows the degree of separation among donkey milk samples of different origin. Moreover, the classification matrix (Fig. 2) indicates that 93.3% of total samples are correctly classified and, in particular 1 sample of farm A was classified incorrectly as farm C sample; 1 of farm B was classified as farm A, and 1 of farm C as farm B. A cross-validation procedure was applied to evaluate the robustness of the classification model. Each sample was in turn omitted from the estimation of model, and then its membership was determined from the resulting model. The cross-validation was again 93.3% correct.

Therefore, we remade the classification of donkey milk samples by further discriminant analysis with a stepwise method, calculating the Mahalanobis squared distances from the

centroid and using the probability of F. The standardized canonical discriminant functions were:

$$F_3 = 0.558\ln(Cl^-) + 0.757\ln(NO_3^-) \text{ (eq.3)}$$

$$F_4 = 0.841\ln(Cl^-) - 0.668\ln(NO_3^-) \text{ (eq.4)}$$

From the Scatterplot given in Fig. 3, where these two discriminant functions were applied to the data set, we can see an improved classification of donkey milks for the farms A, B and C. The classification matrix (Fig. 3) indicates that the 97.8% of total samples are correctly classified and the cross-validation procedure confirms the percentage of cases correctly classified.

Moreover, we have carried out a three-step discriminant analysis procedure to classify a milk sample. In this procedure, each step classifies a milk sample as coming from a particular farm or not, generating a discriminant functions for each farm in each step. The discriminant functions for farm A ( $F_A$ ), farm B ( $F_B$ ), and farm C ( $F_C$ ) were:

$$F_A = 0.774\ln(Cl^-) - 0.402\ln(PO_4^{3-}) - 0.211\ln(SO_4^{2-}) - 0.190\ln(NO_2^-) + 0.954\ln(NO_3^-) \text{ (eq.5)}$$

$$F_B = -0.720\ln(Cl^-) - 0.027\ln(PO_4^{3-}) - 0.214\ln(SO_4^{2-}) + 0.475\ln(NO_2^-) + 1.008\ln(NO_3^-) \text{ (eq.6)}$$

$$F_C = 1.249\ln(Cl^-) - 0.366\ln(PO_4^{3-}) - 0.005\ln(SO_4^{2-}) - 0.682\ln(NO_2^-) + 0.010\ln(NO_3^-) \text{ (eq.7)}$$

The values of the functions at the group centroids are summarized in Table 7. The classification of a milk sample was done by calculating the Mahalanobis squared distances from the centroid and the probabilities of group membership. Mahalanobis squared distances ( $d^2$ ) were calculated for each sample using paired equations for each group. The paired equations for the farm A, for example, were:

$$d_A^2 = [F_A - (-1.678)]^2 \text{ (eq.8)}$$

$$d_{notA}^2 = [F_A - (+0.839)]^2 \text{ (eq.9)}$$

where -1.678 and +0.839 are the values of  $F_A$  at the centroid (Table 7) for farm A and the not-farm A, respectively. The probabilities of group membership (farm A and not-farm A) were computed from the pair of equations:

$$P_A = \frac{10^{(0.5d_A^2)}}{10^{(0.5d_A^2)} + 10^{(0.5d_{notA}^2)}} \quad (\text{eq.10})$$

$$P_{notA} = \frac{10^{(0.5d_{notA}^2)}}{10^{(0.5d_A^2)} + 10^{(0.5d_{notA}^2)}} \quad (\text{eq.11})$$

Similar equation pairs were used for the other two farms.

A milk samples was classified as member or not member of a specific farm according to the largest probability. In the case of the farm A/not-farm A and the farm B/not-farm B models, the 95.6% of total samples are correctly classified, while for the farm C/not-farm C model, the classification was 91.1% correct.

## Conclusions

Donkey's milk is a nutraceutical food, which responds to special needs of children, adults and elderly. It is a unique food with high commercial value, indeed currently 1 L of this milk is sold at 15-20 euro. For its features it could receive from the EU the trademark of Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI), which indicate the relationship with the territory.

The method used here proved to be very easy and fast, relying on the use of instrumentation available to all quality control laboratories. Discriminant Analysis, performed using anion concentrations as independent variables, showed that there is no significant seasonal variability in the anion levels, and that a good classification among donkey's milk samples from different Italian regions can be obtained. The procedure reported here can be used as a method to evaluate traceability of donkey milk to preserve this unique product against fraud or commercial disputes.

## Acknowledgment

The authors thank Prof. Eugenio Cianflone for reading and editing the English version of this paper.

## References

- Brescia MA, Monfreda M, Buccolieri A, Carrino C. 2005. Characterization of the geographical origin of buffalo milk and mozzarella cheese by means of analytical and spectroscopic determinations. *Food Chems.* 89:139-147.
- Calabrese MG, Mamone G, Caira S, Ferranti P, Addeo F. 2009. Quantitation of lysinoalanine in dairy products by liquid chromatography–mass spectrometry with selective ion monitoring. *Food Chem.* 116:799-805.
- Cemek M, Akkaya L, Birdane YO, Seyrek K, Bulut S, Konuk M. 2007. Nitrate and nitrite levels in fruity and natural mineral waters marketed in western Turkey. *J. Food Compos. Anal.* 20:236-240.
- Chiavari C, Coloretti F, Nanni M, Sorrentino E, Grazia L. 2005. Use of donkey's milk for a fermented beverage with lactobacilli. *Lait* 85:481–490.
- Coppola R, Salimei E, Succi M, Sorrentino E, Nanni M, Ranieri P, Belli Blanes R, Grazia L. 2002. Behaviour of *Lactobacillus rhamnosus* strains in ass's milk. *Ann. Microbiol.* 52:55–60.
- Crittenden RG, Andrew AS, LeFournour M, Young MD, Middleton H, Stockmann R. 2007. Determining the geographic origin of milk in Australasia using multi-element stable isotope ratio analysis. *Int. Dairy J.* 17:421–428.
- Dugo Gmo, Pellicanò TM, La Pera L, Lo Turco V, Tamborrino A, Clodoveo ML. 2007. Determination of inorganic anions in commercial seed oils and in virgin olive oils produced from de-stoned olives and traditional extraction methods, using suppressed ion exchange chromatography (IEC). *Food Chem.* 102:599–605.
- European Pharmacopoeia. 2005. Technical guide (5th ed.). December 2005.
- Gapper LW, Fong BY, Otter DE, Indyk HE, Woollard DC. 2004. Determination of nitrite and nitrate in dairy products by ion exchange LC with spectrophotometric detection. *Int. Dairy J.* 14:881–887.
- Gaucheron F. 2005. The minerals of milk. *Reprod. Nutr. Dev.* 45:473–483.
- Gaucheron F, Le Graët Y, Piot M, Boyaval E. 1996. Determination of anions of milk by ion chromatography. *Lait* 76:433–443.
- Kornexl BE, Werner T, Rossmann A, Schmidt HL. 1997. Measurement of stable isotope abundances in milk and milk ingredients: a possible tool for origin assignment and



- 1  
2  
3 quality control. *Zeitschrift Fur Lebensmittel Untersuchung Und-Forschung* 205:19-  
4 24. La Torre GL, Saitta M, Potortì AG, Di Bella G, Dugo G. 2010. High  
5 performance liquid chromatography coupled with atmospheric pressure chemical  
6 ionization mass spectrometry for sensitive determination of bioactive amines in  
7 donkey milk. *J. Chromatogr. A* 1217:5215–5224.  
8  
9  
10 Manca G, Franco MA, Versini G, Camin F, Rossmann A, Tola A. 2006. Correlation  
11 Between Multielement Stable Isotope Ratio and Geographical Origin in Peretta Cows'  
12 Milk Cheese. *J. Dairy Sci.* 89:831–839. Monti G, Bertino E, Muratore MC, Coscia A,  
13 Cresi F, Silvestro L, Fabris C, Fortunato D, Giuffrida MG, Conti A. 2007. Efficacy of  
14 donkey's milk in treating highly problematic cow's milk allergic children: an in vivo  
15 and in vitro study. *Pediatr. Allergy Immunol.* 18:258–264.  
16  
17  
18 Norrington M, Valros A, Munksgaard L. Milk yield affects time budget of dairy cows in tie-  
19 stalls. *J. Dairy Sci.* 95:102–108.  
20  
21  
22 Polidori P, Beghelli D, Mariani P, Vincenzetti S. 2009. Donkey milk production: state of  
23 the art. *Ital. J. Anim. Sci.* 8:677–683.  
24  
25  
26 Reis Lima MJ, Fernandes Silva MV, Rangel AOSS. 2006. Determination of nitrate and  
27 nitrite in dairy samples by sequential injection using an in-line cadmium-reducing  
28 column. *Int. Dairy J.* 16:1442–1447.  
29  
30  
31 Renou J, Deponge C, Gachon P, Bonnefoy J, Coulon J, Garel J, Vérité R, Ritz P. 2004.  
32 Characterization of animal products according to geographic origin and feeding diet  
33 using nuclear magnetic resonance and isotope ratio mass spectrometry: cow milk.  
34 *Food Chem.* 85:63–66.  
35  
36  
37 Sacco D, Brescia MA, Sgaramella A, Casiello G, Buccolieri A, Ogrinc N, Sacco A. 2009.  
38 Discrimination between Southern Italy and foreign milk samples using spectroscopic  
39 and analytical data. *Food Chem.* 114:1559–1563.  
40  
41  
42 Salimei E, Fantuz F, Coppola R, Chiofalo B, Polidori P, Varisco G. 2004. Composition and  
43 characteristics of ass's milk. *Animal Research* 53:67–78.  
44  
45  
46 Sampson HA. 2004. Update on food allergy. *J. Allergy Clin. Immunol.* 113:805–819.  
47  
48 Schaafsma G. 2003. Nutritional significance of lactose and lactose derivatives In Roginski  
49 H, Fuquay JW, Fox PF (Eds.) *Encyclopedia of Dairy Science*, Vol. 3 (pp.1529–1533).  
50 London: Academic Press.  
51  
52 Tafaro A, Magrone T, Jirillo F, Martemucci G, D'alessandro AG, Amati L, Jirillo E. 2007.  
53 Immunological Properties of Donkey's Milk: Its Potential Use in the Prevention of  
54 Atherosclerosis. *Curr. Pharm. Design* 13:3711–3717.  
55  
56  
57 Wack RP, Lien EL, Taft D, Roscelli JD. 1997. Electrolyte composition of human breast  
58 milk beyond the early postpartum period. *Nutrition* 13:774–777.  
59  
60

**Table captions**

2

**Table 1***Repeatability data for the SIC determination of anions under analysis.***Table 2***Sensitivity and linearity parameters for the SIC determination of anions under analysis.*

8

**Table 3***Recovery and precision for anions under analysis.***Table 4***Anion concentrations in Sicilian donkey milks. Minimum and maximum values are expressed as mean values  $\pm$  95% confidence interval ( $n = 3$ ).***Table 5***Kruskal Wallis test by variable "season".***Table 6***Discriminant analysis test (ANOVA) of equality of group means.***Table 7***Values of the discriminant functions at the group centroids.*

22

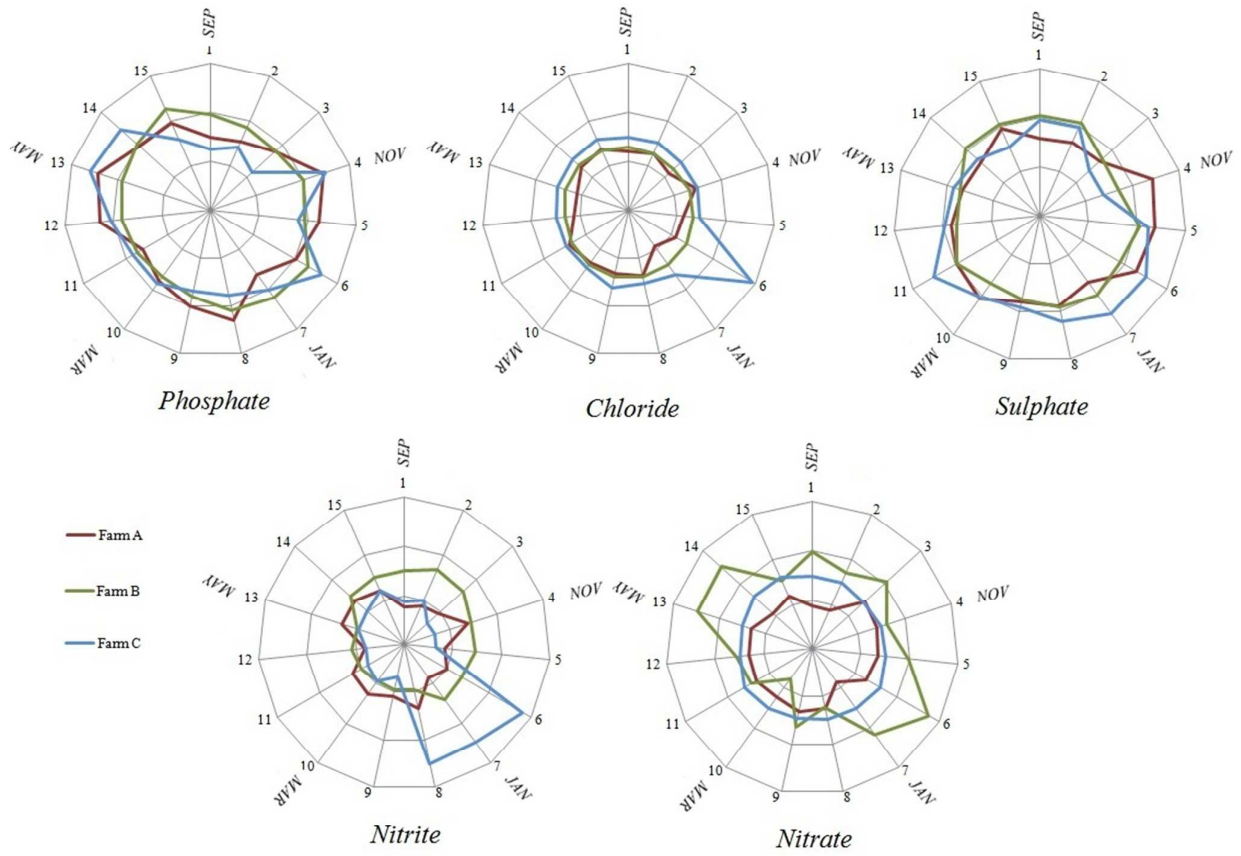
**Figure captions**

2 **Fig. 1.** *Seasonal variation of anion concentrations in donkey milk.*

4 **Fig. 2.** *2D Scatterplot and classification matrix results by using discriminant functions defined in eqs 1 and 2.*

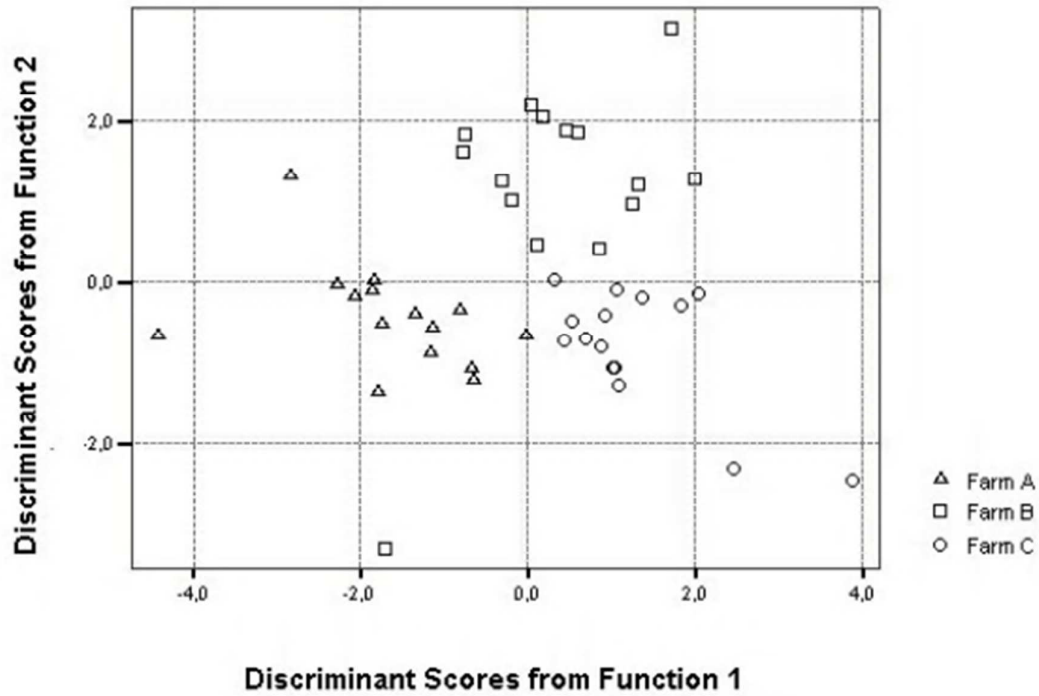
6 **Fig. 3.** *2D Scatterplot and classification matrix results by using discriminant functions defined in eqs 3 and 4.*

For Peer Review Only



**Fig. 1.** Seasonal variation of anion concentrations in donkey milk.

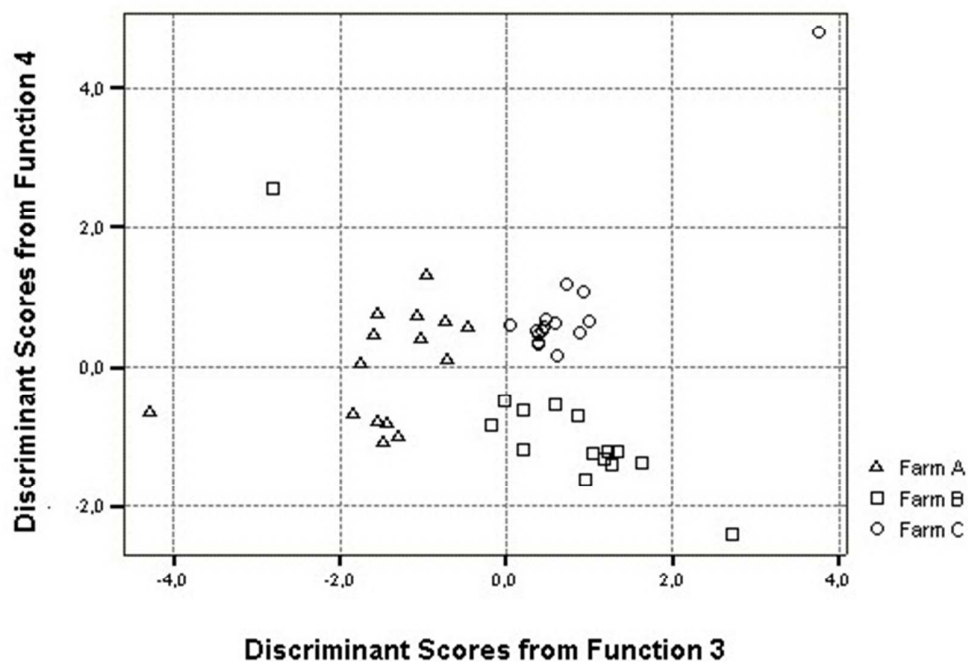
View Only



*Classification matrix*

		Predicted Group Membership			Total
		Farm A	Farm B	Farm C	
Count	Farm A	14	0	1	15
	Farm B	1	14	0	15
	Farm C	0	1	14	15
%	Farm A	93.3	0	6.7	100.0
	Farm B	6.7	93.3	0	100.0
	Farm C	0	6.7	93.3	100.0

**Fig. 2.** 2D Scatterplot and classification matrix results by using discriminant functions defined in eqs 1 and 2.



*Classification matrix*

		Predicted Group Membership			Total
		Farm A	Farm B	Farm C	
Count	Farm A	15	0	0	15
	Farm B	1	14	0	15
	Farm C	0	0	15	15
%	Farm A	100	0	0	100.0
	Farm B	6.7	93.3	0	100.0
	Farm C	0	0	100	100.0

**Fig. 3.** 2D Scatterplot and classification matrix results by using discriminant functions defined in eqs 3 and 4.

**Table 1***Repeatability data for the SIC determination of anions under analysis*

Anion	Intra-day repeatability (RSD%, n=6)		Inter-day repeatability (RSD%, n=10)	
	$T_R$	Area	$T_R$	Area
Cl <sup>-</sup>	1.1	2.4	1.9	3.3
NO <sub>2</sub> <sup>-</sup>	0.9	1.3	1.2	3.7
NO <sub>3</sub> <sup>-</sup>	0.9	1.6	1.6	2.5
PO <sub>4</sub> <sup>3-</sup>	1.4	1.7	2.1	1.3
SO <sub>4</sub> <sup>2-</sup>	1.4	2.3	2.2	3.6

**Table 2***Sensitivity and linearity parameters for the SIC determination of anions under analysis*

Anion	Calibration curves (n=7)	R <sup>2</sup>	Linearity range (µg·L <sup>-1</sup> )	LOD (µg·L <sup>-1</sup> )	LOQ (µg·L <sup>-1</sup> )
Cl <sup>-</sup>	$y=(0.1366\pm 0.0016)x + (-0.0038\pm 0.0054)$	0.9994	7.5-7500	2.4	7.9
NO <sub>2</sub> <sup>-</sup>	$y=(0.0791\pm 0.000)x$	0.9983	25-2500	7.7	25.8
NO <sub>3</sub> <sup>-</sup>	$y=(0.0582\pm 0.0016)x + (0.0184\pm 0.0018)$	0.9895	25-2500	7.5	25.1
PO <sub>4</sub> <sup>3-</sup>	$y=(0.0343\pm 0.0011)x + (-0.0118\pm 0.0190)$	0.9873	37.5-3750	12.2	40.7
SO <sub>4</sub> <sup>2-</sup>	$y=(0.01047\pm 0.0019)x + (-0.0349\pm 0.0323)$	0.9963	37.5-3750	20.2	67.2

*n*, points calibration curves; R<sup>2</sup>, lest square regression coefficient; LOD, limit of detection (S/N=3); LOQ, limit of quantification (S/N=10).



**Table 3***Recovery and precision for anions under analysis*

Anion	Concentration (mg·kg <sup>-1</sup> )	Added (mg·kg <sup>-1</sup> )	Expected (mg·kg <sup>-1</sup> )	Found* (mg·kg <sup>-1</sup> )	Recovery (%)
Cl <sup>-</sup>	510	100	610	590.5 ± 2.2	96.8
		300	810	772.7 ± 3.16	95.4
		500	1010	896.8 ± 5.1	97.7
				Mean value ± S.D.	96.6 ± 1.2
				Precision (%)	1.20
NO <sub>2</sub> <sup>-</sup>	0.03	0.5	0.53	0.5 ± 0.1	90.1
		2	2.53	1.8 ± 0.1	89.6
		5	5.03	4.3 ± 0.7	85.6
				Mean value ± S.D.	88.4 ± 2.5
				Precision (%)	2.8
NO <sub>3</sub> <sup>-</sup>	0.24	1	1.24	1.1 ± 0.7	88.7
		5	5.24	4.6 ± 0.4	87.7
		10	10.24	9.1 ± 0.3	88.8
				Mean value ± S.D.	88.4 ± 0.6
				Precision (%)	0.7
PO <sub>4</sub> <sup>3-</sup>	620	100	720	684.0 ± 0.7	95.0
		300	920	860.2 ± 0.5	93.5
		500	1120	1085.3 ± 0.1	96.9
				Mean value ± S.D.	95.1 ± 1.7
				Precision (%)	1.8
SO <sub>4</sub> <sup>2-</sup>	106	50	156	133.7 ± 0.1	85.7
		100	206	183.5 ± 0.1	89.1
		150	256	224.0 ± 0.4	87.5
				Mean value ± S.D.	87.4 ± 1.7
				Precision (%)	1.9

\*Average of three replicates.

**Table 4**

*Anion concentrations in Sicilian donkey milks. Minimum and maximum values are expressed as mean values  $\pm$  95% confidence interval ( $n = 3$ ).*

	Cl <sup>-</sup> (mg·kg <sup>-1</sup> )	PO <sub>4</sub> <sup>3-</sup> (mg·kg <sup>-1</sup> )	SO <sub>4</sub> <sup>2-</sup> (mg·kg <sup>-1</sup> )	NO <sub>2</sub> <sup>-</sup> (mg·kg <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> (mg·kg <sup>-1</sup> )
<i>Farm A (n=15)</i>					
Min	545.9±9.3	744.5±36.0	125.8±12.2	1.5±0.1	1.7±0.1
Max	863.8±25.3	1210.8±49.4	193.7±15.3	2.7±0.1	2.9±0.1
<b>Mean±S.D.</b>	<b>738.4±86.8</b>	<b>980.1±160.1</b>	<b>151.6±22.2</b>	<b>2.2±0.4</b>	<b>2.4±0.3</b>
<i>Farm B (n=15)</i>					
Min	749.7±11.5	842.3±32.0	135.8±15.2	1.8±0.1	1.5±0.2
Max	831.5±25.1	1156.9±48.6	165.7±12.8	3.3±0.1	5.5±0.4
<b>Mean±S.D.</b>	<b>800.6±27.6</b>	<b>978.6±95.3</b>	<b>151.0±12.4</b>	<b>2.6±0.5</b>	<b>3.7±0.8</b>
<i>Farm C (n=15)</i>					
Min	872.7±23.0	569.4±17.9	109.5±22.0	1.3±0.1	2.8±0.2
Max	1757.9±43.8	1304.4±32.9	200.7±24.4	5.6±0.1	3.2±0.2
<b>Mean±S.D.</b>	<b>967.1±221.5</b>	<b>951.5±236.2</b>	<b>158.3±29.8</b>	<b>2.4±1.0</b>	<b>3.0±0.1</b>

**Table 5***Kruskal Wallis test by variable "season".*

Anion	<i>Farm A</i>		<i>Farm B</i>		<i>Farm C</i>	
	<i>Chi-Square</i>	<i>p-Level</i>	<i>Chi-Square</i>	<i>p-Level</i>	<i>Chi-Square</i>	<i>p-Level</i>
Cl <sup>-</sup>	1.433	0.838	8.833	0.065	7.433	0.115
NO <sub>2</sub> <sup>-</sup>	7.300	0.121	7.367	0.118	8.433	0.077
NO <sub>3</sub> <sup>-</sup>	11.367	0.023	3.700	0.448	4.833	0.305
PO <sub>4</sub> <sup>3-</sup>	4.167	0.384	10.567	0.032	3.414	0.491
SO <sub>4</sub> <sup>2-</sup>	2.867	0.580	5.800	0.215	5.990	0.200

**Table 6***Discriminant analysis test (ANOVA) of equality of group means*

	Cl <sup>-</sup>	PO <sub>4</sub> <sup>3-</sup>	SO <sub>4</sub> <sup>2-</sup>	NO <sub>2</sub> <sup>-</sup>	NO <sub>3</sub> <sup>-</sup>
F statistic	16.853	0.376	0.276	1.679	19.828
Wilks' Lambda	0.555	0.982	0.987	0.926	0.514
p-level	<b>0.000</b>	0.689	0.760	0.199	<b>0.000</b>

Bold values are significant at  $p < 0.001$ .

For Peer Review Only

**Table 7***Values of the discriminant functions at the group centroids*

Origin	Centroid	Origin	Centroid	Origin	Centroid
Farm A	-1.678	Farm B	1.245	Farm C	1.502
Not Farm A	0.839	Not Farm B	-0.623	Not Farm C	-0.751

For Peer Review Only