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

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

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⁻¹) and chlorides (545.93-1757.89 mg kg⁻¹) that as the most abundant anions, followed by and sulphates (109.52-200.69 mg kg⁻¹). The concentrations of nitrites and nitrates are found to be lower at 5.60 mg kg⁻¹ and 5.50 mg kg⁻¹. 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...
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.
Classification of the geographical origin of Italian donkey milk
based on differences in inorganic anions

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

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

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 rediscovered 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).

Considering the unique nutrient profile of donkey’s milk it should be valued as a neutriceutical 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).

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

Sulphates and phosphates, because their low toxicity, are extensively employed in agriculture; the former as pesticides and the latter as fertilizers (Dugo et al. 2007).

Particularly in milk, phosphate is present as inorganic phosphate (soluble phosphate and micellar calcium phosphate (MCP)), organic phosphate as small molecules, covalently bound to the peptide chains of caseins.

The determination of the geographical origin of foodstuffs is becoming of increasing interest to consumers and producers, since it may be used as a criterion to certify quality and typicality. Generally, measurement of multi-element stable isotope ratios by isotope ratio mass spectrometry (IRMS) has been used to determine the geographical origin of milk and milk ingredients (Kornexl et al. 1997; Renou et al. 2004; Manca et al. 2006; Crittenden et al. 2007). Also, classical techniques, i.e. high performance ion chromatography (HPIC), liquid chromatography–mass spectrometry (LC-MS), inductively coupled plasma emission spectroscopy (ICP-AES) and nuclear magnetic resonance spectroscopy (NMR), were used to determine different compounds in combination with chemometric methods to discriminate the geographical origin of milk (Brescia et al. 2005; Calabrese et al. 2009; Sacco et al. 2009.

This research deals with the classification of donkey milk samples from different Italian regions according to their anion content. The anion concentrations were easily determined by Suppressed Ion Chromatography (SIC) following fast milk pre-treatment. The data set which was obtained was sub-divided into three groups and subject to analysis of variance (ANOVA) and Canonical Discriminant Analysis (CDA) to achieve some statistical classification of donkey milk from different Italian areas according to the presence of anions.
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 μS 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 Na₂CO₃) 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⁻¹ 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⁻¹ 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 Na₂CO₃ 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 mg·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
Table 2 summarizes the sensitivity and linearity parameters for the analysis of anions with the method used. The linearity of the method was assessed by analyzing seven standard solutions obtained from the standard mixture. Three replicate analyses were performed at each concentration level. Good linearity was observed in each concentration range, with linear correlation coefficients ($R^2$) better than 0.9873. As per Pharmacopoeia (1999), the limits of detection (LODs) and the limits of quantification (LOQs) were experimentally calculated as a signal-to-noise ratio of 3 and 10, respectively. Among all anions, phosphates had the highest detection limit of 20.16 µg·L$^{-1}$.

The accuracy of the method described for the determination of anions in milk samples was evaluated at three spiking levels, with three replicates for each level. For the recovery test, a sample of donkey milk was previously analyzed and then fortified with note amount of standard anions. The results obtained for each anion are given in Table 3, where it can be noted that the recoveries for each anion were almost constants. From this table, it is also evident that the highest recoveries were determined for chlorides (96.6%) and phosphates (95.1%). For the other three anions, the recoveries were around 88%. All the RSD values were less than 2.8%, which shows the excellent precision of the method used for the determination of anions in milk.

Anion contents in donkey milk

The quantitative results of anions found are show in Table 4. All milk samples were analyzed in triplicate. The obtained data indicate that phosphates (569.4-1304.4 mg·kg$^{-1}$) and chlorides (545.9-1757.9 mg·kg$^{-1}$) are the most abundant anions, followed by sulphates (109.5-200.7 mg·kg$^{-1}$). The concentrations of nitrites and nitrates are found lower than 5.6 mg·kg$^{-1}$ and 5.5 mg·kg$^{-1}$ (maximum values observed).

The chloride concentrations found in these samples are similar to those determined in cow milk (Gaucheron 2005) and twice higher than those determined for human breast milk.
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(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\(^{-1}\), while for nitrites the limit is set at 0.1 mg·L\(^{-1}\). In all milk analyzed samples concentration of nitrate were lower than fixed limit; conversely, all samples showed nitrates at concentration higher than 0.1 mg·L\(^{-1}\). This last data could be attributed to many factors including intake of water, feed or forages which could contain high levels of nitrates, due to nitrogenous fertilizer and herbicides, and industrial and domestic wastes in the environment. So the research on the presence of nitrates 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:

\[
F_1 = 0.976\ln(Cl^-) - 0.446\ln(NO_3^-) - 0.158\ln(SO_4^{2-}) - 0.400\ln(NO_2^-) + 0.678\ln(NO_3^-) \quad (eq.1) 
\]

\[
F_2 = -0.730\ln(Cl^-) - 0.054\ln(NO_3^-) - 0.188\ln(SO_4^{2-}) + 0.555\ln(NO_2^-) + 0.763\ln(NO_3^-) \quad (eq.2) 
\]

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:

\[
F_3 = 0.558\ln(CL^-) + 0.757\ln(NO_3^-) \quad (eq.3)
\]

\[
F_4 = 0.841\ln(CL^-) - 0.668\ln(NO_3^-) \quad (eq.4)
\]

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:

\[
F_A = 0.774\ln(CL^-) - 0.402\ln(PO_4^{3-}) - 0.211\ln(SO_4^{2-}) - 0.190\ln(NO_2^-) + 0.954\ln(NO_3^-) \quad (eq.5)
\]

\[
F_B = -0.720\ln(CL^-) - 0.027\ln(PO_4^{3-}) - 0.214\ln(SO_4^{2-}) + 0.475\ln(NO_2^-) + 1.008\ln(NO_3^-) \quad (eq.6)
\]

\[
F_C = 1.249\ln(CL^-) - 0.366\ln(PO_4^{3-}) - 0.005\ln(SO_4^{2-}) - 0.682\ln(NO_2^-) + 0.010\ln(NO_3^-) \quad (eq.7)
\]

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:

\[
d_A^2 = [F_A - (-1.678)]^2 \quad (eq.8)
\]

\[
d_{notA}^2 = [F_A - (0.839)]^2 \quad (eq.9)
\]
where -1.678 and +0.839 are the values of FA 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:

\[ P_A = \frac{10^{0.5d_{AA}^2}}{10^{0.5d_{AA}^2} + 10^{0.5d_{max}^2}} \]  
(10)

\[ P_{notA} = \frac{10^{0.5d_{max}^2}}{10^{0.5d_{AA}^2} + 10^{0.5d_{max}^2}} \]  
(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.

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Schaafsma G. 2003. Nutritional significance of lactose and lactose derivatives In Roginski
Immunological Properties of Donkey’s Milk: Its Potential Use in the Prevention of
Table captions

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.

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 ± 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.
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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.
Fig. 1. Seasonal variation of anion concentrations in donkey milk.
Fig. 2. 2D Scatterplot and classification matrix results by using discriminant functions defined in eqs 1 and 2.
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

<table>
<thead>
<tr>
<th>Anion</th>
<th>Intra-day repeatability (RSD%, n=6)</th>
<th>Inter-day repeatability (RSD%, n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_R$ Area</td>
<td>$T_R$ Area</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>1.1 2.4</td>
<td>1.9 3.3</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>0.9 1.3</td>
<td>1.2 3.7</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>0.9 1.6</td>
<td>1.6 2.5</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>1.4 1.7</td>
<td>2.1 1.3</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>1.4 2.3</td>
<td>2.2 3.6</td>
</tr>
</tbody>
</table>
Table 2

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

<table>
<thead>
<tr>
<th>Anion</th>
<th>Calibration curves (n=7)</th>
<th>R²</th>
<th>Linearity range (µg·L⁻¹)</th>
<th>LOD (µg·L⁻¹)</th>
<th>LOQ (µg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>y=(0.1366±0.0016)x +(-0.0038±0.0054)</td>
<td>0.9994</td>
<td>7.5-7500</td>
<td>2.4</td>
<td>7.9</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>y=(0.0791±0.000)x</td>
<td>0.9983</td>
<td>25-2500</td>
<td>7.7</td>
<td>25.8</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>y=(0.0582±0.0016)x +(0.0184±0.0018)</td>
<td>0.9895</td>
<td>25-2500</td>
<td>7.5</td>
<td>25.1</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>y=(0.0343±0.0011)x +(-0.0118±0.0190)</td>
<td>0.9873</td>
<td>37.5-3750</td>
<td>12.2</td>
<td>40.7</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>y=(0.01047±0.0019)x +(0.0349±0.0323)</td>
<td>0.9963</td>
<td>37.5-3750</td>
<td>20.2</td>
<td>67.2</td>
</tr>
</tbody>
</table>

n, points calibration curves; R², least 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

<table>
<thead>
<tr>
<th>Anion</th>
<th>Concentration (mg·kg⁻¹)</th>
<th>Added (mg·kg⁻¹)</th>
<th>Expected (mg·kg⁻¹)</th>
<th>Found * (mg·kg⁻¹)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>510</td>
<td>100</td>
<td>610</td>
<td>590.5 ± 2.2</td>
<td>96.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>810</td>
<td>772.7 ± 3.16</td>
<td>95.4</td>
</tr>
<tr>
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<td>1010</td>
<td>896.8 ± 5.1</td>
<td>97.7</td>
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<td>Mean value ± S.D.</td>
<td></td>
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<td>96.6 ± 1.2</td>
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<td></td>
<td>Precision (%)</td>
<td></td>
<td></td>
<td>1.20</td>
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<tr>
<td>NO₂⁻</td>
<td>0.03</td>
<td>0.5</td>
<td>0.53</td>
<td>0.5 ± 0.1</td>
<td>90.1</td>
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<tr>
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<td>2</td>
<td>2.53</td>
<td>1.8 ± 0.1</td>
<td>89.6</td>
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<tr>
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<td>5</td>
<td>5.03</td>
<td>4.3 ± 0.7</td>
<td>85.6</td>
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<tr>
<td></td>
<td>Mean value ± S.D.</td>
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<td></td>
<td>88.4 ± 2.5</td>
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<tr>
<td></td>
<td>Precision (%)</td>
<td></td>
<td></td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>0.24</td>
<td>1</td>
<td>1.24</td>
<td>1.1 ± 0.7</td>
<td>88.7</td>
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<tr>
<td></td>
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<td>5.24</td>
<td>4.6 ± 0.4</td>
<td>87.7</td>
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<td>10</td>
<td>1024</td>
<td>9.1 ± 0.3</td>
<td>88.8</td>
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<tr>
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<td>Mean value ± S.D.</td>
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<td></td>
<td>88.4 ± 0.6</td>
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<td></td>
<td>Precision (%)</td>
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<td></td>
<td>0.7</td>
<td></td>
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<tr>
<td>PO₄³⁻</td>
<td>620</td>
<td>100</td>
<td>720</td>
<td>684.0 ± 0.7</td>
<td>95.0</td>
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<tr>
<td></td>
<td></td>
<td>300</td>
<td>920</td>
<td>860.2 ± 0.5</td>
<td>93.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>1120</td>
<td>1085.3 ± 0.1</td>
<td>96.9</td>
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<tr>
<td></td>
<td>Mean value ± S.D.</td>
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<td></td>
<td>95.1 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Precision (%)</td>
<td></td>
<td></td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>106</td>
<td>50</td>
<td>156</td>
<td>133.7 ± 0.1</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>206</td>
<td>183.5 ± 0.1</td>
<td>89.1</td>
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<tr>
<td></td>
<td></td>
<td>150</td>
<td>256</td>
<td>224.0 ± 0.4</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>Mean value ± S.D.</td>
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<td></td>
<td>87.4 ± 1.7</td>
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</tr>
<tr>
<td></td>
<td>Precision (%)</td>
<td></td>
<td></td>
<td>1.9</td>
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</tr>
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</table>

*Average of three replicates.
Table 4

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

<table>
<thead>
<tr>
<th></th>
<th>Cl&lt;sup&gt;−&lt;/sup&gt; (mg·kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>PO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;3−&lt;/sup&gt; (mg·kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2−&lt;/sup&gt; (mg·kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt; (mg·kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt; (mg·kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Farm A (n=15)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>545.9±9.3</td>
<td>744.5±36.0</td>
<td>125.8±12.2</td>
<td>1.5±0.1</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>Max</td>
<td>863.8±25.3</td>
<td>1210.8±49.4</td>
<td>193.7±15.3</td>
<td>2.7±0.1</td>
<td>2.9±0.1</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>738.4±86.8</td>
<td>980.1±160.1</td>
<td>151.6±22.2</td>
<td>2.2±0.4</td>
<td>2.4±0.3</td>
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<tr>
<td><strong>Farm B (n=15)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>749.7±11.5</td>
<td>842.3±32.0</td>
<td>135.8±15.2</td>
<td>1.8±0.1</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>Max</td>
<td>831.5±25.1</td>
<td>1156.9±48.6</td>
<td>165.7±12.8</td>
<td>3.3±0.1</td>
<td>5.5±0.4</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>800.6±27.6</td>
<td>978.6±95.3</td>
<td>151.0±12.4</td>
<td>2.6±0.5</td>
<td>3.7±0.8</td>
</tr>
<tr>
<td><strong>Farm C (n=15)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>872.7±23.0</td>
<td>569.4±17.9</td>
<td>109.5±22.0</td>
<td>1.3±0.1</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>Max</td>
<td>1757.9±43.8</td>
<td>1304.4±32.9</td>
<td>200.7±24.4</td>
<td>5.6±0.1</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>Mean±S.D.</td>
<td>967.1±221.5</td>
<td>951.5±236.2</td>
<td>158.3±29.8</td>
<td>2.4±1.0</td>
<td>3.0±0.1</td>
</tr>
</tbody>
</table>
Table 5

*Kruskal Wallis test by variable “season”.*

<table>
<thead>
<tr>
<th>Anion</th>
<th>Farm A Chi-Square</th>
<th>Farm A p-Level</th>
<th>Farm B Chi-Square</th>
<th>Farm B p-Level</th>
<th>Farm C Chi-Square</th>
<th>Farm C p-Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl^{-}</td>
<td>1.433</td>
<td>0.838</td>
<td>8.833</td>
<td>0.065</td>
<td>7.433</td>
<td>0.115</td>
</tr>
<tr>
<td>NO_{2}^{-}</td>
<td>7.300</td>
<td>0.121</td>
<td>7.367</td>
<td>0.118</td>
<td>8.433</td>
<td>0.077</td>
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<tr>
<td>NO_{3}^{-}</td>
<td>11.367</td>
<td>0.023</td>
<td>3.700</td>
<td>0.448</td>
<td>4.833</td>
<td>0.305</td>
</tr>
<tr>
<td>PO_{4}^{3-}</td>
<td>4.167</td>
<td>0.384</td>
<td>10.567</td>
<td>0.032</td>
<td>3.414</td>
<td>0.491</td>
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<tr>
<td>SO_{4}^{2-}</td>
<td>2.867</td>
<td>0.580</td>
<td>5.800</td>
<td>0.215</td>
<td>5.990</td>
<td>0.200</td>
</tr>
</tbody>
</table>
Table 6

Discriminant analysis test (ANOVA) of equality of group means

<table>
<thead>
<tr>
<th></th>
<th>Cl(^{-})</th>
<th>PO(_4^{3-})</th>
<th>SO(_4^{2-})</th>
<th>NO(_2^{-})</th>
<th>NO(_3^{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F statistic</strong></td>
<td>16.853</td>
<td>0.376</td>
<td>0.276</td>
<td>1.679</td>
<td>19.828</td>
</tr>
<tr>
<td>Wilks' Lambda</td>
<td>0.555</td>
<td>0.982</td>
<td>0.987</td>
<td>0.926</td>
<td>0.514</td>
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<tr>
<td><strong>p-level</strong></td>
<td><strong>0.000</strong></td>
<td>0.689</td>
<td>0.760</td>
<td>0.199</td>
<td><strong>0.000</strong></td>
</tr>
</tbody>
</table>

Bold values are significant at \(p < 0.001\).
Table 7

Values of the discriminant functions at the group centroids

<table>
<thead>
<tr>
<th>Origin</th>
<th>Centroid</th>
<th>Origin</th>
<th>Centroid</th>
<th>Origin</th>
<th>Centroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>-1.678</td>
<td>Farm B</td>
<td>1.245</td>
<td>Farm C</td>
<td>1.502</td>
</tr>
<tr>
<td>Not Farm A</td>
<td>0.839</td>
<td>Not Farm B</td>
<td>-0.623</td>
<td>Not Farm C</td>
<td>-0.751</td>
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</tbody>
</table>