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The use of multi element profiling to differentiate between cow and buffalo milk

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Abstract

The multi element profile of milk from 12 cows and 6 water buffaloes was investigated, to establish whether dairy products derived from the two species could be distinguished. Multi-element data were obtained using ICP-MS. Following assessment against the team's QA/QC criteria, or where the levels were below the LOD for the procedure, 16 elements (P, S, K, Ca, V, Cr, Mn, Fe, Co, Zn, Ga, Rb, Sr, Mo, Cs and Ba) were submitted for statistical analysis. Using linear discriminant analysis (LDA) it was possible to differentiate between milk from the two species, produced under identical environmental and animal husbandry conditions, on one farm. The sources of food and water available for consumption by the animals were also analysed and the results showed that there was no correlation between the elemental composition of the dietary components and the profiles observed in the milk.

Keywords: Food; Milk; Cow; Buffalo; Trace elements; ICP-MS; Authenticity; Statistical analysis

1. Introduction

Milk has always been an important foodstuff; hence it well have been one of the earliest commodities to be subjected to extension or adulteration (Holeman, 1984), such as addition of water (Commission Decision (EEC), 1991, Papps, Voutsinas, & Kondyli, 1994). There are also safety issues if milk from different animals is used, because many people are allergic to cows' milk and therefore choose milk, cheese and yoghurt from other animals, such as sheep and goats. If a significant quantity of cow's milk is added to such products, without proper labelling, health problems can result. Another source of risk is the use of mastitic milk as a replacement feed (Aiello, Napoli, Di Donna, Spina, & Sindona, 2006). Therefore, there is a need to characterise milk from different animals, both as a food commodity itself and also as a component in other dairy products.

Previous works have shown that trace element profiling can be used to identify the geographical origin of foodstuff (Benincasa, Lewis, Perri, Sindona, & Tagarelli, 2007; Brescia, Caldarola, Buccolieri, Dell'Atti & Sacco, 2003; Brescia, Monfreda, Buccolieri, & Carrino, 2005). One research group reported that multi-element profiling could distinguish the animal species from which a foodstuff had derived (Coni, Bocca, Ianni, & Caroli, 1995; Coni et al., 1996). However, from the data, it was not clear if the geographical location of the farms had been taken into account in this study. It is very likely that at least part of the differences in the trace elements profiles measured in the milks were due to differences in the underlying geology/hydrology of the participating farms.

In the study presented here, samples of milk from a mixed herd of cows and water buffaloes, having equal access to identical forage and water (herded in the same field and had been given similar regimes of veterinary medicinal care) were analysed by ICP-MS to see if physio-

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logical differences between the species would result in differences in the multi-element composition of the product. These 'biomarker' elements could then be used to identify situations when fraudulent labelling of the milk, and associated by products, e.g., mozzarella, had occurred. The data might also provide an important insight into the dietary requirements of the two animal species.

2. Materials and methods

2.1. Materials and apparatus

Ultrapure HNO_3 (Aristar grade, Merck-BDH, Poole, Dorset, UK) certified for impurities was used in this work. Single and multielement standards (Certipur, Merk, Darmstadt, Germany) were also analytical-reagent grade. Aqueous solution were prepared using ultrapure water, with a resistivity of 18.2 MΩ cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA). All glassware was decontaminated with nitric acid (2%, v/v) over night, rinsed with ultrapure water and dried.

Microwave digestion was carried out using a PerkinElmer 'Multiwave' system fitted with 50 mL quartz vessels capable of 70 bar working pressure (75 bar cut off).

The determination of the elements of interest (P, S, K, Ca, V, Cr, Mn, Fe, Co, Zn, Ga, Rb, Sr, Mo, Cs and Ba) was carried out utilizing an Elan 6000 ICP-MS instrument (Perkin–Elmer Ltd., Beaconsfield, Buckinghamshire, UK). The ICP-MS operating conditions are listed in Table 1.

2.2. Sampling

Eighteen milk samples were obtained from a Grillo farm in the Sila mountain (Calabria, Italy), six from water buffaloes and twelve from cows.

Manual milking was employed to avoid potential contamination due to metallic containers and tank lorries. Milk samples were directly put into acid-washed plastic containers and immediately stored at −20 °C until required for analysis. Samples of the animal feed (fresh and dried) and drinking water (river and pool) available to the animals were also obtained for analysis.

Table 1
Instrumental parameters and operating conditions for the Elan 6000 ICP-MS

RF power (W)	1000
Nebulizer (carrier gas) flow rate (L/min)	0.75
Lens voltage (V)	6.00
Analog stage voltage (V)	−1850
Pulse stage voltage (V)	950
Discriminator threshold (V)	70
Quadrupole rod offset (V)	0
Resolution (amu)	0.70
Detector	Dual
Speed of peristaltic pump (rpm)	24
Sweeps/reading	40
Replicates	6
Dwell time	50 ms
Scan mode	Peak hopping

2.3. Analytical procedure

Milk samples (4 mL), fresh and dried forage (0.5 g), water from river and pool (4 mL), were quantitatively transferred to the quartz vessel of a microwave digestion system and concentrated nitric acid (5 mL) added. The operating conditions used for the microwave digestion system is presented in Table 2.

After digestion the digested liquor was quantitatively transferred to a graduated polypropylene test-tube and made up to volume (10 mL) with ultrapure water. 1 mL of the resulting solution was transferred a second time to a test tube and made up to volume (5 mL) using ultrapure water. In order to check the performance of the instrument, hence the instrument drifts, before performing ICP-MS analysis, 100 μl of a solution containing Indium was added to each test tube as internal standard to make up a final concentration of 50 μg/L. To validate the experimental procedure, two multi-elemental certified reference materials were randomly distributed in the analytical sequence, NIST 1547 (peach leaves) and NIST 8435 (whole milk powder) and were randomly distributed between the other samples.

The analytical batch consisted of a set of calibration standards, that were analysed at the beginning of the run, the samples, and, all distributed between them, a minimum of four procedural blanks, one procedural blank spiked with a standard solution containing the elements of interest and the certificate reference materials. To check the quality of the run, at the end of the run, a mid-range calibration standard was analysed a second time. The whole analysis was considered valid if the value of the same calibration standard analysed at the beginning and at the end of the run gave a value lower than 20%. Furthermore, the performance of the instrument was good as the response of the internal standard, contained in each solution analysed, gave always the same response throughout the whole analysis.

The limit of detection were defined as three times the standard deviation of the signal from reagent blanks, after correction for sample weight and dilution. The blanks were used to calculate this value only. So, all the elements that were below this value were not accepted.

Table 2
Microwave digestion program

Step	Power (W)	Time (min)
1	0–500	2
2	500	5
3	500–1000	2
4	1000	20
5	0	15

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2.4. Calibration procedure

A five point calibration curve covering the range 0.01–100 μg/L with 50 μg/L of indium as internal standard was used for the quantitative analysis of samples. Solutions of appropriate concentration were obtained by dilution of a 1000 μg/mL element standard solution of P, S, K, Ca, V, Cr, Mn, Fe, Co, Zn, Ga, Rb, Sr, Mo, Cs, Ba.

2.5. Statistical analysis

Principal Component Analysis (PCA) was performed by Statistica 7.1 (StatSoft 2005 Edition) and Linear Discriminant Analysis (LDA) was executed by V-Parvus 84 2004 (Forina, Lanteri, Armanino, Cerrato Oliveros, & Casolino, 2004).

3. Result and discussion

3.1. Quality control and quality assurance data

Initially, about 50 elements were investigated but only 16 were submitted for statistical analysis. The criteria utilized to select those elements were as follow: recovery data were accepted if results were in the range of 60–140%, with 75% within 80–120% and for CRM values, within 40%. The results must be not below the limit of detection (LOD). The replicate agreement was considered acceptable if the value of the RSD was minor of 20%. Table 3 details the LOD values and the percentage recovery of a known amount of analyte spiked for the 16 elements investigated. Table 3 also presents mean, standard deviation ($n = 3$) and percentage recovery of the 12 elements for which the CRMs quote a certified value.

Table 4
Mean concentration and standard deviation of elements (μg/kg) in milk samples by ICP-MS

Element	Water buffalo Mean value	Cow Mean value
P	1.19×10^6 (14)	7.75×10^5 (12)
S	3.57×10^5 (15)	2.66×10^5 (19)
K	6.41×10^5 (11)	1.19×10^6 (18)
Ca	1.74×10^6 (15)	1.22×10^6 (15)
V	4.10 (17)	2.93 (20)
Cr	0.34 (7)	9.38 (18)
Mn	2.47 (24)	31.9 (17)
Fe	301 (18)	325 (13)
Co	2.10 (20)	1.44 (19)
Zn	6488 (20)	3814 (20)
Ga	3.50 (17)	2.24 (13)
Rb	1440 (19)	2088 (15)
Sr	749 (11)	698 (17)
Mo	16.9 (16)	29.0 (24)
Cs	3.56 (17)	2.51 (20)
Ba	330 (17)	226 (14)

In order to check the sensitivity and the reproducibility of the digestion procedure, one cow's milk and one water buffalo's milk samples were digested three times. The relative standard deviations obtained were very good: the RSD values for the element concentrations in cow's milk were between 0.5% and 7%, whereas Mn, Cs, Rb and Co gave a value between 10% and 11.5%. The analysis for water buffaloes produced a slightly higher RSD values that were between 1.5% and 10%, whereas Mn, Fe, Ga, S, K and Ca gave a value between 11% and 13.8%.

Table 4 summarises the mean value (μg/kg) and the standard deviation of all samples analysed in this study, for each element investigated.

Table 3
Limit of detection (LOD), percentage recovery of a spike solution and quality assurance material performance data (NIST 8435 and NIST 1547)

Element	Spike recovery (%)	LOD (μg/g)	N8435			N1547		
			Found (μg/g)	Certified (μg/g)	Accuracy (%)	Found (μg/g)	Certified (μg/g)	Accuracy (%)
^{31}P	99	1.2	5840 ± 240	7800 ± 490	74.9	1280 ± 65	1370 ± 70	93.7
^{34}S	100	82	2000 ± 170	2650 ± 350	75.5	0.86 ± 0.21	n/a	n/a
^{39}K	107	0.57	11160 ± 420	13630 ± 470	81.9	21200 ± 210	24300 ± 300	87.3
^{43}Ca	109	1.1	7820 ± 350	9220 ± 490	84.8	13500 ± 150	15600 ± 200	86.8
^{51}V	97	0.001	0.011 ± 0.004	n/a	n/a	0.29 ± 0.03	0.37 ± 0.03	77.6
^{53}Cr	118	0.004	0.42 ± 0.05	(0.5)	84.0	0.85 ± 0.14	(1)	85.0
^{55}Mn	125	0.005	0.174 ± 0.085	0.17 ± 1.1	102.4	75 ± 3	98 ± 3	76.1
^{56}Fe	117	0.16	174 ± 15	n/a	n/a	174 ± 13	218 ± 14	79.7
^{59}Co	95	0.001	0.068 ± 0.007	n/a	n/a	0.068 ± 0.008	(0.07)	97.1
^{66}Zn	100	0.079	25.0 ± 2.9	28.0 ± 3.1	89.3	16.8 ± 0.8	17.9 ± 0.4	93.7
^{71}Ga	100	0.001	0.018 ± 0.005	n/a	n/a	0.083 ± 0.011	n/a	n/a
^{85}Rb	116	0.001	12.9 ± 0.9	(16)	80.9	17.2 ± 0.4	19.7 ± 1.2	87.2
^{88}Sr	118	0.010	3.64 ± 0.5	4.35 ± 0.5	83.7	42 ± 4	53 ± 4	78.6
^{95}Mo	102	0.005	0.21 ± 0.12	0.29 ± 0.1	71.4	0.051 ± 0.004	0.060 ± 0.001	85.0
^{133}Cs	99	0.001	0.017 ± 0.004	n/a	n/a	0.071 ± 0.004	n/a	n/a
^{138}Ba	120	0.001	0.58 ± 0.17	0.58 ± 0.2	99.5	101 ± 5	124 ± 4	81.8

Certified values without standard deviation are reported in parenthesis.

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3.2. Statistical analysis of multi-element data

Principal component analysis (PCA) is the basic tool for data analysis. PCA is very important to gather an overview of data, especially in the preliminary steps of a multivariate analysis. It is a powerful visualization tool and provides a way to reduce the dimensionality of the data and allows elimination of unnecessary information. With PCA, matrix decomposition decreases a large number of variables into a smaller set of components by investigating the correlation between variables. These components give linear combination of variables which account for more of the variance than any other combination (Hair, Anderson, & Tatham, 1987; Sharaf, Illman, & Kowalski, 1986; Vandeginste et al., 1998). Score plots represent the projections of the objects (samples) in the planes defined by principal components, whereas loading plots represent the projection of the original variables in the same planes. Samples close to each other in the score plot can be considered similar, and, for instance, object that are projected in the left in the graph have high values for variables placed to the left in the loading plot. The absolute value of the loading in a component (between 0 and 1) describes the importance of the contribution of the component, so the more a variable is far from origin, the greater its contribution is in the statistic model generated by PCA. Moreover, the variables that are near each other in the loading plot are positively correlated, whereas variables that are projected opposite to each other are negatively correlated.

In the present work PCA have been applied to the concentration of 16 elements of each single sample. The plot of scores of the samples and loadings of the variables on the two first principal components are plotted in Fig. 1.

Cow and buffalo milks are clearly separated on the first principal component score, which shows 46.68% of the total variance, with water buffalo milk samples represented by low scores and cows milk represented by high scores. The examination of the loading plot provides insights into the discrimination of the variables mainly contributing to PC1. Ca, P, Ga, Zn, Mn, Ba and S have the highest absolute loading values on the PC1, all of them being negative. Accordingly, these variables are higher in the water buffalo milk samples (negative scores on PC1). On the contrary, K and Rb are the elements present at higher concentration in cow milk samples.

One of the most commonly used classification techniques is linear discriminant analysis (LDA). This tool allows classifying unknown samples after checking of possible differentiation of samples of known origin. LDA derives linear combinations of the independent variables that best discriminate between the defined groups, by maximizing the variance between groups and by minimizing the variance within each group in such a way that outsider can be identified more lightly than by PCA. It must be pointed out that LDA require the data matrices for each category to have an high ratio between the number of training samples and the number of variables used. Discriminant analysis using a low ratio of samples to variables generates an unstable model and analysis is also weakened by the presence of redundant information (highly correlated variables), which yield a less robust model. Accordingly, in the present study PCA have again been applied to the concentration of the nine elements of each single sample mainly contributing to separation of the groups (Ca, P, Ga, Zn, Mn, Ba, S, K and Rb).

The first two principal components account for 87.12% of the total available information, so coordinates of scores for these PCs can be submitted to LD analysis, using two groups corresponding to the two types of milk, as input a priori. The scores of the first two roots produced from LDA showed a clear separation between cow and buffalo milks (Table 5).

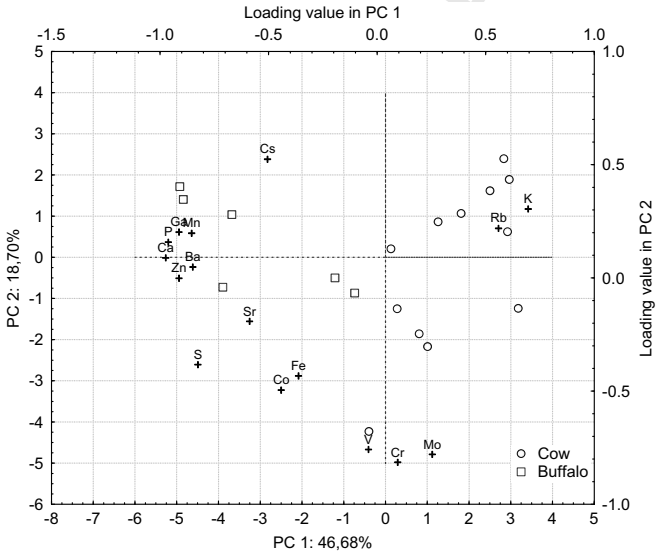

Fig. 1. Biplot of principal component scores and loadings (○ cow, □ buffalo, + variable).

Table 5
Scores of the first two roots produced from LDA

Sample	Root 1	Root 2
Cow 1	−5.24223	−0.60863
Cow 2	−8.35870	0.94960
Cow 3	−11.88722	2.71386
Cow 4	−10.71974	2.13012
Cow 5	−6.32821	−0.06564
Cow 6	−9.50743	1.52397
Cow 7	−4.43730	−1.01110
Cow 8	−5.65215	−0.40367
Cow 9	−8.65207	1.09629
Cow 10	−12.94881	3.24466
Cow 11	−6.25332	−0.10309
Cow 12	−13.36469	3.45260
Buffalo 1	6.72712	−6.59331
Buffalo 2	6.29100	−6.37524
Buffalo 3	0.73804	−3.59876
Buffalo 4	5.38709	−5.92329
Buffalo 5	1.54363	−4.00156
Buffalo 6	5.15109	−5.80529

In this case, an acceptable sample/variable ratio (18:2) was used and a robust model should be obtained. In fact, from a statistical point of view, the high value (36.33) of the F (2.15) parameter indicates a significant difference among the means of the groups, whereas the information from data treatment is characterized by a high degree of reliability since the p level is extremely low (<0.00001).

The effectiveness of a classification technique in predicting the group of unknown object (sample) can be achieved by several validation procedures. The common validation technique is cross-validation. The sample set was divided into training set including 16 samples and into validation set which contain 2 samples. The first one was used to generate the model whereas the validation set was used to validate the model. The training and validation sets were randomly selected from all samples. The prediction capacity of the LDA model was determined by analyzing the validation set of samples that had not been used at any time to construct the model. In other words, all milk samples but two were used for calculating discriminant functions, and then the samples of the validation set was used as unknown, and classified. The procedure was repeatedly carried out for all samples. The proposed model predicts correctly all observations. The results obtained should be considered as a first approach to the classification of the origin of milk useful for further prediction. A wider application of the method, although it works satisfactory in the examined case, will require a larger number of samples from selected origins.

3.3. Dietary inputs

Two sources of water (river and pool) and two sources of forage (fresh and dried) were available to the cows and water buffalo. The concentration of the elements in the water coming from the river is higher than the concentration of the elements in the water coming from the pool. Several elements couldn't be quantified in the forage. However, the fresh forage seem to have an higher value of these elements.

No significant correlation was found between mean concentration of elements in the water sources and the milk types ($p = 0.90$) nor between the element profiles in milk and forages ($p = 0.92$). Hence the difference observed between cows and buffalo are not due to food or water.

3.4. Physiological differences

Even though the digestive tract of the two species are very similar, it has been reported that buffalo have longer retention time of feed in their digestive system and greater digestive efficiency than cattle (Appleton, Dryden, & Kondos, 1976; Koch, Jung, Crouse, Varel, & Cundiff, 1995; Schaefer, Young, & Chimwano, 1978).

The higher efficiency would have a pronounced effect on the animal's mineral absorption behaviour, and is reflected in the data presented here. It has been reported that longer feed retention would allow buffaloes more time to digest the high levels of fibre in feeds such as sedges and grasses, releasing minerals and vitamins which would otherwise have not been available for absorption in the large and small intestines (Ensminger, Oldfield, & Heinemann, 1990; Commission Regulation (EEC), 1992).

Currently, all mineral (and vitamin) requirements for water buffalo have been based on beef cattle requirements. However, the data presented in this work indicate that the mineral requirements of water buffalo should be re-assessed.

4. Conclusions

The results presented in this work show that the discrimination between cow's and water buffalo's milk can be achieved by a simple and rapid method, such as ICP-MS, which allows a simultaneous quantitative determination of the elements of interest present in the foodstuff. As the herd containing the two species were restricted to a single geographical locale, the observed differences can not be attributed to the animals having access to different water or food sources.

The linear discriminant analysis model classifies correctly the origin of the all samples of examined milks, indicating that these analytical parameters may warrant further investigation as indicators of fraudulent marketing practices.

Future work will involve to build up a bigger set of data in order to see if it is possible to discriminate between the two milks coming from different farms; to investigate the physiological attributes that result in such a pronounced difference in the mineral absorption between the two animal species; whether the differences described in this work, are still apparent after processing into dairy products such as cheese.

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