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Accessible Technologies for the Verification of Origin of Dairy Products as an Example Control System to Enhance Global Trade and Food Safety

Final Report of a Coordinated Research Project

Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

IAEA
International Atomic Energy Agency

ACCESSIBLE TECHNOLOGIES FOR THE VERIFICATION OF ORIGIN OF DAIRY PRODUCTS AS AN EXAMPLE CONTROL SYSTEM TO ENHANCE GLOBAL TRADE AND FOOD SAFETY

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FINAL REPORT OF A COORDINATED RESEARCH PROJECT

PREPARED BY THE
JOINT FAO/IAEA CENTRE OF NUCLEAR TECHNIQUES IN FOOD AND AGRICULTURE

INTERNATIONAL ATOMIC ENERGY AGENCY
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FOREWORD

Globalization and the increasing complexity of trade in food provide both opportunities and risks to countries around the world. There are increasing opportunities for the Member States of the IAEA and the Food and Agriculture Organization that produce safe, high quality food to obtain premium prices for it on the global market. The risks are associated with inadvertent or fraudulent mislabelling, causing reputational damage that can adversely affect a Member State's international food exports. Food fraud is of paramount importance when food safety is also compromised. The consequences include lower prices and/or loss of market access for a Member State and potential health risks for consumers. When food safety is compromised, it is imperative that a product's traceability system can rapidly identify the source of the contaminant or adulterant, enabling it to be removed from the supply chain.

One significant constraint to controlling these risks is the capacity of Member State laboratory services to support food traceability systems with independent means of verification. Nuclear and complementary techniques for food origin and food authentication analysis can help to mitigate this constraint by providing information on the food product itself to support labelling claims, identify when the labelling is inaccurate and/or fraudulent and directly provide information to help trace the origin of products if necessary.

This coordinated research project addressed some of the challenges that developing countries are facing in providing analytical support for food traceability systems, using milk as an example commodity. Milk and milk products have been involved in fraud issues that have also endangered public health (e.g. milk adulteration with melamine to increase the apparent protein content). Consequently, dairy commodities are of high priority for improved traceability and authenticity control due to their relatively simple processing procedures, high level of trade and frequent use as an ingredient in products destined for relatively vulnerable consumer groups, such as infant formula for young children. The methodology developed in this coordinated research project is intended to act as a template that can be transferred to other food commodities as required. The benefit to laboratories is enhanced capability to apply state of the art nuclear and related methods for determining the provenance of foodstuffs. The project also explored mechanisms to enhance networking among research institutions involved in research on food authenticity and origin.

The project was implemented by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture between 2013 and 2018 and involved nine research contracts, four research agreements and one technical contract.

The IAEA officers responsible for this publication were S. Kelly, R. Frew and A. Cannavan of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture.

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SUMMARY

1. BACKGROUND

This publication is an output from the FAO/IAEA Joint Division's CRP D52038 "Accessible Technologies for the Verification of Origin of Dairy Products as an Example Control System to Enhance Global Trade and Food Safety". The CRP started on the 26th of November 2013 and finished on the 13th of September 2018. The purpose of the CRP was to assist Member States to develop the methodology to implement a sustainable system, utilising nuclear and related techniques, for the independent verification of the origin of food.

Food safety and quality are vital aspects of food security. The food supply is vulnerable to a range of food hazards (microbiological, chemical, physical) that may arise at any stage of the food supply chain. The dramatic increase in the volume of global trade and complexity of supply chains has caused a number of issues related to food authenticity and safety over recent decades. Many commodities, especially those that attract premium prices, may be subject to fraud such as adulteration or counterfeiting. Furthermore, this poses serious health risks due to the unknown identity of the adulterants used and the origin of counterfeits, which may be produced in unsanitary conditions or in premises that are unlicensed for food production.

In addition to the well-publicized food safety incidents such as aflatoxins in maize, dioxins in pork, melamine in dairy products, Salmonella in peanuts and fipronil in eggs, new hazards and risks are continually emerging. These may be related to unintentional contamination with, e.g., food additives or microbes, or intentional contamination (adulteration for economic gain or with the intent to harm consumers). Other issues may also pose threats to food safety which are not yet well understood or characterized, such as organised crime syndicates, the effects of climate change on food production, or emerging technological processes such as the use of nanoparticles in food.

Questions concerning origin are among the first to be asked when a food safety incident arises. In addition, consumers in key markets are increasingly concerned with the origin of their food and are willing to pay more if they can be assured of its provenance.

One of the primary tools for ensuring food safety and authenticity is a traceability system. Traceability systems are paper based or electronic systems that pass information along with the commodity. This provides the consumer with confidence that the products they are purchasing come from a supplier with the appropriate food safety and quality control measures. However, all such systems are subject to failure, either inadvertently or deliberately through acts of food fraud. The incidence of fraud is difficult to measure but estimates from the EU Framework 7 TRACE project suggested levels of 15 – 20% are likely in the European market.

It is widely recognized that there is a need for mechanisms to independently verify the origin of food and hence audit and/or support the traceability control systems. Nuclear techniques have been shown to be very effective in detecting the adulteration or counterfeiting of food products, and in discriminating foods from different geographical origins. These techniques have the potential to provide independent verification of information based traceability systems and provide additional information on the integrity of the food product itself.

Despite many studies that demonstrate its usefulness, there has been very limited uptake of this nuclear technology to date. The reasons for this include:

- The high cost of entry, as relatively large amounts of background information need to be collected using relatively expensive analytical techniques.

- The interpretation of the data and the level of certainty attainable have been hampered by the limited availability of reference data and the lack of standardized, robust, and accessible multi variate and spatial data analysis tools.

- Most of the stakeholders (regulators, producers) are unaware of the capabilities of this technology and so it has not gained widespread acceptance.

- The current bespoke nature of the technology requires a relatively high level of expertise for implementation.

Recent developments in instrumentation (e.g., laser based analysers for light isotopes and trace metals) reduce analytical costs considerably and may facilitate accessible systems and uptake. However, while a multi isotope/element approach with sufficient data of high quality can provide high levels of certainty in assigning origin, the more limited data obtained from the new technologies needs to be assessed and verified to determine the fitness for purpose of these approaches.

This CRP aimed to bridge gaps by developing and demonstrating an end to end system for verification of origin using nuclear and complimentary techniques. Ultimately the users of this technology will be food producers, regulators, and policy makers within the Member States. The methodology, therefore, needs to be implementable as a whole system that is robust in the hands of people with a wide range of expertise, does not impose undue costs, and will be accepted by regulators and consumers in the markets.

The results of the project described in this publication will assist producers to better communicate the qualities and origin related attributes or *terroir* of different food commodities, which can strengthen sustainable food systems through labelling claims such as Geographical Indications, e.g., Jamaican Blue Mountain coffee, Indian Darjeeling tea and Moroccan Taliouine saffron. In addition, verifying the integrity of food can help to prevent fraud in the form of false descriptions, counterfeits, substitutions, and adulteration and thereby reduce barriers to trade. The outcome will be enhanced food safety and facilitation of global trade. Others to benefit from the outcomes of this project will be the analytical service and science providers in the participating countries who will receive training and experience in the development and application of the methods. They will also benefit widely from the networking opportunities and interactions with international researchers.

1.1. Conclusions reached while closing the CRP D52038

- It was recorded at the final meeting that the CRP D52038 had successfully demonstrated the feasibility of using stable isotope and trace element (SITE) analysis, combined with other nuclear and complementary techniques, to establish the geographical origin of liquid milk and/or powdered milk produced in the participating Member States.

- Each of the contract holders had demonstrated acquired competence in nuclear and related techniques and so there has been a measurable increase in capacity for this technology in the participating Member States.

- The project also raised the awareness in Member States of SITE analysis and its wider applications to food traceability (production methods and geographical origin) and

authenticity and its potential to reduce barriers to trade and enhance consumer confidence in the provenance of food products, especially where a regional or identity or geographical indication is claimed.

- The project has enhanced the Member State capabilities in SITE analysis and has generated several new methods, standard operating procedures, and training opportunities. This has also resulted in a significant number of dairy authenticity and traceability datasets for the first time.

- Over the 5 years of the project the 10 research contract holder Member States generated 17 scientific publications in trade, national and international journals; gave 21 oral communications related to the project; presented 23 poster communications; supported 8 PhD, 8 Masters and 4 undergraduate students; trained 28 personnel; formed 10 new links to respective national dairy industries and 14 new academic links; generated 19 in house method protocols; and hosted 3 related workshops.

- The CRP has been successful in helping participants secure further investment by their respective Member States in SITE capabilities and helped leverage and secure new national and European Commission funding.

- One of the major collaborative achievements from the project was the joint publication of the development of a method for the rapid elemental analysis of milk powders using laser ablation inductively coupled plasma mass spectrometry (LA ICP MS) and its potential use in geographic sourcing (Hoffmann et al., (2018), Talanta, 186, 670 677).

- Some of the technology developed in the CRP has already been disseminated to laboratories in other Member States through expert missions, fellowships, and scientific visits under IAEA's Technical Cooperation program and as SOPs/protocols.

- The CRP also identified new pertinent areas of research such as claims related to geographical indications, production methods and ethical compliance, that could be addressed through future projects.

- There were a number of success stories from the CRP such as Slovenia developing a "made in Slovenia" milk mark (label) verifiable through the database developed in this CRP.

1.2. Stakeholder Workshop

The last day of the fourth and final research coordination meeting concluded with a Stakeholder Dissemination Workshop at the Jožef Stefan Institute, Ljubljana, Slovenia organised by Professor Nives Ogrinc, and her team, to present the outputs from CRP D52038. The stakeholder event was attended by Slovenian government officials, consumer groups, representatives of the dairy industry, retailers, and researchers. The meeting was used to highlight the real world applicability of the research conducted in CRP D52038. Examples were presented from New Zealand, Slovenia, Singapore, and the U.S.A. of nuclear techniques such as stable isotope analysis being applied in industry control systems and government surveillance and enforcement exercises.

2. OBJECTIVE

The information reported in this publication brings together the results of the work completed under the CRP D52038. The information is useful for research on, and technology transfer to facilitate, determination of the origin of milk and dairy products to support, and if required, independently verify food traceability systems. This publication presents a source of method protocols that can be directly applied or adapted to other food commodities.

3. SCOPE

This publication consists of research papers utilising stable isotope and trace element (SITE) analysis, in combination with multivariate statistics, to characterise the origin of authentic milk and dairy products sampled by the participating institutes. The SI and TE data have been produced primarily by isotope ratio mass spectrometry (IRMS) and inductively coupled plasma mass spectrometry (ICP MS), respectively. Other analytical techniques used and reported here include; stable isotopes in water from milk measured by cavity ring down spectroscopy (CRDS); trace elements measured by energy dispersive x-ray fluorescence (EDXRF) spectroscopy; strontium isotope ratios ($^{87}Sr/^{86}Sr$) measured multi collector ICP MS; milk fatty acid profiles measured gas chromatography – mass spectrometry (GC-MS); and metabolite profiles measured by nuclear magnetic resonance (NMR) spectroscopy.

4. STRUCTURE

The report presents the studies by each Member State participating in the CRP to characterise their own national milk production into regional zones using SITE profiling in combination with multivariate statistics. There are three exceptions; 1) the publication from Lithuania which reports the variation in the stable isotope composition of milk produced from a single cow to better understand the intra and inter annual variation in a single animal at a single geographic location; 2) the publication from the island state of Singapore, which relies heavily on imported milk and consequently their milk samples are from major importing countries; and 3) a second publication from Slovenia which describes the measurements of an interlaboratory comparison material (ICM) by participating institutes in this CRP.

5. REVIEW OF THE RESEARCH PAPERS PRESENTED IN THIS PUBLICATION

This subsection presents a brief summary of the papers reported in this publication and the conclusions reached at the final research coordination meeting (RCM). It also provides a brief summary of the stakeholder workshop held on the last day of the fourth and final RCM and a summary of the scientific publications in international peer reviewed journals that resulted directly from this CRP.

Paper 1 (Argentina): The aim of this work was to verify the usefulness of SITE 'fingerprints' to differentiate the origin of milk samples from different areas of Argentina, linking the 'milk fingerprint' with those corresponding to soil, water, and forage. Samples from four production areas were analysed and milk provenance was assessed using 16 selected variables (Na, Mg, Al, V, Co, Ni, As, Se, Rb, Sr, Mo, Hg, δ^2H, $\delta^{18}O$, $\delta^{13}C$ and K/Rb). Generalized Procrustes analysis demonstrated the consensus between soil, water, forage, and milk, in addition to significant differences between the studied production areas.

Paper 2 (Bangladesh): The project work aimed to establish a method for distinguishing the geographical origin of raw milk from seven climatic zones of Bangladesh and also the microbiological safety of the milk sampled. A stepwise canonical discriminant analysis of multi

element data demonstrated that Ca, Mg, Na, Zn, Cu, Cr, and Ni concentrations were effective in distinguishing samples from South Central, South and North Eastern Bangladesh. Whilst Pd, Cd, Ni, Zn and Cu can be applied to distinguish fresh pasteurised milk from ultra-high temperature (UHT) processed milk.

Paper 3 (China): Some dishonest manufactures adulterate UHT and pasteurized milk with reconstituted powdered milk for economic gain. In this study, a Partial Least Squares Discriminant Analysis (PLSDA) model, using ^{1}H NMR and ^{13}C NMR spectral data, was developed to distinguish between reconstituted milk and UHT milk. Milk metabolites of interest were identified by 1D and 2D NMR analysis as L carnitine, succinate, and acetate. The results showed that, combined with chemometrics, NMR can be used to successfully detect reconstituted powdered milk in UHT milk.

Paper 4 (Lithuania): This paper describes experiments to better understand the seasonal and inter annual variation in the carbon, nitrogen and oxygen stable isotopic composition of milk produced by a single cow at one geographical location. Measurements of stable isotope ratios in the cow's milk water, artesian water and precipitation were performed in addition to C, N, and O stable isotope measurements of the feed. The main water source for the cow was artesian water during the winter, while during the summer grass water influenced oxygen stable isotope values in the milk water. Stable oxygen isotope ratios in milk water were relatively lower in winter/transitional seasons and higher in summer showing the dependence on the main water source, whereas carbon and nitrogen stable isotope ratios reflected the main feed source.

Paper 5 (Morocco): Twelve agro-ecological milk production zones were selected based on their different climatic conditions (Gharb region, Middle atlas, and Meknes region, Loukouss, north, south (Agadir and Taroudant), Settat and Casablanca). The results demonstrated that stable isotope ratios of H, C, N and O of milk were linked to the territory, particularly the type of vegetation and the environment. From analysis of the O and H isotopes three groups were observed related to altitude and distance from sea. Multi element analysis was not found to improve distinction of the different geographic origin studied and this may be explained by the use of feed and/or compound feeds from other regions.

Paper 6 (Russia Federation): IRMS and ICP MS methods were developed and used to measure H, C, N and O stable isotope ratios and the concentration of macro, micro and trace elements in milk, respectively. The effectiveness of the applied methods to differentiate provenance was evaluated on 400 authentic milk samples collected from different locations in the Russian Federation. A quantitative assessment of the reliability of different classification models was conducted, including linear discriminant analysis, support vector machines, random forest, stochastic gradient busting, and artificial neural network. Repeated k fold cross validation was used to optimize non-linear models. The highest classification accuracy obtained was 96% on an independent test set of 100 samples. The overall precision for allnon-linear models was in the range of 93 to 96%.

Paper 7 (Slovenia 1): The results of this work include the first database of authentic Slovenian cow, sheep and goat milk and cheese, and includes the stable isotopic composition of oxygen in milk; the isotopic composition of hydrogen, carbon, nitrogen, oxygen, and sulphur in casein; the content of fatty acids, and elemental composition. The data also includes strontium isotope ratios in milk that can improve the verification of origin. The developed and validated methods were further used to verify the situation on the Slovenian retail market. In addition, the use of the oxygen isotope values of lactose as an internal standard was found to be a promising method to detect dilution of milk with water.

Paper 8 (Slovenia 2): This paper deals with the interlaboratory study of stable isotope ratios of carbon, nitrogen and sulphur in a rice flour inter comparison material (ICM) to demonstrate core measurement capabilities in the project. The characterisation was performed on rice flour reference material (RM) prepared by the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA). The development of new rice flour RM was selected in order to promote stable isotopes for the use as markers of origin/authenticity. The performance of nine participants was very good, illustrating their ability to obtain accurate results for carbon, nitrogen and sulphur isotope ratios within the calibration range afforded by internationally agreed reference materials. This was despite the fact that none of the participants used exactly the same approach in terms of instrumentation or data treatment.

Paper 9 (Sri Lanka): The present study establishes baseline stable isotope and trace element data for authentic milk sampled from different production zones in Sri Lanka (low country dry zone, low country wet zone, coconut triangle, mid country to upcountry). The stable isotope ratios of carbon and nitrogen and percentage of carbon and nitrogen were assessed for whole milk, milk fat, casein, and whey by IRMS. Trace elements in whole milk samples were analysed using ICP MS. Discriminant and PCA analysis of SITE data from the milk samples collected permitted the four regions to be clearly differentiated.

Paper 10 (Singapore): Singapore's heavy reliance on imports to meet the population's requirements means the island state is especially susceptible to food safety and food fraud related incidents. While carbon and nitrogen isotope signatures showed potential in differentiating the milk samples by geographical origin, it was the combination of both isotope signatures and elemental concentrations that enabled the geographical origin of the milk samples to be definitively differentiated. An iterative stepwise linear discriminant analysis procedure applied to the isotope ratios and element concentrations of 57 milk samples separated them into their six different countries of origin.

6. PUBLICATIONS RESULTING FROM RESEARCH CONDUCTED IN THIS CRP

This is a list of publications, in peer reviewed scientific journals and book chapters, that have resulted directly from research conducted within the coordinated research project.

Hoffman T., Jaćimović R., Bay L. J., Griboff J., Jagodic M., Monferrán M., Ogrinc N., Podkolzin I., Wunderlin D. & Almirall J. (2018) "Development of a method for the elemental analysis of milk powders using laser ablation inductively coupled plasma mass spectrometry (LA ICP MS) and its potential use in geographic sourcing". Talanta, 186, 670 677. https://www.sciencedirect.com/science/article/abs/pii/S0039914018303965

Griboff J., Baroni M.V., Horacek M., Wunderlin D.A. and Monferran M.V. (2019). "Multielemental + Isotopic Fingerprint Enables Linking Soil, Water, Forage and Milk Composition, Assessing the Geographical Origin of Argentinean Milk". Food Chemistry, 283 549 558. https://www.sciencedirect.com/science/article/pii/S0308814619301359

Huque R., Jolly Y. N., Choudhury T. R., Munshi M. K., Hussain M. S., Khatun A., Roy B. K., Islam M., Hossain M. A. and Hossain A. (2018) "Evaluation of elemental, microbial and biochemical status of raw and pasteurized cow's milk". International Food Research Journal, 25, 1693 1701. http://www.ifrj.upm.edu.my/25%20(04)%202018/(48).pdf

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Attributes of Cow's Milk in Respect to Regional Discrimination in Bangladesh". Archives of Current Research International, 12,1 12. http://www.journalacri.com/index.php/ACRI/article/view/17261

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Liang, K., Zhao, Y., Han, J., Liu, P., Qiu, J., Zhu, D., Qin, Y., Lu, L. and Wang, X. (2018) "Fatty acid composition, vitamin A content and oxidative stability of milk in China". Journal of applied animal research, 46, 566 571. https://www.tandfonline.com/doi/abs/10.1080/09712119.2017.1360186

Potocnik, D., Necemer, M., Mazej, D., Jacimovic, R. and Ogrinc, N. (2016) "Multi elemental composition of Slovenian milk: analytical approach and geographical origin determination". Acta Imeko, 5, 15 21. https://acta.imeko.org/index.php/acta imeko/article/view/IMEKO ACTA 05%20%282016%29 05 .

Nečemer, M., Potočnik, D. and Ogrinc, N. (2016) "Discrimination between Slovenian cow, goat and sheep milk and cheese according to geographical origin using a combination of elemental content and stable isotope data". Journal of food composition and analysis, 52, 16 23. https://www.sciencedirect.com/science/article/abs/pii/S0889157516301120

Eftimov, T., Korošec, P., Potočnik, D., Ogrinc, N., Heath, D. and Seljak, B.K. (2017) "How to perform properly statistical analysis on food data? An e learning tool: Advanced Statistics in Natural Sciences and Technologies". Book chapter Science within Food: Up to date Advances on Research and Educational Ideas. https://pdfs.semanticscholar.org/4fe4/eb829f5408dc90eaff0eeb6b0b4421034464.pdf

PAPERS PRESENTED

EVALUATING THE ORIGIN OF ARGENTINEAN MILK BY MULTI ELEMENT AND ISOTOPIC ANALYSIS

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Abstract

The aim of this work was to link the multi element and isotopic characteristics of soil, water and milk from the main production areas in Argentina. Authentic milk samples collected from four production areas were evaluated by measuring the concentration of 26 elements and the hydrogen (δ^2H), carbon (δ^{13}C), nitrogen (δ^{15}N) and oxygen (δ^{18}O) stable isotope composition. The geographic region of origin of the Argentinean milk samples could be discriminated by chemometric analysis of 16 selected variables; the concentration of Na, Mg, Al, V, Co, Ni, As, Se, Rb, Sr, Mo, Hg, the ratio K/Rb and the stable isotope values δ^2H, δ^{18}O, δ^{13}C and δ^{15}N). generalized procrustes analysis (GPA) showed good consensus between soil, water, and milk. Furthermore, GPA illustrated differences between studied areas. In addition, canonical correlation analysis (CCA) confirmed significant correlations between the measured parameters for milk and drinking water and between milk and soil. Currently, this approach looks promising to verify the regional geographical origin of Argentinean milk, establishing the link between different elements/ stable isotopes from soil and water into milk, which should prove useful in predicting the origin of milk produced in diverse regions from soil and water data.

1. INTRODUCTION

Milk can be consumed directly, transformed into several dairy products such as cream, butter, cheese, etc., or incorporated in the production of a variety of foods, such as cakes, biscuits, cookies, ice cream, chocolates, etc. In the last few years, with the development of a globalised food market and the increased interest of consumers to know the characteristics, quality and geographical origin of foods, many countries have adopted/introduced regulations to guarantee the traceability of food. The Protected Denomination of Origin (PDO) and Controlled Denomination of Origin (DOC) systems have been promoted, and applied to control and, ultimately, ensure the origin and quality of food as well as to prevent fraud [1]. One of the more recent requirements to achieve PDO or DOC certifications is to obtain a chemical characterisation of the food that links it to the *terroir* of the production area, which may be used as a means to both protect and promote the food product.

The isotopic and inorganic chemical composition of a food reflects the local geochemistry of both soil and water, which is influenced by geology, temperature, climate, distance from the sea, elevation, latitude, homeostasis and technological processing [2, 3, 4]. Thus, the inorganic composition of foods can be relatively more stable in time, compared to the organic constituents, which are subject to a more rapid turnover by microbial action, biochemical reactions, oxidation reduction and hydrolysis. Trace element content and isotopic ratios of plants that can be used as forage are assumed to be related to the chemistry of the local water and soil layers in which they grow, although anthropogenic inputs and agricultural practices can have some influence. In so far as the soil layer reflects the underlying geology, the isotopic and elemental composition of a crop should be correlated with the geological characteristics of

the production areas [5]. Consequently, the measurement of elemental composition is considered to be an effective hypothesis driven tool to link a food to its place of origin. In addition to the essential elements that plants need for growing (B, N, Mg, P, S, Cl, K, Ca, Mn, Fe, Ni, Cu, Zn, and Mo) [6], plants can assimilate other bio available elements from soil and water. Thus, non-essential elements, such as alkaline metals, especially rubidium (Rb) and caesium (Cs), can be easily mobilized in the soil and transported into plants, becoming good indicators of geographical identity [5]. However, the availability of soil elements depends on several factors, such as soil pH, humidity, porosity, clay and presence of humic complexes, etc. Consequently, the range of soils present, in addition to different agricultural practices (organic, low input, traditional, etc.), and the bioavailability of soil constituents may supply unique fingerprints in the final food product that characterize its geographical identity [5].

Argentina's main dairy production areas are in the so called 'Pampa Húmeda', which are wet plain lands, particularly in the Provinces of Córdoba and Santa Fe. Almost 70% of the dairy farms in Argentina are located in this area.

Authentication studies have shown that one single method, or one single parameter, is generally insufficient to indicate a food's origin, and to support traceability systems [7]. Thus, our current knowledge indicates that it is necessary to evaluate at least two different groups of parameters (e.g., trace elements and isotopes), combining them with multivariate statistics, to build models that permit differentiation of food from various geographic origins [3, 8, 9].

In this project we investigated the use of multivariate statistics to enable classification rules that help verify different geographic origins (spatial differences). The sum of all these results provide the scientific basis to underpin PDO/DOC regulations and applications for PDO/DOC status for foods produced in Member States.

2. MATERIALS AND METHODS

2.1. STUDY AREAS

2.1.1. Province of Córdoba: San Justo department Balnearia (farm 1); Freire (farm 7); and Rio Cuarto department Vicuña Mackena (farm 8). This area represents 86% of dairy farms in the province of Córdoba and 87% of total milk production in the province; the region under study involves 30% of national milk production and 28% of dairy farms across Argentina.

2.1.2. Province of Santa Fe: San Cristóbal department Suardi (farms 3 and 4). This zone corresponds to the 'Cuenca Central Lechera Santafesina'. In this area 87% of dairy farms are in the province of Santa Fe, accounting for 89.6% of total milk production in the province. The region under study involves 37% of national milk production and 36% of dairy farms across Argentina.

2.1.3. Province of Catamarca: Santa Rosa department (farm 5). This area was selected as 'reference site' due to its very low amount of dairy farming.

2.2. SAMPLE COLLECTION AND PREPARATION

From each of the 5 farms selected, samples of animal drinking water, soil and liquid milk were taken during October 2016 and October 2018. The sampling protocol was similar to that previously used by our group [3,4, 8, 9].

2.2.1. *Animal drinking water*

At least three independent samples (1.5 L each, n= 15) were collected at each farm from the cattle drinking reservoirs, transferred into pre cleaned (acid washed) plastic bottles, acidified with ultrapure nitric acid (HNO_3), and stored at 4°C until analysis. Prior to measurements, samples were filtered using 0.45 µm nitrocellulose filters (Sartorius, Göttingen, Germany). For the isotope analysis the water dry residue was produced by evaporating the water at low temperature <50 °C. The residue was decarbonated by addition of 5% HCl and then dried again. The decarbonated water dry residue was introduced into tin capsules, weighed and analysed by bulk carbon and nitrogen stable isotope analysis through an elemental analyser connected to a stable isotope ratio mass spectrometer (EA IRMS).

2.2.2. Soil

At least three independent samples (n=15) were collected at each farm using a plastic shovel and transferred into clean 1 L plastic containers. Subsequently, sediments were dried at 40°C, sieved to 63 µm using acrylic meshes. The analyses of labile or bioavailable elements in sediments was performed on 1 g of the < 63 µm dried material, which was processed by acid leaching, using ultra-pure hydrochloric acid (HCl, 0.5 N) [10]. For isotope analysis the soil samples were decarbonated by addition of 5 % HCl and dried again, prior to weighing them into tin capsules for bulk carbon and nitrogen stable isotope analysis by EA IRMS [11].

2.2.3. Liquid milk

Three independent samples (1.5 L each, n= 15) were taken at each farm. Sampling was performed at the input to the cooling tank where milk provided by the entire group of cows from a particular farm was mixed. These samples were transported frozen to the laboratory and stored at −80 °C until freeze drying. For multi elemental analysis, a duplicate sub sample of 0.25 g of freeze dried milk was introduced into a Perfluoroalkoxy alkane (PFA) digestion vessel (Savillex) and mixed with 7 mL of ultrapure nitric acid and 1 mL ultrapure hydrogen peroxide (Merck Química, Buenos Aires, Argentina). Acidified samples were left to predigest for 30 minutes at room temperature, and then placed on hot plate for 24 h at 240 °C. Hereinafter, samples were cooled to room temperature, filtered through 0.45 µm diameter filter (Sartorius), and stored at 4 °C until analysis by inductively coupled plasma–mass spectrometry [11].

2.3. SAMPLE ANALYSIS

2.3.1. Multi element analysis

The concentrations of the following macro, micro and trace elements Li, Be, Na, Mg, Al, K, Ca, V, Cr, Fe, Mn, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Mo, Ag, Cd, Ba, Hg, Tl y Pb were measured by ICP MS (Agilent Technology 7500 cx Series), equipped with an ASX 100 autosampler (CETAC Technologies, Omaha, NE). Quality assurance (QA) and quality control (QC) of multi elemental analyses were performed using certified reference materials (CRMs): IAEA 153 (milk powder bottle 187), IAEA 155 (Whey powder bottle 370) and NIST 1643e (water). Recoveries from CRMs were 101 ± 9%, 100 ± 11%, and 101 ± 3%, respectively. [11].

2.3.2. Stable Isotope analysis

The multi isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) were measured using a Delta V Isotope Ratio Mass Spectrometer, connected via a ConFlo IV interface with an elemental analyser (EA) (both Thermo Fisher Scientific). $\delta^{2}H$ and $\delta^{18}O$ measurements were carried out with the same instrumentation but with the EA running in high temperature conversion mode. Before measurement, the dried and homogenized milk samples were defatted with petroleum ether using a Soxhlet apparatus. Between 0.95 and 1 mg of the remaining defatted dry mass (DDM) was homogenized and weighed into tin capsules for $\delta^{13}C$ and $\delta^{15}N$ analysis. 0.2 mg of the DDM was weighed into silver capsules for $\delta^{2}H$ and $\delta^{18}O$ analysis. Stable isotope values (δ) were expressed in parts per thousand (‰), relative to Vienna Pee Dee Belemnite (VPDB) and atmospheric N2 (AIR) for $\delta^{13}C$ and $\delta^{15}N$; and Vienna Standard Mean Ocean Water) for $\delta^{2}H$ and $\delta^{18}O$. Replicate measurements of internal laboratory standards (milk powder and casein) show that the measurement errors for both carbon and nitrogen isotope analyses were better than ± 0.2‰, better than ± 0.3‰ for O and better than±3‰ for H isotope measurements [11].

2.4. STATISTICAL ANALYSIS

Multivariate statistical analysis (MVA) methods were applied to data sets obtained from the measurement of the different matrixes (water, soil and milk). These included linear discriminant analysis (LDA), generalized procrustes analysis (GPA), and canonical correlation analysis (CCA). Concentrations of elements were used as chemical descriptors for milk, water, and soil samples, and LDA in stepwise mode was performed. LDA was carried out to determine if milk, soil, and water samples could be statistically differentiated with respect to their geographical origins. The most significant variables were selected by stepwise analysis according to their F values. 'Leave one out' cross validation was used to assess the reliability of the classification models. GPA and CCA were used to assess the relationship between milk and geological data (soil and water). GPA works by constructing a consensus configuration by applying transformations on groups of related data sets in order to superimpose them. The Gower algorithm was used to minimize the within sample variance by applying translation, scaling, and rotation to produce a p dimensional configuration average Yc. Subsequently, a q dimensional average group space is constructed from Yc using principal component analysis (PCA). Consequently, GPA algorithms and theory may be applied to match milk stable isotope and multi element data with the corresponding soil and cattle drinking water data. On the other hand, CCA allowed the evaluation of the relationship between soil, water and milk studied during this work. Analysis of variance (ANOVA) was performed with each variable and, in the case of significance ($P < 0.05$); a multiple comparison test was performed to reveal paired differences between the means.

3. RESULTS AND DISCUSSION

Results from both multi elemental and isotope analyses are summarized in Table 1 below. The results of water, soil and milk analyses as well as the correlation between these three matrixes, according to the region of origin of each of the farms studied are presented in Figure 1. The concentrations of the inorganic elements in water, soil and milk were analysed using discriminant analysis (backward stepwise mode), achieving 100% correct classification on the three types of samples, according to the sampling region (Northeast of Córdoba (CBA NE) southern Córdoba (CBA South), Catamarca and Santa Fe). Six variables were necessary to classify water samples according to their provenance: As, B, Mn, Al, Mg and Se. Twelve variables were necessary to classify soil samples: Li, Be, Mg, V, Cr, Mn, Ni, Rb, Sr, Ag, Cd and Ba. Finally, ten variables were necessary to classify milk samples: Li, Pb, Cu, Fe, Mg, Ca, Na, B, Al and Ni. Figure 1 shows the distribution of the samples according to their geographical

origin, while box plots show the relative concentration for some selected elements. Considering water samples, Ba allows differentiation of the four studied regions, while Cu allows differentiation of soil samples and Sr permitting differentiation of milk samples from three studied areas.

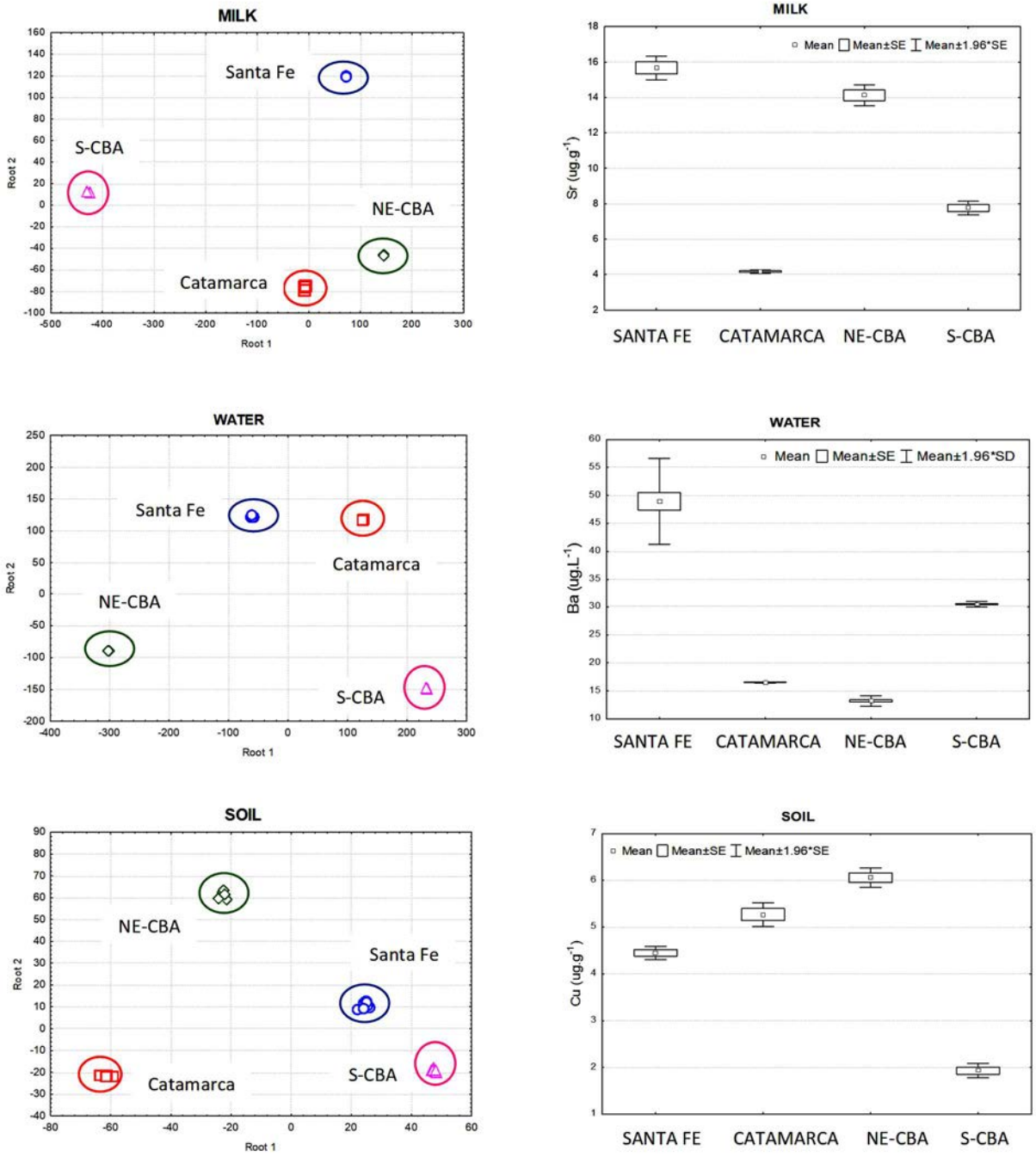

FIG. 1. Distribution of water, soil and milk samples according to the first two functions of the linear discriminant analysis (backward stepwise). Box Plot representing selected elements that permit a high degree of differentiation between the production areas. Abbreviations: Sr = strontium, Ba = barium, Cu = copper, SE = standard error, SD = standard deviation.

TABLE 1. MEANS AND STANDARD DEVIATIONS OF MEASURED ELEMENTS AND ISOTOPE RATIOS CORRESPONDING TO WATER, SOIL AND MILK ACCORDING TO REGION OF ORIGIN

	water				soil				milk			
variable	CBA-NE	Santa Fe	CATAMARCA	CBA-S	CBA-NE	Santa Fe	CATAMARCA	CBA-S	CBA-NE	Santa Fe	CATAMARCA	CBA-S
Ag	<LOD	<LOD	<LOD	<LOD	0.07 ± 0.01 ^{a}	0.09 ± 0.02 ^{a}	0.10 ± 0.01 ^{a}	0.07 ± 0.01 ^{b}	<LOD	<LOD	<LOD	<LOD
Al	11 ± 8 ^{a}	44 ± 80 ^{a}	21 ± 19 ^{a}	24 ± 16 ^{a}	1906 ± 315 ^{a}	1697 ± 407 ^{b}	1754 ± 101 ^{b}	1393 ± 42 ^{b}	8 ± 4 ^{b}	15 ± 7 ^{c}	3.9 ± 1.6 ^{a}	15 ± 10 ^{c}
As	52 ± 28 ^{b}	95 ± 27 ^{c}	25 ± 12 ^{a}	272 ± 69 ^{d}	0.42 ± 0.07 ^{b}	0.44 ± 0.14 ^{b}	0.51 ± 0.03 ^{c}	0.31 ± 0.04 ^{a}	<LOD	<LOD	<LOD	0.045 ± 0.004 ^{a}
B	1116 ± 292 ^{b}	3141 ± 390 ^{c}	154 ± 51 ^{a}	12629 ± 660 ^{d}	2.9 ± 1.5 ^{a}	2.8 ± 0.8 ^{a}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Ba	13 ± 4 ^{a}	49 ± 16 ^{c}	36 ± 13 ^{b}	16 ± 9 ^{a}	55 ± 7 ^{b}	55 ± 8 ^{b}	59 ± 1 ^{c}	35 ± 6 ^{a}	1.2 ± 0.2 ^{a}	1.8 ± 0.6 ^{b}	1.4 ± 0.2 ^{a}	1.9 ± 0.3 ^{b}
Be	<LOD	<LOD	<LOD	<LOD	0.29 ± 0.04 ^{b}	0.33 ± 0.05 ^{c}	0.41 ± 0.02 ^{d}	0.17 ± 0.03 ^{a}	<LOD	<LOD	<LOD	<LOD
*Ca	28 ± 8 ^{a}	81 ± 23 ^{b}	25 ± 6 ^{a}	80 ± 13 ^{b}	2.5 ± 0.3 ^{c}	2.4 ± 0.5 ^{c}	2.2 ± 0.1 ^{b}	1.1 ± 0.1 ^{a}	7.9 ± 1.4 ^{a}	8.9 ± 1.9 ^{b}	10.3 ± 0.2 ^{c}	7.7 ± 2.2 ^{a}
Cd	0.06 ± 0.01 ^{a}	0.08 ± 0.04 ^{a}	<LOD	1.06 ± 0.08 ^{b}	0.1 ± 0.03 ^{b}	0.14 ± 0.03 ^{c}	0.16 ± 0.01 ^{d}	0.045 ± 0.008 ^{a}	<LOD	<LOD	<LOD	<LOD
Co	<LOD	0.71 ± 0.34 ^{b}	<LOD	0.11 ± 0.01 ^{a}	2.7 ± 0.4 ^{b}	3.4 ± 0.4 ^{c}	4.7 ± 0.2 ^{d}	1.2 ± 0.2 ^{a}	0.006 ± 0.001 ^{a}	0.008 ± 0.001 ^{b}	0.006 ± 0.001 ^{a}	0.009 ± 0.003 ^{b}
Cr	0.04 ± 0.01 ^{a}	5.82 ± 0.47 ^{b}	0.33 ± 0.09 ^{a}	31 ± 7 ^{c}	0.47 ± 0.11 ^{b}	0.53 ± 0.18 ^{b}	0.33 ± 0.03 ^{a}	0.30 ± 0.04 ^{a}	0.10 ± 0.04 ^{a}	0.09 ± 0.04 ^{a}	0.11 ± 0.06 ^{a}	0.09 ± 0.04 ^{a}
Cu	0.8 ± 0.6 ^{a}	3.7 ± 0.5 ^{c}	1.9 ± 2.1 ^{b}	3.8 ± 1.9 ^{c}	4.1 ± 0.9 ^{b}	4.8 ± 1.2 ^{c}	5.1 ± 0.5 ^{c}	1.7 ± 0.4 ^{a}	0.48 ± 0.08 ^{c}	0.37 ± 0.17 ^{b}	0.28 ± 0.05 ^{a}	0.34 ± 0.08 ^{b}
Fe	78 ± 90 ^{b}	30 ± 35 ^{a}	31 ± 3 ^{a}	24 ± 24 ^{a}	862 ± 124 ^{a}	1119 ± 445 ^{b}	1083 ± 28 ^{b}	872 ± 0.003 ^{a}	5.1 ± 1.2 ^{a}	5.6 ± 2.1 ^{a}	8.6 ± 1.1 ^{c}	7.0 ± 1.2 ^{b}
Hg	<LOD	<LOD	<LOD	<LOD	0.21 ± 0.09 ^{b}	0.12 ± 0.02 ^{a}	0.48 ± 0.06 ^{c}	0.13 ± 0.01 ^{a}	0.75 ± 0.09 ^{a}	<LOD	<LOD	0.88 ± 0.14 ^{a}
*K	10 ± 3 ^{a}	45 ± 10 ^{b}	6 ± 1 ^{a}	70 ± 4 ^{c}	1.4 ± 0.3 ^{c}	1.1 ± 0.2 ^{b}	1.1 ± 0.1 ^{b}	0.8 ± 0.1 ^{a}	11.2 ± 2.3 ^{a}	11.7 ± 2.2 ^{a}	14.2 ± 2.5 ^{b}	10.2 ± 3.5 ^{a}
Li	75 ± 16 ^{b}	224 ± 65 ^{d}	26 ± 9 ^{a}	170 ± 50 ^{c}	0.99 ± 0.36 ^{c}	0.75 ± 0.34 ^{b}	0.71 ± 0.32 ^{b}	0.45 ± 0.07 ^{a}	0.13 ± 0.03 ^{a}	0.84 ± 0.41 ^{b}	0.29 ± 0.01 ^{a}	0.61 ± 0.46 ^{b}
*Mg	10 ± 3 ^{a}	42 ± 10 ^{b}	8 ± 3 ^{a}	111 ± 25 ^{c}	0.64 ± 0.11 ^{c}	0.51 ± 0.14 ^{b}	0.37 ± 0.06 ^{a}	0.32 ± 0.04 ^{a}	0.79 ± 0.11 ^{a}	0.89 ± 0.17 ^{b}	1.04 ± 0.04 ^{c}	0.79 ± 0.23 ^{a}
Mn	53 ± 34 ^{b}	35 ± 47 ^{a}	13 ± 1 ^{a}	23 ± 26 ^{a}	275 ± 54 ^{b}	310 ± 31 ^{c}	473 ± 15 ^{d}	121 ± 11 ^{a}	0.22 ± 0.02 ^{a}	0.39 ± 0.09 ^{c}	0.23 ± 0.05 ^{a}	0.28 ± 0.08 ^{b}
Mo	29 ± 14 ^{a}	50 ± 25 ^{a}	19 ± 1 ^{a}	734 ± 198 ^{b}	<LOD	<LOD	<LOD	<LOD	0.8 ± 0.3 ^{b}	0.4 ± 0.1 ^{a}	<LOD	0.5 ± 0.2 ^{a}
*Na	444 ± 199 ^{b}	1237 ± 87 ^{c}	44 ± 18 ^{a}	3427 ± 238 ^{d}	0.11 ± 0.05 ^{c}	0.07 ± 0.05 ^{b}	0.03 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	2.5 ± 0.7 ^{a}	5.0 ± 1.3 ^{c}	4.3 ± 0.1 ^{b}	3.2 ± 0.9 ^{a}
Ni	0.31 ± 0.12 ^{a}	1.1 ± 0.6 ^{b}	0.9 ± 0.1 ^{b}	0.90 ± 0.13 ^{b}	2.1 ± 0.3 ^{b}	2.4 ± 0.6 ^{c}	2.63 ± 0.08 ^{c}	0.5 ± 0.1 ^{a}	0.08 ± 0.02 ^{a}	0.10 ± 0.04 ^{b}	0.07 ± 0.03 ^{a}	0.08 ± 0.02 ^{a}
Pb	0.23 ± 0,01 ^{a}	0.48 ± 0.29 ^{b}	0.51 ± 0.59 ^{b}	0.07 ± 0.02 ^{a}	5.4 ± 0.5 ^{b}	6.1 ± 0.5 ^{c}	7.7 ± 0.9 ^{d}	2.8 ± 0.2 ^{a}	0.042 ± 0.008 ^{a}	0.047 ± 0.004 ^{a}	0.055 ± 0.013 ^{a}	0.055 ± 0.018 ^{a}
Rb	2.4 ± 0.6 ^{b}	11.5 ± 2.2 ^{c}	1.5 ± 0.3 ^{a}	20.1 ± 0.7 ^{d}	1.1 ± 0.2 ^{b}	0.91 ± 0.09 ^{a}	0.85 ± 0.24 ^{a}	0.93 ± 903 ^{a}	3.3 ± 0.6 ^{a}	4.3 ± 0.9 ^{b}	6.9 ± 0.9 ^{d}	5.2 ± 1.9 ^{c}
Se	<LOD	4 ± 1 ^{a}	<LOD	41 ± 3 ^{b}	0.5 ± 0.1 ^{b}	0.49 ± 0.21 ^{b}	0.69 ± 0.07 ^{c}	0.24 ± 0.17 ^{a}	<LOD	<LOD	0.41 ± 0.03 ^{a}	<LOD
Sr	637 ± 241 ^{b}	2289 ± 559 ^{c}	277 ± 114 ^{a}	4086 ± 924 ^{d}	40 ± 7 ^{b}	40 ± 5 ^{b}	36 ± 11 ^{b}	12 ± 1 ^{a}	11.6 ± 2.2 ^{a}	16.3 ± 2.9 ^{b}	11.6 ± 3.3 ^{a}	12.2 ± 0.9 ^{a}
V	1.3 ± 0.9 ^{a}	159 ± 21 ^{c}	42 ± 19 ^{b}	501 ± 111 ^{d}	3.1 ± 0.4 ^{a}	4.2 ± 1.1 ^{b}	4.1 ± 0.2 ^{b}	2.8 ± 0.3 ^{a}	0.02 ± 0,01 ^{a}	0.07 ± 0.03 ^{b}	0.04 ± 0.01 ^{a}	0.14 ± 0.10 ^{c}
Zn	7 ± 5 ^{a}	10 ± 5 ^{a}	10 ± 7 ^{a}	28 ± 19 ^{b}	8.1 ± 2.1 ^{b}	8.3 ± 4.9 ^{b}	5.9 ± 0.6 ^{a}	5.8 ± 1.5 ^{a}	24 ± 4 ^{a}	24 ± 3 ^{a}	28 ± 2 ^{b}	23 ± 5 ^{a}
Ca/Sr	46 ± 4 ^{b}	35 ± 3 ^{b}	143 ± 63 ^{c}	20 ± 2 ^{a}	64 ± 6 ^{a}	61 ± 12 ^{a}	91 ± 52 ^{b}	99 ± 6 ^{b}	694 ± 87 ^{b}	544 ± 56 ^{a}	1018 ± 400 ^{c}	626 ± 135 ^{b}
K/Rb	3983 ± 471 ^{b}	3886 ± 170 ^{b}	3688 ± 558 ^{a}	3520 ± 291 ^{a}	1285 ± 324 ^{c}	1141 ± 186 ^{b}	1000 ± 324 ^{a}	870 ± 113 ^{a}	3530 ± 973 ^{d}	2770 ± 189 ^{c}	2424 ± 561 ^{b}	2002 ± 55 ^{a}
δ ^{2}H	ND	ND	ND	ND	ND	ND	ND	ND	-52.3 ± 3.3 ^{b}	-46.3 ± 4.1 ^{b}	-48.4 ± 1.1 ^{b}	-67.4 ± 33.0 ^{a}
δ ^{13}C	-8.6 ± 1.5 ^{b}	-7.2 ± 1.6 ^{c}	-12.6 ± 1.9 ^{a}	-3.2 ± 1.3 ^{d}	-19 ± 1 ^{c}	-19 ± 1 ^{c}	-21.9 ± 1.4 ^{a}	-20 ± 1 ^{b}	-16.8 ± 1.1 ^{c}	-18.7 ± 0.5 ^{a}	-18.9 ± 1.6 ^{a}	-18.2 ± 1.2 ^{b}
δ ^{15}N	4.55 ± 0.22 ^{a}	17.6 ± 1.1 ^{c}	4.9 ± 0.1 ^{a}	8.2 ± 0.3 ^{b}	ND	ND	ND	ND	4.9 ± 0.3 ^{b}	5.7 ± 0.5 ^{c}	5.0 ± 0.7 ^{b}	4.2 ± 0.3 ^{a}
δ ^{18}O	ND	ND	ND	ND	ND	ND	ND	ND	20.8 ± 0.7 ^{b}	20.2 ± 0.7 ^{a}	20.1 ± 0.1 ^{a}	20.6 ± .1.0 ^{b}

[a] ND, not determined; <LOD, below limit of detection. Water element values are reported in μg.L $^{-1}$ except for Na, K, Ca, and Mg, which are in mg. L $^{-1}$. Soil and milk element values are reported in μg.g $^{-1}$ dry weight except for Na, K, Ca, and Mg, which are in mg.g $^{-1}$ dry weight. Isotopes ratios are expressed in δ units (‰, per mil). LODs: (water): Ag 0.13 μg.L $^{-1}$; Be 0.03 μg.L $^{-1}$; Cd 0.013 μg.L $^{-1}$; Co 0.013 μg.L $^{-1}$; Hg 0.4 μg.L $^{-1}$; Se 0.4 μg.L $^{-1}$. (soil and milk): Ag 0.006 μg.g $^{-1}$; Cd 0.03 μg.g $^{-1}$; As 0.01 μg.g $^{-1}$. B 0.06 μg.g $^{-1}$; Be 0.03 μg.g $^{-1}$ Hg 0.015 μg.g $^{-1}$; Mo 0.06 μg.g $^{-1}$; Se 0.03 μg.g $^{-1}$. Different letters in the same row indicate significant differences $p < 0.05$.

Figure 2 shows a generalised Procrustes analysis (GPA), with the general configuration of the geographic regions according to the three studied matrixes (milk, soil and water). From Figure 2 it can be seen that there is a clear difference between samples from three studied areas and the reference area (96 % considering only two components), with a high consensus between three analysed matrixes (soil, water and milk). To evaluate correlations between studied matrixes, using a formal mathematical method, we applied Canonical Correlation Analysis (CCA). The first CCA was calculated between milk and water, using 20 variables, obtaining no significant correlation (r2 = 0.97, P <0.139). The second CCA was estimated between milk and soil, using 20 variables in common, which showed a significant correlation (r^2 = 0.98, P <0.001), which also showed a significant correlation (r2 = 0.97, P <0.001). Considering these results, we see that both GPA and CCA demonstrated the influence of the geographic region of origin in the composition of milk. Furthermore, Figure 3A shows canonical variables establishing the correlation between milk and soil. Additional correspondence between levels of Cr in milk and soil samples is shown in Figure 3B from different geographical areas.

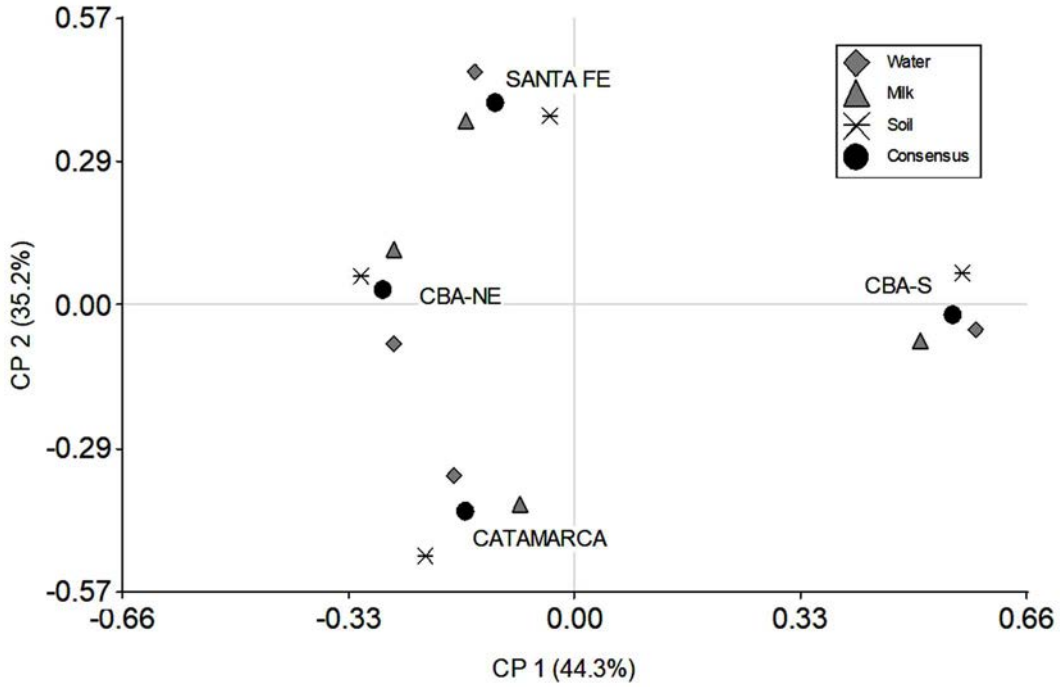

FIG. 2. Generalised Procrustes analysis (GPA) Consensus configuration among the multi element and isotopic profiles of milk, soil and water.

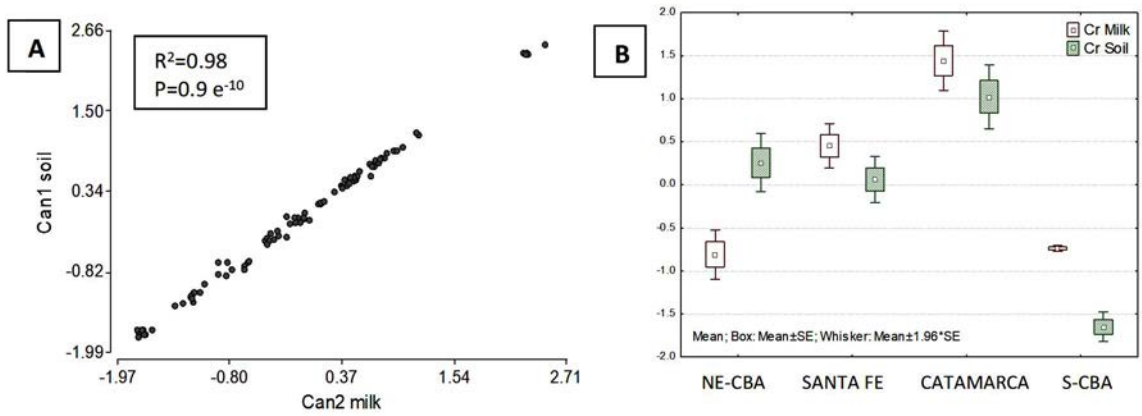

FIG. 3. Canonical variables showing the correlation between milk and soil (A). Correspondence between levels of Zn in milk and soil samples (B) from different geographical areas. Standardized values. Mean ± SE.

4. CONCLUSION

The use of selected elements ratios (K/Rb and Ca/Sr) was useful and complementary to multi elemental and isotopic data to differentiate soil, water and milk from the studied areas (SF; CBA NE; CBA S and CAT). Soil, water and milk were well differentiated by LDA. CCA showed a positive and significant correlation between forage composition and milk at the multivariate level. CCA also showed significant positive correlations between milk and soil, showing that milk composition is influenced by the soil characteristics of the production area. GPA shows an integrated overview on both links between studied matrixes (water soil milk) and differences between studied areas. Multi elemental and multi isotopic fingerprints, in addition to chemometrics, allowed a clear differentiation of milk produced in the four areas in Argentina, presenting an interesting methodological approach for future studies on food provenance in Argentina and other IAEA Member States.

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ASSESSMENT OF THE AUTHENTICITY AND SAFETY OF BANGLADESHI MILK USING NUCLEAR AND COMPLEMENTARY MICROBIOLOGICAL TECHNIQUES

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Abstract

Milk is a widely consumed commodity at risk of intentional adulteration affecting consumer confidence with respect to its nutritional value, safety, and provenance. The authenticity of milk has become an important issue because regulatory authorities, policy makers, processors, retailers, and consumers are interested in knowing the milk's origin and quality. In view of these concerns, the present study was undertaken on authentic fresh cow's milk from six geographic regions of Bangladesh to characterise its biochemical, trace element, and isotopic features to permit discrimination of origin using a multivariate statistical approach. In addition, because of the nature of raw milk and the challenging conditions under which it is produced and stored in Bangladesh, microbiological analysis was also performed to assess pathogenic safety issues of milk and milk products. Regarding trace element analysis, both nuclear, instrumental neutron activation analysis (INAA), and spectrophotometric, flame and graphite furnace atomic absorption spectroscopy (AAS) measurements were used to determine the concentration of Pb, Cd, Cr, Cu, Co, Ni, As, Mn, Zn, Fe, Ca, Mg, Na and K in fresh cows' milk from six climatic regions; South Central, South Western, Western, North Western, South Eastern and North Eastern Bangladesh. Tests were performed on International Atomic Energy Agency (IAEA) certified reference milk powder (IAEA 153) to validate the reliability of measured analytical data. Principal component analysis (PCA) classified the set of element data into three components where PC1 was mainly correlated with concentrations of Pb, Cd, Cr, Cu, Co, Mg and Cu is negatively correlated with other elements. Chemometric approaches, such as canonical discriminate analysis (CDA) identified five elements (Zn, Cr, Mg, Cu and Ni) as the important variables for discrimination between inter regional production areas of Bangladeshi (BGD) raw milk. The nitrogen and carbon stable isotope (δ^{15}N and δ^{13}C) study of defatted milk samples permitted partial separation among South Western, North Eastern and South Eastern regions of production origin. The stepwise CDA identified the nitrogen isotope data as the prevalent component contributing 90.3% of variance toward the distinction of milk origin and thus providing an indication of diversity of dietary materials of the individual cows as well as dairy farms. Furthermore, the values of δ^{13}C and δ^{15}N have been found to be independent of season of production and that there is a common isotopic pattern between milk of summer and winter season. Alongside, the study of stable isotope and trace element (SITE) data the biochemical constituents, protein, lactose, casein, fat, and non-fat solids (SNF) in raw milk were determined and showed provincial discrimination among South central, West, and North west region of Bangladesh. In addition, to address safety concerns the total microbial load in milk samples was measured and found to be higher in the milk of South Eastern region of Bangladesh compared to the other regions and therefore pasteurization, at least, of raw milk is recommended for preventing human health hazards. Besides raw milk, ready to eat cheeses collected from local markets were found to have a significant bacterial load especially, *L. monocytogenes.*

Pathogenicity of isolated *L. monocytogenes* using cytotoxicity assay on Vero cell lines revealed the severe health risk associated with the cheese consumption in Bangladesh.

1. INTRODUCTION

Food authentication is an emerging issue that may be defined as the correspondence between the (bio)chemical characteristics of a food and its label information, which may include food origin and production claims e.g., geographical indication or organic [1]. Due to increasing awareness concerning food quality and safety, consumers continuously demand the reassurance of origin and nutritional value of their foods. The declaration of basic information such as the name of the food, ingredients declaration, net content and origin in value added foods is of particular interest since these products are often the target of fraudulent misdescription and/or economically motivated adulteration. The provenance evidence of a food product is a very important criterion in the context of food safety issues and protection of the consumer's right in accordance with national and international regulations and guidelines [2]. Generally, high quality food products from specific geographical locations, where the product represents the regional identity, culture and *terroir* can be sold at premium prices. Customers are also willing to pay a premium price for qualitatively superior products. Consumers rely heavily on food labelling and there is an assumption that the information will be truthful allowing them to make informed decisions about the food they are buying. Unfortunately, there is a real risk that consumers may be misled by fraudulent mislabelling and the true extent of this fraud, by its nature, is hard to assess.

With the development of the economy and abundant production of food, consumers in Bangladesh have more choices and increased purchasing ability for better quality products. Certified products, i.e., organic food and Geographical Indication Products are recognised by some consumers, which has brought higher profits to these premium quality products. However, at the same time Bangladesh is facing challenges in the food sector, with respect to production and trade, and false description, substitution of cheaper ingredients and adulteration, as well as counterfeit labelling. Incidents of fraud and fake food are frequently reported in the public media, which seriously affects market development, and also has adverse impacts on the confidence of consumers, brand reputations and certified food as well as the potential increased and significant risk posed to human health by fake products produced in unlicensed or unsanitary conditions.

Milk is a high value added product, rich source of natural nutrients for the human diet, and contains more than twenty different trace elements. Many of them are essential elements and play important roles in human nutrition [3, 4]. Bangladesh has immense prospects for expanding the dairy sector. Milk production was 6.9 million tons in 2013 14. Thus, there is a production deficit of 7.12 million tons [5]. The huge gap between supply and demand is largely met by imported milk powder from abroad [6]. Growth in demand for milk and dairy products is 10 % per year and growth of local production is only 7 9% per year [5]. So, there is a good opportunity for the local dairy industries to contribute to this shortfall. However, increased demand for milk has also made it prone to fraudulent activity. Moreover, milk is most susceptible to be mislabelled and its country of origin can sometimes be in question. The authenticity of milk has become a focal point, attracting the attention of scientists, producers, consumers, and policymakers in Bangladesh. The authenticity of dairy products is also linked to its geographic origin and processing technology used [7]. Thus, there is a pressing need for accurate standardized food authentication techniques to determine milk origin and safety. The capability

to certify a food or its origin or authenticity is of significant economic importance to many stakeholders in developing countries like Bangladesh. Isotopic and elemental fingerprinting provides a robust analytical tool to determine the origin of particular food and simultaneously provide information on heavy metal concentrations, which is also of concern in Bangladesh e.g., arsenic and cadmium.

Considering the importance of milk authenticity, the project work undertaken in CRP D52038 aimed to provide an extensive overview for discriminating milk according to geographical regions of production in Bangladesh based on multi element and stable isotope compositions. One of the first questions asked after a food safety incident is 'where is this food from?' and being able to independently verify any labelling or documentary evidence of geographical origin is an extremely important tool. In addition, government and commercial dairy enterprises collect milk from smallholders from across the country and then process and distribute the milk to all major cities in the country therefore it is also vital to know the quality of the milk along with its provenance. Thus, the microbial quality assessment of the milk samples taken during this study has also been investigated.

2. MATERIALS AND METHOD

2.1 SAMPLING SITES

Bangladesh is a tropical country and lies mainly on the deltas of large rivers flowing from the Himalayas. On the basis of its climatic conditions, it can be divided into seven distinct climatic zones. From these seven climatic regions fresh cow's milk was collected from six of them South Central (12 farms), South Western (10 farms), Western (9 farms), North Western (6 farms), South Eastern (10 farms) and North Eastern (10 farms). All samples (1 L) were collected from the bulk milk tanks of the farms and placed into clean and sterilised plastic bottles within 20 min of milking. Samples were taken at ambient temperatures during the morning milking on the day of sampling and immediately placed in a cool box for storage and transfer to the laboratory. The sampling procedure was repeated three times under the same conditions on three different test dates. At the laboratory the milk samples were stored frozen at 20ºC and until the trace elemental analysis was performed. Three replicate measurements were performed on each sample.

2.2 ELEMENTAL ANALYSIS OF MILK BY ATOMIC ABSORPTION SPECTROPHOTOMETRY (AAS)

2.2.1. Instrumental, Chemicals, and Sample digestion

A Varian AA240FS Atomic Absorption Spectrometer (AAS) was used for the determination of Pb, Cd, Cr, Cu, Co, Ni, Mn, Zn, Fe, Ca, Mg, Na, K. Only As was measured using a Varian AA240 fitted with a VGA 77 hydride vapor generator for HG-AAS. The purity of acetylene and argon was 99.99% and 99.999%, respectively. The rest of the metals were measured using hollow cathode lamps. Standard solutions (Spectropure, USA) for each element were at the highest purity level (99.98%) and supplied by Varian Inc, USA. Concentrated nitric acid (HNO_3, Supra pure) and all other Chemicals were supplied by E. Merck (Germany) and all solutions were prepared using 18 MΩ/cm deionized water generated from an E pure system (Thermo Scientific, USA). Milk samples were placed in an XP vessel and digested with concentrated HNO_3 using a microwave accelerator reaction system (MARS'5, CEM Corporation, USA) according to a US EPA procedure (3051A). When digestion was complete the solution was diluted to 10 mL using ultra-pure water and then measured using AAS to determine the concentration of metals present in the milk samples.

2.2.2 Calibration and Accuracy of AAS measurements

The method was validated for accuracy and precision. A certified reference milk powder material IAEA 153 was used to evaluate the accuracy of the method. Table 1 shows the average concentrations of the metals measured were between 92.09% to 99.89% of their certified values, indicating acceptable accuracy (Table 1). The linearity range, detection and quantification limits were also determined. A five point calibration curve was generated using different concentrations of stock standards for each metal to estimate the linearity range. The calibration points for Cd, Co, Cr, Cu, K, Mg, Mn, Na, Ni, Pb and Zn ranged from 0.1 to 1 mg/L. The calibration points for As, Ca, and Fe ranged was from 1.0 to 10 mg/L. Instrument detection limit (IDL) at the 99% confidence limit was calculated using the following formula: IDL = t (0.01, df) s; Where, df = degree of freedom, SD = standard deviation and t= critical value for the student t-Test at 99% confidence level. Here, the value of t is 2.821 (for a corresponding df = 9). Therefore, IDL = 2.821 x SD; Instrument Quantification limit (IQL) = IDL x 10; Method detection limit (MDL) = 4 x IDL; and Limit of Quantification (LOQ) =10 x IDL.

TABLE 1. ACCURACY OF AAS MEASUREMENTS AGAINST MILK POWDER REFERENCE MATERIAL IAEA153

Heavy metals	Certified value (mg/kg)	Measured value (mg/kg)	Mean Recovery (%)
Ca	12870	12555 ± 1130	97.6
Cu	0.57	0.56 ± 0.04	98.3
Fe	2.53	2.33 ± 0.21	92.1
K	17620	17600 ± 1584	99.9
Mg	1060	1020 ± 82	96.2
Mn	0.19	0.18 ± 0.01	94.7
Na	4180	4030 ± 363	96.4
Zn	39.56	37.95 ± 3.03	95.9

2.3 INSTRUMENTAL NEUTRON ACTIVATION ANALYSIS (INAA)

2.3.1. Sample preparation

Approximately 5 g of sample was heated at 600˚C in a furnace for about 14 hr to convert it into dry ash. About 50 mg of each dried ash sample was weighed in polyethylene bag and heat sealed (double pack). For relative standardization approach, standard reference material NIST 1633b (Coal Fly Ash) was used in this study. The standard was prepared in the same way as the samples. Samples and standard were placed in a vial for irradiation.

2.3.2 Neutron irradiation methods

The 3 MW TRIGA Mark–II research reactor at the Bangladesh Atomic Energy Commission was used to perform two irradiation schemes with the pneumatic transfer system. The first short irradiation scheme was separately administered to each sample with a thermal neutron flux of 5.28×10^{12} n cm^{2} sec^{1} for 60 seconds at 250 kW. The second longer irradiation was administered simultaneously to all the samples and standards with a neutron flux of 2.11×10^{13} n cm^{2} sec^{1} for 8 minutes at 2.4 MW. Three IRMM 530RA Al 0.1% Au (0.1 mm foil) monitor foils were

irradiated at the same time to determine the neutron flux gradient within the sample stack. The foils were placed at the bottom, middle and top of the sample/standard stack for the long irradiation exposure.

2.3.3 Gamma counting method

A high purity germanium (HPGe) detector (CANBERRA) coupled to a digital gamma spectrometer (ORTEC, DSPEC Jr^{TM}) was used for gamma ray counting after irradiation of the samples and standards. For the shorter time irradiated samples counting was performed for 300 seconds after a decay time of about 300 seconds and a second counting for 600 seconds after a decay time of 2 to 3 hours. For the longer time irradiated samples, the first counting period was 40 minutes after a decay time of 2 days. The second counting period was for 2 hours after a decay time of 7 to 10 days and the third counting period was 8 to 12 hours after a decay time of 14 to 21 days. Short lived and long-lived radionuclides were determined from the short and long irradiation periods separately by comparison with NIST SRM 1633b (coal fly ash) for standardisation of results.

2.4 STABLE ISOTOPE ANALYSIS BY ELEMENTAL ANALYSER ISOTOPE RATIO MASS SPECTROMETRY (EA IRMS)

2.4.1. Carbon and nitrogen stable isotope analysis

A Carlo Erba NA1500 (CE Elantech, Inc, Lakewood, NJ, USA.) EA coupled to a Delta Plus Advantage (ThermoScientific, Bremen) IRMS was used to simultaneously measure the total (bulk) carbon and nitrogen stable isotope ratios. Approximately 1 mg of homogenised samples were weighted into tin capsules and kept dry in a desiccator prior to $\delta^{13}C$ and $\delta^{15}N$ analysis. The measurements were made by the combustion of sample materials into N_2 and CO_2 gas using helium carrier gas enriched with oxygen at the time of sample introduction. The furnace contained Cr_2O_3 held at 1050° C and the flash combustion generated a transient localised temperature of approximately 1800°C. These conditions completely oxidized all organic material in the sample. Separation of gases was achieved with a 2 m Porapack GC column maintained isothermally at 70°C. This enabled at least 60s of baseline separation between N_2 and CO_2. These gases were then carried to a the IRMS. The measured isotopic values of carbon and nitrogen obtained were normalised and reported as per mil against the international scale for carbon and nitrogen relative to VPDB and Air respectively. Normalisation was made by 3 point calibration with two international reference materials and a laboratory standard. Time based drift corrections were calculated from the laboratory standard interspersed regularly within the sample sequence. The δ values of glutamic acid reference materials of USGS 40, USGS 41 and an in house control standard of EDTA (Elemental Microanalysis Ltd, UK) that was normalised against IAEA reference material. Typical precision for control materials was ± 0.2 ‰ for $\delta^{15}N$ and ± 0.1 ‰ for $\delta^{13}C$.

2.4.2. Hydrogen stable isotope analysis

A thermal conversion element analyser (TC/EA) coupled to a Delta V IRMS (ThermoFisher Bremen) was used to determine the $\delta^{2}H$ of solid materials and liquid standards. Solid materials were prepared in loosely wrapped silver cups. The quantitative high temperature conversion pyrolyzes the samples producing H_2 and CO gas for analysis. Samples were admitted to the TC/EA using a Costech zero blank autosampler to eliminate the interference of atmospheric moisture. The Thermo TC/EA was equipped with a stainless steel packed GC column (Varian 0.6 m × 6 mm O.D. × 4.0 mm I.D.) with 5A molecular sieve and was coupled to the Delta V

Advantage IRMS via a ConFlo III interface. The packed GC column was maintained isothermally at 88 °C during the measurement sequence. The TC reduction furnace was comprised of a heat resistant aluminium oxide (Al_2O_3) ceramic tube packed with glassy carbon chips and quartz wool at the bottom. Solid samples were pyrolyzed to H_2 and CO gases in this reduction furnace at 1400 °C and the corresponding H_2 and CO gases were separated in the packed GC column prior to reaching the detector. Upon measuring δ^2H values, formation of H_3^+ as an ion molecule collision by product, makes the quantitative correction factor for H_3^+ necessary [8]. The H_3^+ correction commenced routinely on a daily basis by introducing reference gas pulses to the mass spectrometer's detector at ascending stepwise intensities to assure and monitor stability and linearity of the ion source. The H_3^+ factor was transferred to Isodat NT® (version 2.5) software gas configuration and used to correct the reported δ^2H values automatically before further data normalisation.

2.5 ASSESSMENT OF MICROBIAL QUALITY

The microbial status of the authentic milk samples collected for the study were assessed immediately after arrival at the laboratory. The method of Sharp and Lyles [9] was used to enumerate the standard plate count (SPC) to assess the total viable bacterial count (TVBC). The Nutrient agar used to determine TVBC was supplied by Difco™, USA, at a pH of 7.0 to 7.4. This was also used for isolation purposes. Pasteurized milk samples were directly inoculated on these media without any dilution, whereas raw milk samples were diluted appropriately using 0.9% sodium chloride solution. Then 100 µl of each sample was inoculated into culture media with a sterile pipette and spread, using a fresh sterile glass spreader, onto the respective media for each sample. Plates were incubated at 37ºC for 24 to 48 hours. After the incubation period, plates displaying colonies were counted. The TVBC was obtained by multiplying the average number of colonies in a particular dilution by the dilution factor. Microorganisms associated with the milk samples were expressed as the number of organisms of colony forming units per millilitre (cfu/ml). Total coliform count (TCC) was enumerated at 37ºC using MacConkey agar (Acumedia, USA) medium. A selective medium, mannitol salt agar (MSA) was used for Staphylococcus bacteria. Biochemical tests according to Bergey's manual [10] and morphological characteristics were carried out to enumerate staphylococci. Total Listeria count was enumerated in the same way using agar base (Oxford formulation) medium supplemented with SR0206E or SR0140E. Viable cell counts (cfu/ml) were the average of at least three independent experiments.

2.5.1. Cytotoxic effect analysis

2.5.1.1. Isolation of L. monocytogenes

L. monocytogenes from different types of cheese was isolated as described by Hitchins [11]. Briefly, 10 g of cheese sample, was taken and homogenized in 90 mL of Buffered *Listeria* Enrichment Broth (BLEB), incubated at 30°C for 4 h, and a selective agent was added [0.5% (w/v) acriflavin and nalidixic acid and 1.0% (w/v) cycloheximide, Hi media, Mumbai, India]. Selective enrichment media was used further for isolation by continuing incubation at 30 °C for 48 hours. Finally, a loopful of each sample was streaked on *Listeria* Oxford agar (Hi media, Mumbai, India) and incubated at 37 °C for 20 hours.

2.5.1.2. Identification of L. monocytogenes

Brown-green coloured colonies with a black halo were examined for their size, colour, consistency, shape and microscopic examination after Gram staining. Those colonies showing typical characteristics of *Listeria* were selected and purified by repeated streaking, given an isolate number such as 1 to 13 and maintained at 4 °C. The isolates were subjected to biochemical assays (utilization of dextrose, xylose, mannose, mannitol, starch, SIM, lacithinage, at 37°C) as recommended in USFDA/BAM/CFSAN described by Hitchins [11]. The standard methods described by G. James [12] were adopted for their identification as *L. monocytogenes*.

2.5.1.3. Detection of L. monocytogenes isolates based on the Vero cell cytotoxicity assay

The method described by Farber and Speirs [13] was used to prepare the cell free culture supernatant (CFCS). Each identified *L. monocytogenes* isolate was inoculated into 5 mL of Tryptic Soy Broth (TSB) (Himedia, Mumbai, India) and placed in an incubator at 35 °C for 24 hours. 2 mL of broth culture was re inoculated into 20 mL of TSB and incubated at 35 °C for 16 hours in a shaking water bath set at low speed. A centrifuge was used to separate the cellular material (10 000 × g) at 4 °C for 30 min. The supernatant was filtered through 0.22 μm pore size membrane (Nalgene, India). The sterility of CFCS was checked by streaking on Tryptic Soy Agar (TSA) plates (Hi media, Mumbai, India) and incubated at 35°C for 24 hours. The sterile CFCS were then stored at −20°C.

2.5.1.2 Cell culture Procedure

Cytotoxic effects were examined in the cell and tissue culture laboratory, Centre for Advanced Research in Sciences, University of Dhaka. In brief, a Vero cell line, a kidney epithelial cells extracted from an African green monkey, was propagated and maintained in DMEM (Dulbecco's Modified Eagles' medium, Gibco, USA) supplemented with 1% penicillin streptomycin (1:1) and 0.2% gentamycin and 10% foetal bovine serum (FBS, Gibco, USA). When the cells were confluent, they were routinely sub cultured using 0.25% trypsin EDTA solution (Biochrom, UK). Cells were maintained at 37°C under a humidified 5 % CO_2 atmosphere (Frión Herrera et al. 2014). A confluent Vero cell monolayer with the cell density of 1.5×10^4 cells/well was seeded in a 96 well plate. After incubation at 37°C in a 5 % CO_2 incubator for 24 hours, 25 μL of cell free culture supernatant (CFCS) (filtered) was added to each well (Ting et al. 2010). Cytotoxicity was examined under an inverted light microscope after 48h of incubation. Triplicate wells were used for each sample.

2.6 STATISTICAL ANALYSIS

All measurements were determined from triplicate experiments and the results were expressed as the mean ± 1 sample standard deviation (σn – 1). The Data were elaborated and analysed by the Statistical Package for Social Sciences (SPSS software, Version 18). Differences were considered significant at the level of $P<0.05$. The experimental data was analysed to evaluate the canonical discriminant functions, boxplots and correlation coefficients of milk samples with respect to their geographical origin of production.

3. RESULTS AND DISCUSSION

3.1 PRINCIPAL COMPONENT ANALYSIS OF MULTI ELEMENT DATA

Unsupervised principal component analysis (PCA) was used to reduce the large set of variables to a smaller set that still contains most of the information in the large set. It enables clustering

of variables into different groups (principal components) where such variables belonging to one component are highly correlated with each other [14] and the removal of redundant variables. Varimax rotation was used to maximize the sum of the variance of the factor coefficients.

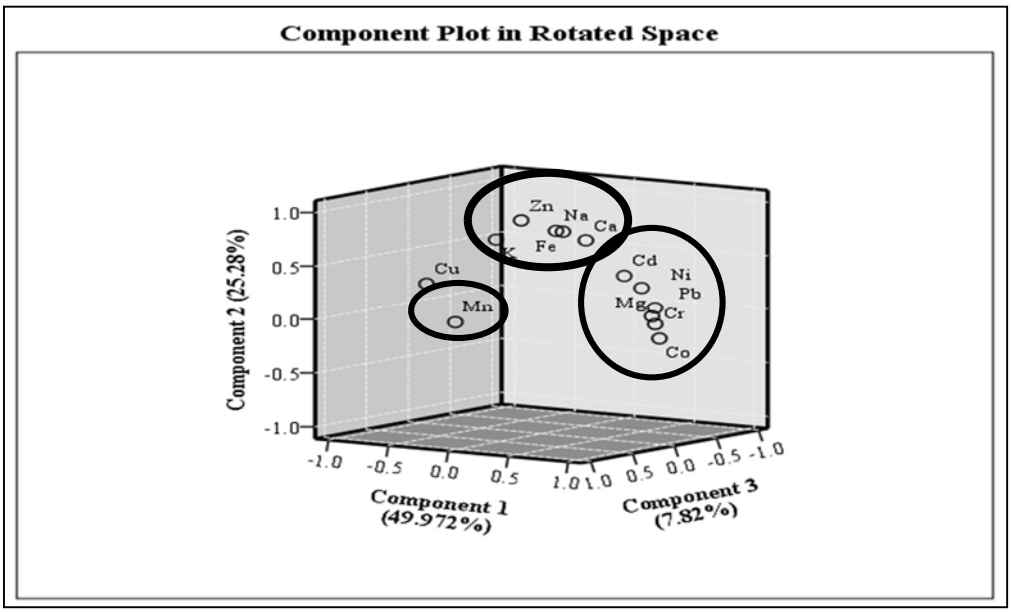


FIG. 1: Principal component analysis of the multi element data derived from milk produced in the 6 different climatic regions of Bangladesh.

TABLE 2. KAISER MEYER OLKIN (KMO) AND BARTLETT'S TESTS APPLIED TO THE TRACE ELEMENT DATA

KMO and Bartlett's Test	
Kaiser Meyer Olkin Measure of Sampling Adequacy	0.813
Bartlett's Test of Sphericity Approx. Chi Square	940.533
df	78
Sig.	.000

In the PCA analysis around 83 % of the total variability can be explained by the first three principal components (Figure 1). PC1 explained about 50 % of the variability and was mainly correlated with the concentrations of Pb, Cd, Cr, Cu, Co, Mg of samples where Cu is negatively correlated with other elements. The concentrations of Na, Ca, Fe, Zn and K dominated in PC2, which explained about 25 % of the data variability. PC3 contained the concentrations of Mn accounting for approximately 8 % of the data variability. The Kaiser Meyer Olkin (KMO) Measure of sampling adequacy value was found to be 0.813, exceeding the recommended value of 0.6 [15] indicating that the variables were correlated enough for appropriate PCA and interpreted as meritorious according to the guideline of Kaiser [16]. Similarly, the significance level of 0.000 ($p<0.01$) of Bartlett's test of sphericity in this study confirmed that PCA can be applicable for determining factors responsible for the overall separation of BGD milk samples according to their production origin (Table 2).

3.2 CANONICAL DISCRIMINATION ANALYSIS

A stepwise canonical discriminant analysis (CDA) of the trace elements measurements was performed to discriminate raw milk samples collected from the six different climatic zones of

Bangladesh. The discriminant analysis revealed that the milk samples from South Central, South Eastern and North Eastern were found to be the most distinguishable from those of the other regions sampled in the present study (Figure 2). This also suggests that the multi element concentrations in the milk samples from the three climatic zones (South Western, North Western and Western) may not be significant enough to give good geographical distinction, and a separate analysis of these regions could possibly improve the discrimination if higher resolution was required through an iterative process. An earlier report also found South Central region milk was distinctive based on multi element analysis compared to other regions [17].

The current results of the CDA are expressed by the group centroids of the respective climatic regions of origin. Out of 14 studied elements, five elements (Zn, Cr, Mg, Cu and Ni) were found to be the most important factors allowing a complete discrimination. The present results showed that the geographic origins of about 99.7% of samples could be distinguished accurately by the 3 functions; function 1 (93.9 % of variance), function 3 (5.8 % of variance) and function 3 (0.3 % of variance). The first function represents the most powerful differentiating dimension and it was mainly correlated with the concentrations of Mg, Cr and Ni. The second and third functions also represent additional significant dimensions of differentiation and were correlated with Zn and Cu respectively.

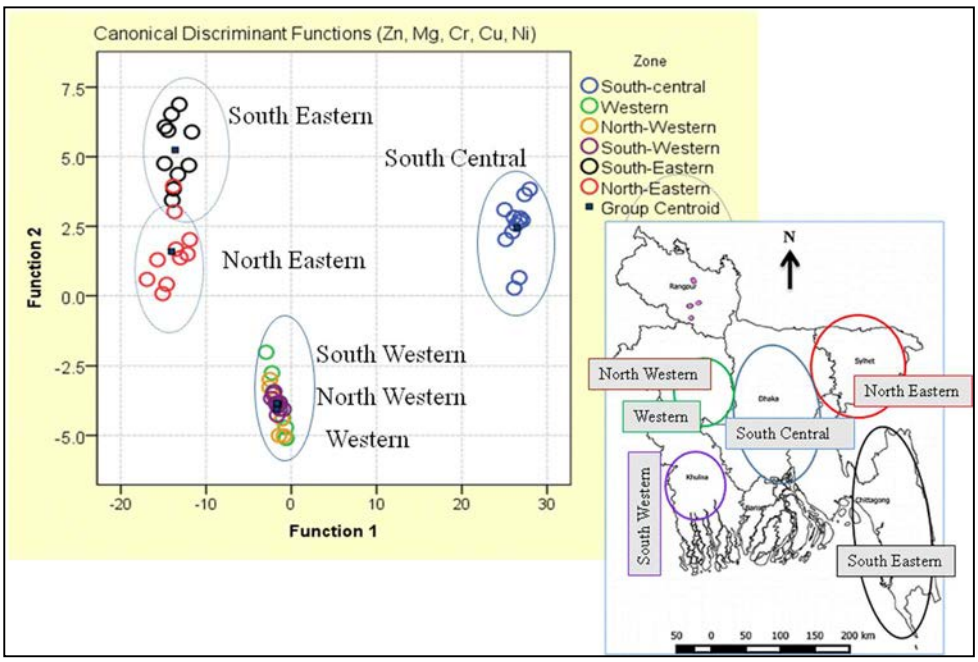


FIG. 2. A plot showing discrimination of milk according to its origin using significant elemental parameters. Function 1 represents 93.9 % of the variability, while function 2 represents 5.8 % of the variability, in the data.

TABLE 3. DISCRIMINANT ANALYSIS TEST (ANOVA) OF EQUALITY OF GROUP MEANS

	Zn	Cr	Mg	Cu	Ni
Wilks' Lambda	0.493	0.013	0.183	0.106	0.011
F statistic	10.48	786.61	45.55	86.25	948.78
p level	0.000	0.000	0.000	0.000	0.000

The test for significant differences between the groups on the individual predictor variables was evaluated based on the Wilk's lambda factor. It also shows which variables contribute significantly to the separation of the groups. Smaller values of Wilks' lambda indicate greater discriminatory ability of the variable. In the present CDA analysis Cr and Ni showed smaller values of Wilks' lambda indicating greater discriminatory ability (Table 3). Wilks' lambda was found to be significant ($P< 0.000$) by F test for all independent variables. To assess the discriminatory power and the stability of the model, a 'leave one out' cross validation was performed. The results showed that 80.7% original grouped cases correctly classified and 73.7% of cross validated grouped cases were correctly classified. Similar studies by Osorio et al., [18] classified milk and cheese successfully according to their geographical origin and feeding regimes on the basis of minor and trace elements by applying a stepwise canonical discriminant analysis. Similarly, Potocnik et al., [19] found partial regional discrimination of Slovenian milk taking into account Ca, S, P, K and Cl with a lower prediction ability of 66.7%. They also suggested that if element data is used with other chemical indices such as stable isotope analysis, a clearer picture regarding discrimination of geographical region could be achieved.

3.3 STABLE ISOTOPE ANALYSIS

Besides multi element analysis, the stable isotope ratios of carbon and nitrogen have also been used to discriminate between different geographical origins of foods, as these bio elements are influenced to varying degrees by climate, water availability, altitude, latitude, soil properties, agricultural practice, and anthropogenic activity close to production regions [20]. The present work aimed to discriminate cow's milk according to its production regions by measuring the stable isotopes of carbon and nitrogen. Samples from three climatic regions of Bangladesh were used for stable isotope analysis. Isotopic abundances were also investigated in different processed milk available in Bangladesh. The stable isotope measurement (δ^{15}N and δ^{13}C) was performed on defatted and dried milk samples collected from South Western , North Eastern and South Eastern Bangladesh to establish if the geographical origin of cow's milk could be determined. Unfortunately, samples from other three studied regions (South Central, North Western and Western) were not included in this stable isotope analysis due to limited resources.

The degree of separation among BGD cow's milk samples of different origin achieved using canonical discriminant analysis is shown in Figure 3. The first function explained 90.3% of the variance in the data and was correlated with the δ^{15}N of the milk defatted dry mass (DDM). The second discriminant function accounted for 9.7 % of the variation and it was correlated with δ^{13}C. A cross validation procedure was conducted to evaluate the robustness of the classification model. The leave one out cross validation result was that 67.4 % of milk samples were correctly classified based on the δ^{15}N and δ^{13}C values of the milk's defatted dry mass. All samples from South Western region were clearly classified except four samples (Figure 3). Out of these four samples one sample was classified incorrectly as a South Eastern sample and other three samples were classified relatively close to North Eastern region. Samples from the South Eastern and North Eastern regions overlapped each other. These two regions, however, are very similar to each other in in terms of elevated terrain and climate. There were no significant differences in δ^{15}N values between these two regions. However, δ^{13}C values of milk from the South Eastern region were significantly higher than the North Eastern and South Western regions. Besides hill tracks the South Eastern region was also located in a coastal belt. higher δ^{13}C values in milk of this region may be due to the influence of marine saline conditions. Animals grazing on coastal planes [21], and in salt marshes [22] may possess isotopic values influenced by the consumption of plants within coastal ecosystems. Saline conditions can increase δ^{13}C values in plants due to

an influence on stomatal opening and therefore on the exchange with atmospheric carbon [23 25].

It is well known that the $\delta^{13}C$ of different animal tissues is highly influenced by the composition of the food they ingest, normally consisting of different plant species [26]. The $\delta^{13}C$ values of the DDM from milk produced in the South Western and North Eastern regions showed no significant differences but the $\delta^{15}N$ values were significantly different between these two regions. Animals of the South Western and North Eastern regions are fed similar diets (mostly C_3 plants) but the climatic conditions of these two regions is quite different. It has been reported that animals with the same diet but reared in different regions, with different climatic condition, possess different $\delta^{15}N$ values [27 – 28]. The results from this study maintained the same trend in the case of the $\delta^{15}N$ values in milk from South Western and North Eastern Bangladesh.

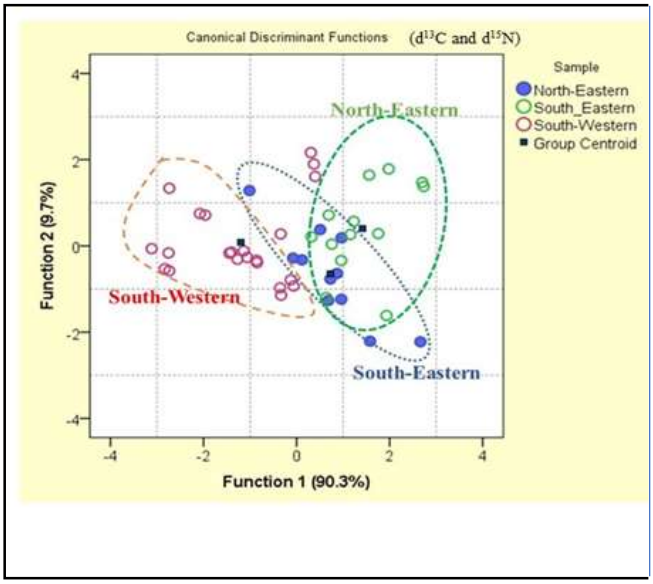


FIG. 3. A plot showing discrimination of milk according to their origin using $\delta^{15}N$ and $\delta^{13}C$. Function 1 represents 90.3 % of variability, while function 2 represents 9.7 % of variability.

3.4 SEASONAL VARIATION IN STABLE ISOTOPE RATIOS OF RAW MILK

To investigate seasonal variation in the isotope ratios of BGD milk; samples were collected during the winter and summer seasons. The measurement of $\delta^{13}C$ and $\delta^{15}N$ indicated that there was no significant ($P < 0.05$) seasonal variation in raw milk samples (Figure 4).

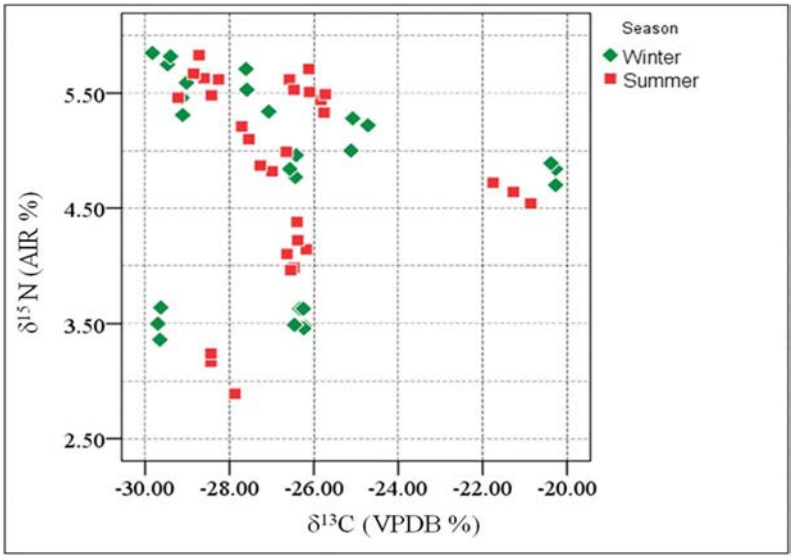
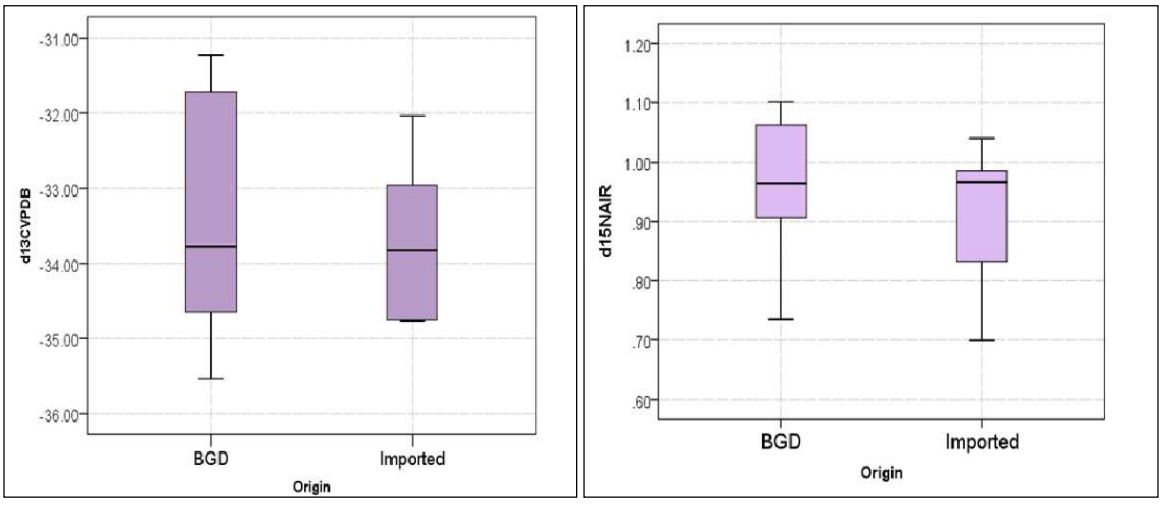


FIG. 4. Scatter plot to show seasonal variation in $\delta^{13}C$ and $\delta^{15}N$ ratios.

3.5 STABLE ISOTOPIC VARIATION IN LOCAL AND IMPORTED MILK POWDER

Box plots are used to show overall patterns of variables for a particular group. Here they provide a useful way to visualize distribution, outliers, and the median value of variables for larger groups of data. In the present study the box plot of stable isotope data ($\delta^{13}C$ and $\delta^{15}N$) for locally produced and imported milk powder, available in Bangladesh, is shown below in Figure 5. In case of the $\delta^{13}C$ box plot (Figure 5A), the interquartile range (IQR) is larger (3.1) in locally produced powder milk (BGD milk) than the IQR (2.3) of imported milk powder indicating BGD milk samples appeared to have larger variability than imported milk powder. However, their median values are similar. In the case of $\delta^{15}N$ values, the IQR and median of both BGD and imported milk powder appeared to be similar in magnitude (Figure 5B). Based on the independent sample t test for $\delta^{13}C$ and $\delta^{15}N$, it can be stated that there were no significant differences in both mean of $\delta^{13}C$ and $\delta^{15}N$ values between BGD and imported milk powder.



A **B**

FIG. 5. Isotopic variation between locally produced and imported milk powder.

3.6 REGIONAL DISCRIMINATION OF COW'S MILK USING (BIO)CHEMICAL PARAMETERS

A multivariate statistical analysis (MVA) of the measured values of the constituents of authentic samples from a variety of sources, has permitted the geographical characterization of BGD milk. Canonical Discriminant analysis was applied to the milk constituents (protein, lactose, casein, fat, and SNF) in order to classify milk samples into groups according to their geographical origin. The MVA was conducted because of the significant variation in (bio)chemical composition of cow's milk of different regions of Bangladesh that had been found in our earlier study reported by Khatun et al. [29].

The plot of the first to canonical discriminant functions (Figure 6) displayed good regional separation, suggesting that these five components contained sufficient information to assess the geographical origin of milk that could potentially be used to identify its origin if there were public health effects related to the milk produced in those studied areas. The values for milk collected in South central region were dispersed broadly, whereas those of West and North west region samples converged within a very small region. Several studies have effectively used combined chemical and statistical analyses to determine the geographic origin of milk and milk products [30 31]. However, it should be noted that this is a relatively small dataset and the stability of such a model with respect to seasonal and annual variations needs to be assessed and/or characterised e.g., in relation to changes in feeding practice.

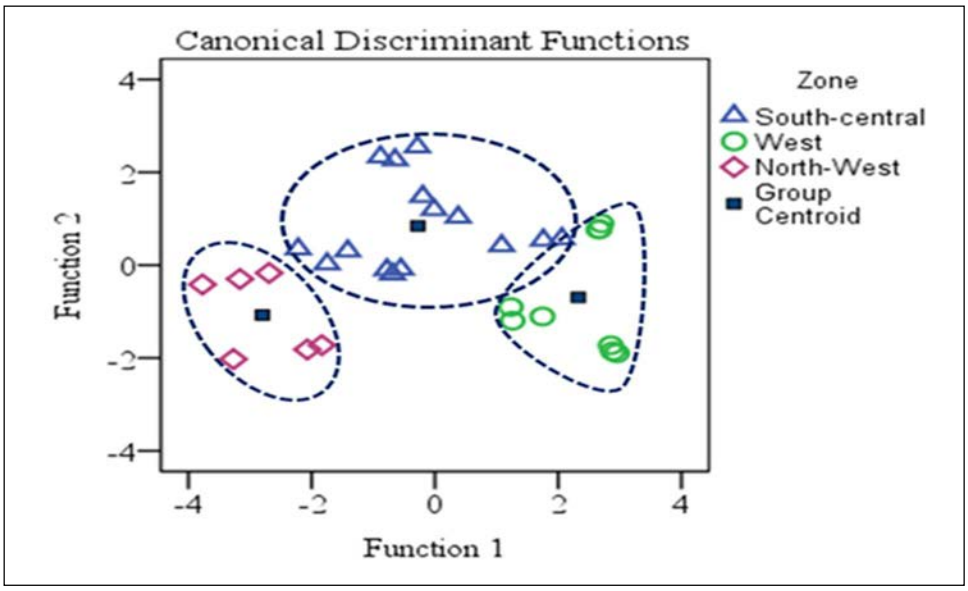


FIG. 6. Plot of the first and second discriminant functions of different geographical regions of production of BGD milk based on the measurement of its (bio)chemical constituents (protein, lactose, casein, fat and SNF). Function 1 represents 81.5 % of variability, while function 2 represents 18.5 % of the variability in the sample data.

3.7 MICROBIOLOGICAL SAFETY EVALUATION OF RAW COW'S MILK

Raw milk inherently favours microbial growth, including bacterial pathogens, due to the mixture of nutrients present, and the challenges for appropriate cold storage in developing countries with hot climates. The bacterial loading in milk directly influences the quality and safety of dairy products and therefore it demands attention through appropriate surveillance of the microbiological status of raw milk all over Bangladesh.

During the course of the CRP, several microbiological analyses were carried out to investigate the level of microbial activity in authentic raw cow's milk samples collected from the six geographic regions within Bangladesh. The constructed radar chart (Figure 7) shows the average total viable bacterial counts (TBC), total coliform counts (TCC), total *Staphylococcal* counts (TSC), and total *Listeria* counts (TLC) in the collected raw milk samples. There is a wide variation of different types of bacterial load in the raw milk produced in the six studied regions. Several reasons have been noted for raw milk contamination with pathogens, either directly through organisms or indirectly. Indirect contamination may be due to some reasons such as (i) the udder and teats may be contaminated by a cow's own faecal matter or the faecal matter of other cows (ii) milking equipment in contact with surfaces that have faecal contamination and (iii) environmental contamination. Our preceding reports showed higher microbial load in cow's milk of the South Central region compared to West and North west part of Bangladesh [29]. In the comprehensive investigation of six regions reported here, the lowest value of TBC, TSC, and TLC was observed in milk from North Western region, whereas South Eastern raw milk showed the highest microbial count. Other regions lie within these ranges (Table 4). Most of the reputable dairy companies of Bangladesh obtain their raw milk from the dairy farms of the Western and North western regions.

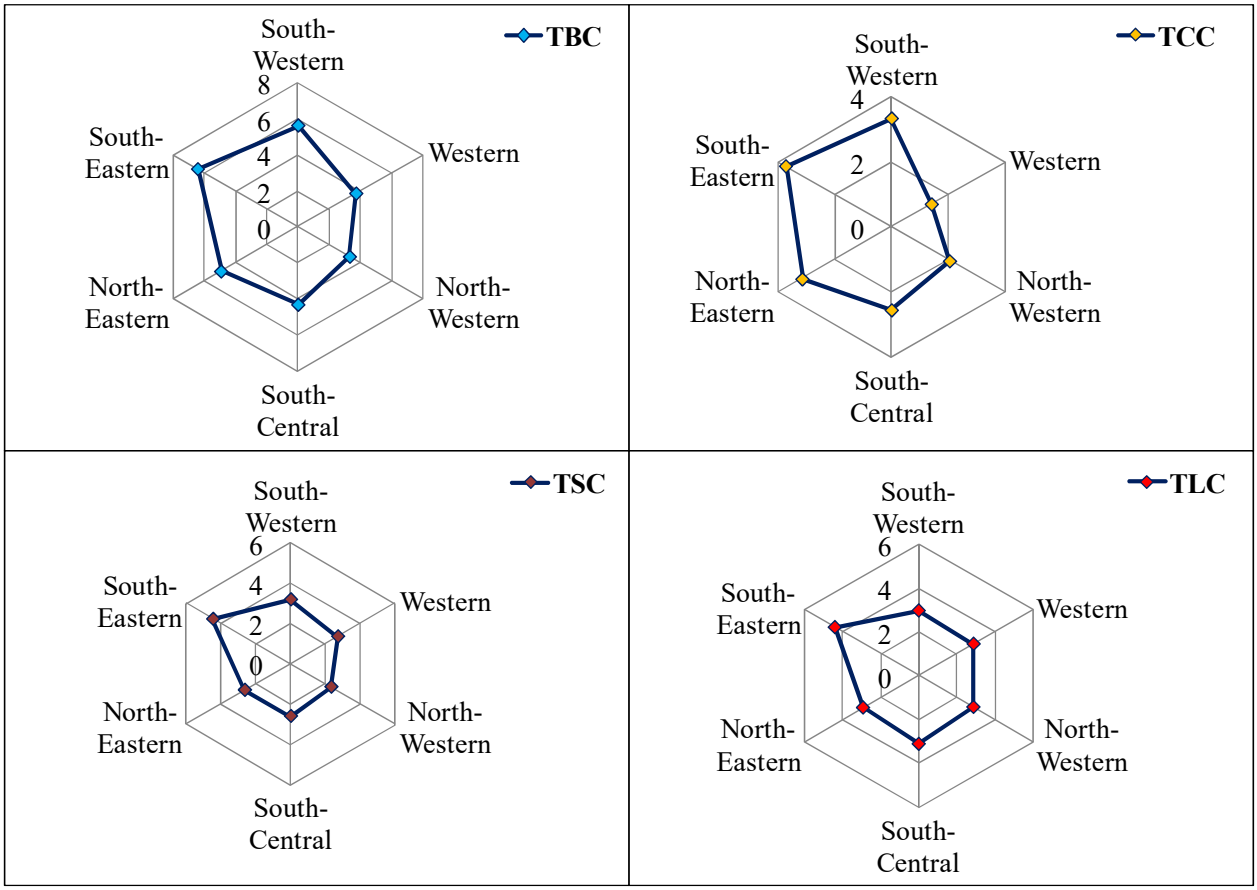


FIG. 7. Microbiological quality analysis of authentic raw milk samples collected from six different regional zones within Bangladesh.

TABLE 4. MICROBIOLOGICAL SAFETY EVALUATION OF RAW COW'S MILK

Climatic Zone	TBC	TCC	TSC	TLC
	cfu/gram			
South Western	4.35×10^5	2.16×10^3	1.57×10^3	9.04×10^2
Western	5.19×10^3	2.6×10^1	5.02×10^2	7.59×10^2
North Western	2.01×10^3	1.14×10^2	2.06×10^2	7.39×10^2
South Central	2.06×10^4	3.37×10^2	4.18×10^2	1.29×10^3
North Eastern	8.64×10^4	1.46×10^3	4.31×10^2	8.27×10^2
South Eastern	2.68×10^6	5.58×10^3	2.80×10^4	2.50×10^4

Dairy farmers from these latter regions do their utmost to maintain the appropriate hygiene standards during milk collection that helps to prevent milk from becoming contaminated with faecal matter. Moreover, dairy cows of these regions are kept in rural areas with low levels of industrialization and pollution. However, according to the recommended value ($\leq 2 \times 10^4$cfu/ml) for TBC by Bangladesh Standard and Testing Institution and United States Public Health Service [31 32], the bacterial load in South Eastern milk (2.68×10^6 cfu/ml) exceeds safe limits. Chowdhury et al. [33] surveyed some hygiene practices adopted in commercial dairy farms in Chittagong, Bangladesh. They observed some deficiencies such as insufficient cleaning of calf pens and feeding utensils that might have increased the likelihood of the spread of different diarrheal and respiratory disease pathogens among calves. Moreover, the use of surface water (from ponds, rivers and lakes *etc.*) as cow's drinking water will increase the risk of introduction of many water borne diseases into the farm. Thus, the whole hygiene and dairy management system affects the microbial status of fresh raw milk. The incidence of coliforms in raw milk has gained considerable attention due to their association with contamination of faecal origin; hence it is defined as a hygienic indicator to reflect the general microbiological quality in routine testing [34]. In the present study, the total coliform counts were too high for all the studied samples. Banik et al. [35] found the average coliform count in raw cow's milk obtained from daily markets in different regions of Bangladesh ranged from 1.0×10^4 to 2.7×10^5cfu/ml which were comparatively higher than the present study. Among the results, the western region raw milk was relatively safe having only 2.6×10^1 cfu/ml compared to South eastern milk that was found to possess 5.58×10^3 cfu/ml, which is much higher than the recommended maximum level of not more than 10 bacteria per millilitre of raw milk [36]. Recently, Alam et al. [37] reported the existence of faecal coliform (E. coli) in the Chittagong district of the South eastern region. The study identified some practices that have a high impact in contaminating milk with E. coli; such as adding water hyacinth leaves into milk, washing milk vat/containers everyday with pond and surface water, washing milk vat/container everyday with water supplied from city corporation, selling milk after 1 2h of collection, multiple farm sourced milk, milk vats and containers made of dried mud, and addition of banana tree leaves to milk. In the present study, TSC count ranged from 2.06×10^2 to 2.80×10^4cfu/ml in different regions. The lowest range of TSC counts was found in the North West region, which may result from relatively better hygienic status found in the well organised dairy farm production system. In addition, other factors may be the use of relatively higher quality milking utensils and the better quality of drinking water. The presence of *Listeria* spp. causes listeriosis, which in turn is a major cause septicaemia, meningitis and encephalitis in the infected person [38]. There are several factors that may contribute to milking areas being contaminated by Listeria. These include but are not limited to bad quality silage, infrequent and/or insufficient cleaning of exercise areas, low

standards of cow hygiene and insufficient lighting in milking areas. It is clear that the samples tested were exposed to wide-ranging microbial contamination through unhygienic handling and storage conditions and this poses a significant health risk to consumers. Based on the findings of this study, it could be recommended that raw milk is not consumed without boiling and great care should be taken when consume raw milk. In addition, the milk producers should be educated on personal and environmental hygiene. However, ideally all milk should be pasteurised and transported with adequate refrigeration prior to consumption, thus preventing human health hazards.

3.8 MICROBIOLOGICAL SAFETY EVALUATION OF HARD CHEESE

Microbiological analysis (total bacterial count, total coliform count, total Staphylococcal counts and total Listeria counts) of five locally produced hard cheeses was carried out to assess the microbiological quality of ready to eat cheeses. The results showed that all cheeses contained a high bacterial load especially Listeria (Figure 8). However, the samples were completely free from coliforms and Staphylococcal bacteria. In the present study, the total count of Listeria exceeded the standard limit of $>10^2$ according to the guide figures of the Food Safety Authority of Ireland. Listeria is the most consistently pathogenic species that may pose a significant risk to consumers. Therefore, the detection of Listeria spp. in ready to eat food can indicate inadequate heat treatment or post processing contamination from the production environment. The presence of Listeria spp. could also indicate the presence of *L. monocytogenes*. Cheeses made with pasteurised milk are of lower *L. monocytogenes* risk because during pasteurisation milk heated to a specific temperature for a specific period of time, kills *L. monocytogenes* effectively. Hard cheeses made with unpasteurised milk are likely to be less acidic and contain more moisture that induces the presence, growth and survival of pathogenic microorganisms including *L. monocytogenes*. It is generally accepted that good quality cheese will not exceed 100 cfu/g of *Listeria monocytogenes* throughout its shelf life [39]. A study has been conducted for the detection and confirmation of a toxin producing Listeria, *L. monocytogenes.* To know the effect of pathogenic *L. monocytogenes* isolates on continuous cell lines, the cytotoxicity assay was performed on Vero cell lines, a kidney epithelial cells extracted from an African green monkey. Vero cell cytotoxicity assay (in vitro) resulted in a positive assay in 5 strong haemolysin producing *L. monocytogenes* isolates. According to assay results, *L. monocytogenes* isolates, including the reference strain, showed pathogenic effect on Vero cell lines and the Vero cells changed from spindle (characteristic of normal Vero cells) to round and shrivelled cells that floated on the medium of culture wells (Figure 9).

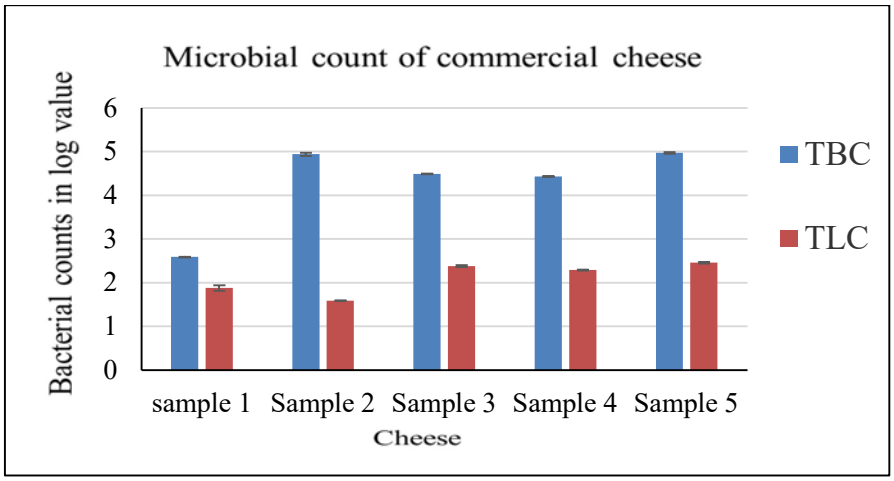


FIG. 8. Microbial quality assessment in of five ready to eat hard cheeses.

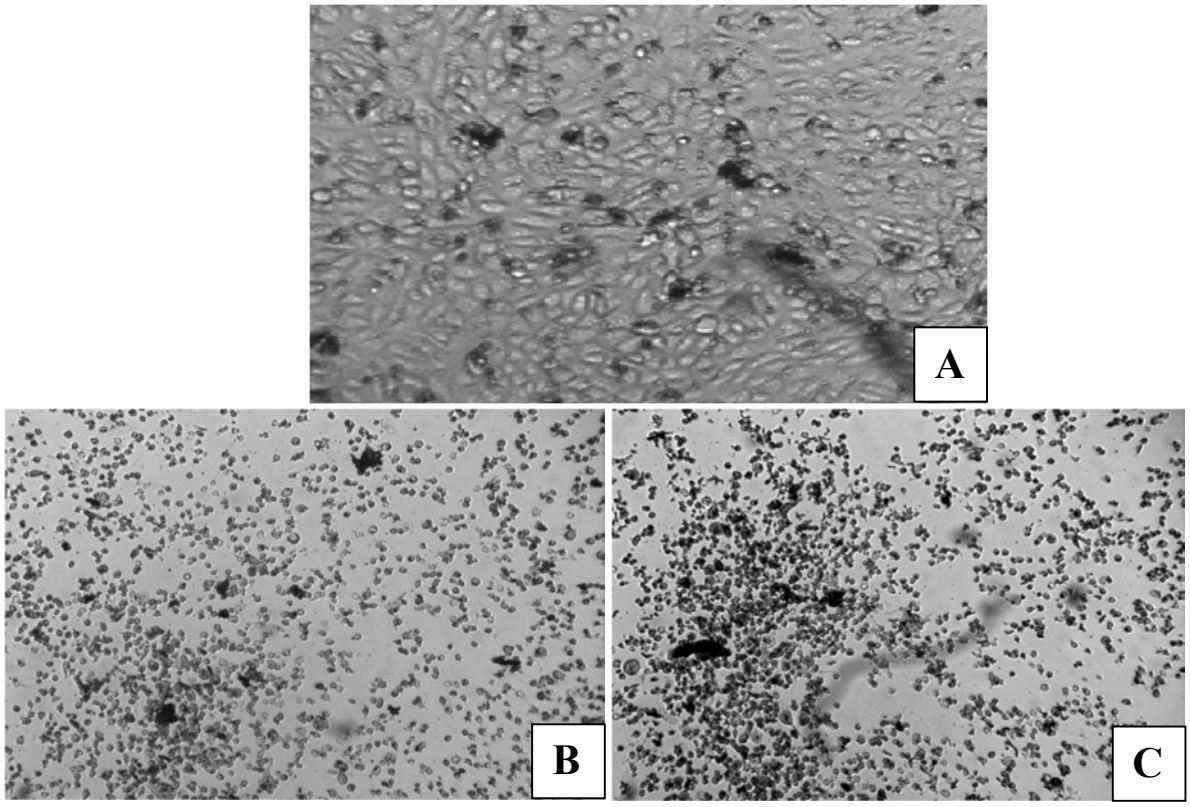


FIG. 9. Cytotoxicity test for L. monocytogens, isolated from hard cheese. A: Vero cell (kidney epithelial cell) from African Green Monkey, B: haemolytic effect by positive control L. monocytogensATCC 7644 culture strain on Vero cell, C: haemolytic effect by isolated L. monocytogenson Vero cell.

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TRACEABILITY AND IDENTIFICATION OF ORGANIC PORK BASED ON ^{1}H NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF POLAR METABOLITES

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Abstract

A study was performed to establish an organic pork traceability model aimed at identifying the authenticity of retail organic pork in the market. The experimental work centred around characterisation of polar metabolites, extracted from porcine muscle tissue, by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The NMR spectra were then investigated with chemometric techniques such as Principal Component Analysis (PCA) and discriminant analysis (DA) after optimizing data reduction, pre-treatment and testing conditions. Results showed 18 polar metabolites could be used as biomarkers for organic pork characterisation, and only glycine was found to differ significantly between organic and conventional pork. The cumulative contribution rate of the first three principal components explained 76.74% of the data variance. The PCA analysis showed the organic and conventional pork were well separated. The rate of correct classification of the discriminant model was 70% established through cross validation. The traceability model for organic versus conventional pork was therefore deemed to be relatively reliable based on this feasibility study.

1. INTRODUCTION

The main nutritional components fat, protein, and carbohydrate are abundant in pork, which is a traditionally and widely consumed meat in China [1]. As one the main varieties of meat, pork accounts for 67% of total of total meat consumption [2]. Organic pork is more desirable for consumers because the pigs, from which it is derived, should be raised in an uncontaminated environment, fed with organically planted crops grown without the use of pesticides, chemical fertilisers and synthetic growth regulators. During the pig's growth, any hormone, antibiotics and artificial additives are forbidden [3]. Consequently, consumers are paying more attention to organic pork with its improvements in animal welfare and production standards. However, the significant retail price difference between organic and conventionally produced pork means that there is a clear economic incentive to mislabel conventionally produced pork as organic. Current quality assurance technology for organic pork in China is based on animal ear tags, which may result in error or intentional fraud during slaughter and cutting. Therefore, there is a clear requirement for an independent analytical test to confirm the authenticity of organic pork to detect fraudulent mislabelling and protect the interests of consumers and honest traders [4].

NMR spectroscopy an established laboratory technique that has been applied widely for organic compound structure identification and qualitative and quantitative analysis in the fields of physical chemistry, biology and medicine [5]. More recently, NMR technology has helped us understand metabolic processes [6], with particular capability for analysing metabolites, NMR is popular in studying the metabolome and metabonomics [7, 8]. In this study, the aim was to determine if polar metabolites present in pork tenderloin could be used as suitable biomarkers for differentiating porcine tissues derived from pigs reared under different production regimes.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL MATERIALS

Organic and conventional pigs were raised in a pig farm located in Shunyi district in Beijing. Rearing strategies were as follows: pigs with an average birth weight of 1.5 kg were fed on Beibeiru, a milk formulated for piglets who had not been vaccinated up to middle pig breeding stage. When the animals had matured to middle pig breeding stage, all animals were inoculated with two essential vaccines against foot and mouth disease and pseudo rabies, respectively. The pigs were then kept in an indoor area of 1.5 m^2 per animal. Organic rations included 75 % organic corn, 25 % organic soybean and 0.3 % sodium chloride. Conventional rations included 70 % corn, 20 % soya, 10 % concentrated feed supplement and 0.3% sodium chloride. In addition, organically produced pigs had access to an outdoor area of 3 m^2 per animal for walk from 9:00 am to 4:00 pm where there was also the possibility to forage. For sampling, 10 organic and 10 conventional pigs were slaughtered, and pork tenderloins were transported to the laboratory and kept at 20℃. The pork tenderloin was then thawed prior to sample preparation where it was cut into 2 mm slices, freeze dried and powdered with a high impact ball mill. The powdered pork tenderloin was either directly extracted or stored at 20℃ prior to extraction.

2.2. INSTRUMENTS AND REAGENTS

The following equipment was used for the study: A 400 MHz nuclear magnetic resonance spectrometer (Bruker BioSpin GmbH, Germany), S210 Seven Compact pH meter (Mettler Toledo apparatus), FD 1C 50 freezer dryer (Beijing Boyikang Company), BiofugeStratos centrifuge (Heraeus, Germany), Vortex Genius 3 vortex mixing apparatus (IKA Group), ultrasonic bath (Fisher Company, China), Milli Q Water Purification Systems (Millipore, China) and JA2003 Electronic precision balance (Tianjin Tianma Apparatus Company, China). The primary reagents included 70 % perchloric acid solution (Sigma Aldrich), potassium hydroxide (Sinopharm Chemical Reagent Company), disodium hydrogen phosphate, sodium dihydrogen phosphate, TSP (Sodium Trimethylsilylpropionate) and deuterium oxide (D_2O) purchased from J&K Scientific Ltd.

2.3. SAMPLE ANALYSIS

2.3.1. Sample pre treatment

Approximately 1.0 g dried and powdered pork tenderloin was extracted with 12 mL of 0.6 M perchloric acid solution, 3 mL of ultrapure water. The mixture was vortexed for 30 seconds, ultrasonicated for 10 minutes and finally centrifuged at 15000 rpm for 10 min. After centrifugation the supernatant was removed and placed in another tube before being adjusted to pH 7.0 using 5 M and 0.5 M potassium hydroxide solution. The solution was then placed in an ice bath for 30 min and centrifuged again at 15 000 rpm for 10 min. The supernatant was adjusted to pH 7.0 again with 0.5 M potassium hydroxide solution. The solution was then freeze dried, and the residue dissolved in 650 µL of D_2O by vortex mixing. The D_2O solution was centrifuged at 15 000 rpm for 10 min and 600 µL of the supernatant was placed into a 5 mm i.d. NMR tube for analysis.

2.3.2. ^{1}H NMR analysis conditions

^{1}H NMR spectra were recorded on a Bruker high resolution NMR spectrometer, performed at a proton (^{1}H) frequency of 400 MHz. Experimental parameters were as follows: the spectra were obtained with 32 scans over a spectral width of 10.9993 ppm, an O1P of 4.7 ppm and an

acquisition time of 7.4 seconds. The presaturation method was applied to suppress the proton signal of water. The spectra were Fourier transformed (FT) with a FT size of 65 k. The receiving gain was 36 and relaxation delay was 30 seconds. Spectra were manually phase and baseline corrected, and chemical shifts were normalised using trimethylsilylpropanoic acid (TSP) as 0 ppm shift on the signal scale.

2.3.3. Qualitative and quantitative analysis

A typical 1H NMR spectrum of a pork tenderloin extract is shown in Figure 1 below. The abscissa and ordinate values represent chemical shift (ppm) and peak relative intensity. The complete spectra chemical shifts were normalised by setting TSP to 0 ppm. In addition, the peak area of TSP was set to 1 for quantification of metabolites of interest. Qualitative analysis aimed at metabolites of interest was carried out by reference to chemical shifts and peak attributes reported in the corresponding literature. 18 polar metabolites in the ^{1}H NMR spectra were identified (Figures 2 to 9 below) and their corresponding chemical shift and quantitative signal are shown in Table 1. According to the NMR principle, the proton concentration of the metabolite was proportional to the peak area of the corresponding proton signal. Consequently, the concentration of the polar substance was calculated from the peak area ratio of the relevant polar metabolites to the TSP proton concentration [9]. The calculation of the metabolite concentration was made according to the formula below:

$$\frac{A(\text{Met})}{A(\text{Tsp})} = \frac{N(\text{Met}) \cdot C(\text{Met})}{N(\text{Tsp}) \cdot C(\text{Tsp})} = \frac{N(\text{Met}) \cdot W(\text{Met}) / M(\text{Met})}{N(\text{Tsp}) \cdot W(\text{Tsp}) / M(\text{Tsp})}$$

$$W(\text{Met}) = W(\text{Tsp}) \cdot \frac{(A(\text{Met}) / N(\text{Met})) \cdot M(\text{Met})}{(A(\text{Tsp}) / N(\text{Tsp})) \cdot M(\text{Tsp})}$$

where Met is the metabolites, Tsp is the internal standard substance (TSP), A, N, C, W, and M are the peak area, proton number, molecular number, quantity and molar mass, and W (Met) is the mass of metabolites. All extracted samples were measured in triplicate.

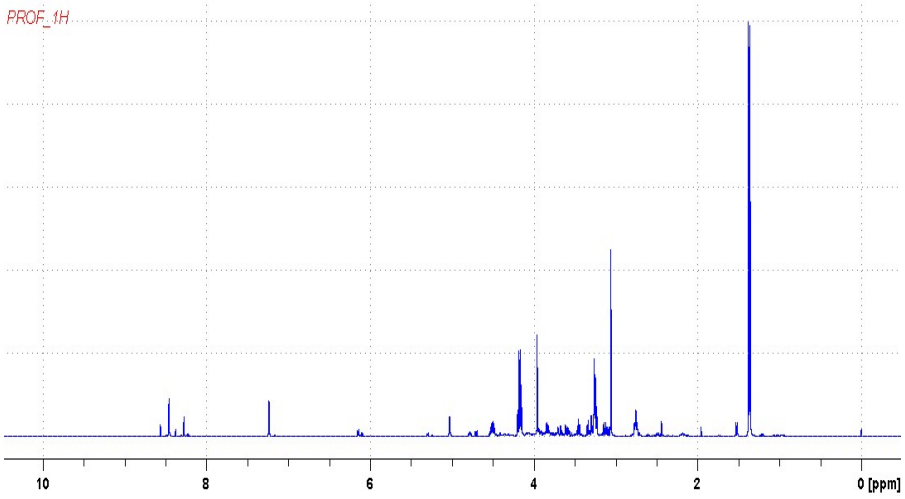

FIG. 1. The ^{1}H NMR spectra of polar metabolites extracted from pork meat tenderloin.

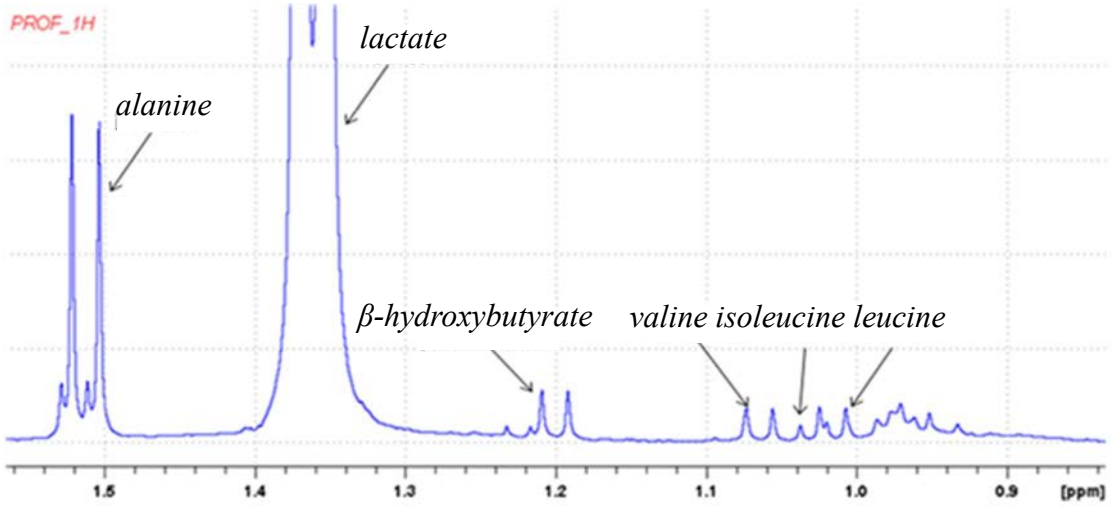

FIG. 2. The ^{1}H NMR spectra of alanine, lactate, β hydroxybutyrate, valine, isoleucine and leucine.

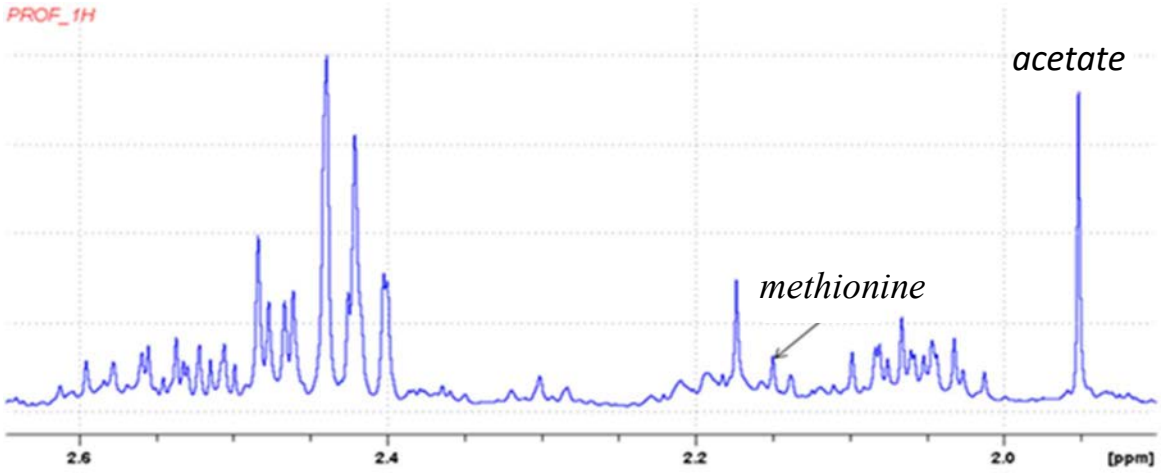

FIG. 3. The ^{1}H NMR spectra of methionine and acetate.

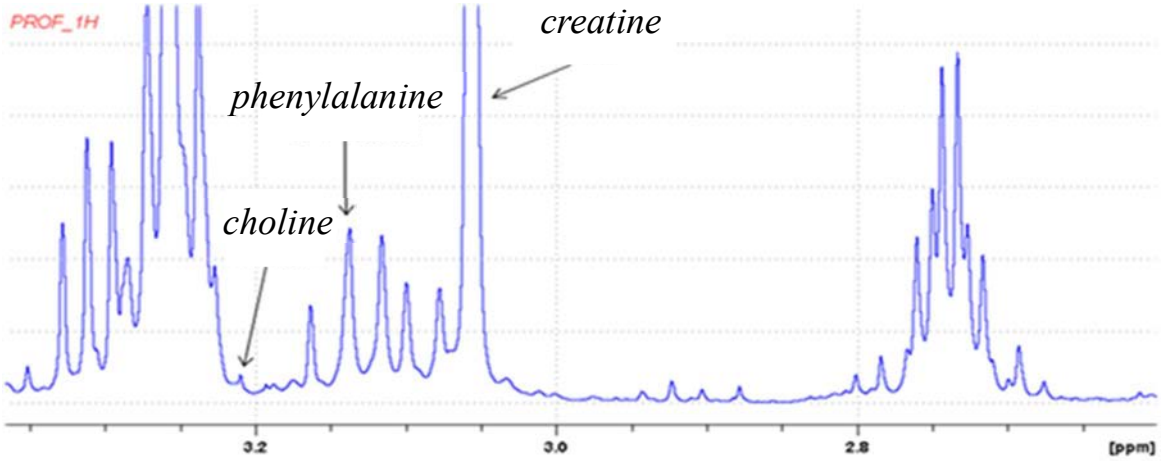

FIG. 4. The ^{1}H NMR spectra of choline, phenylalanine and creatine.

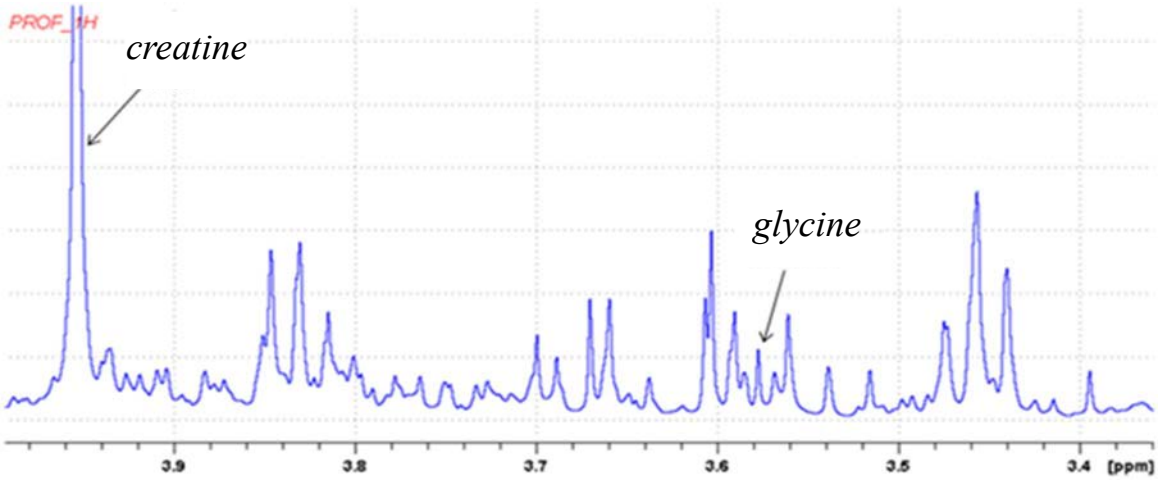

FIG. 5. The [1]H NMR spectra of creatine and glycine.

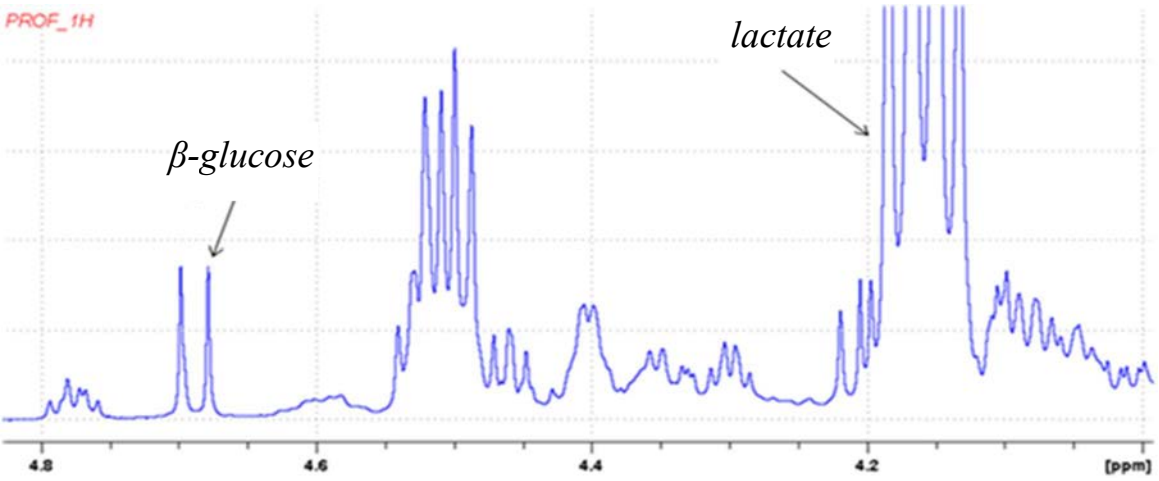

FIG. 6. The [1]H NMR spectra of β glucose and lactate.

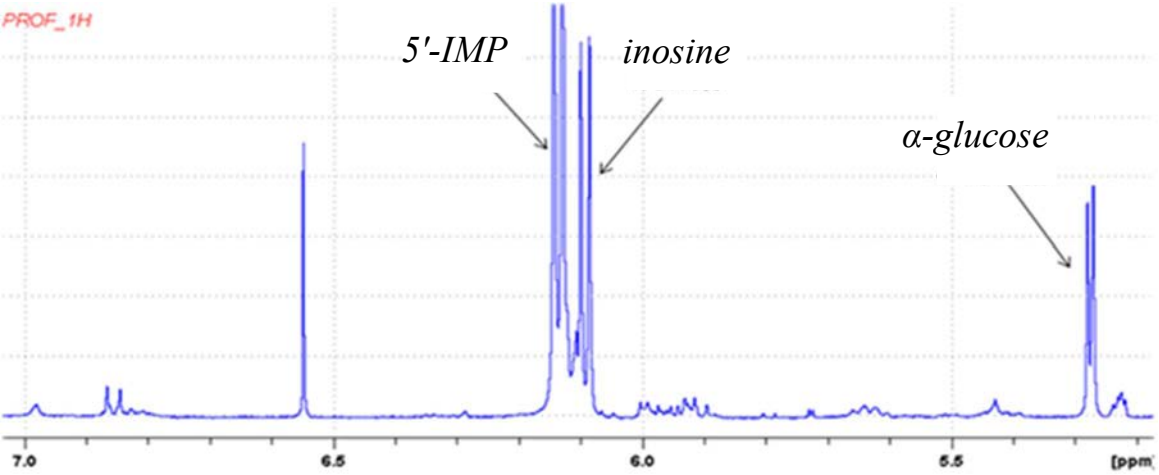

FIG. 7. The [1]H NMR spectra of 5' IMP, inosine and α glucose.

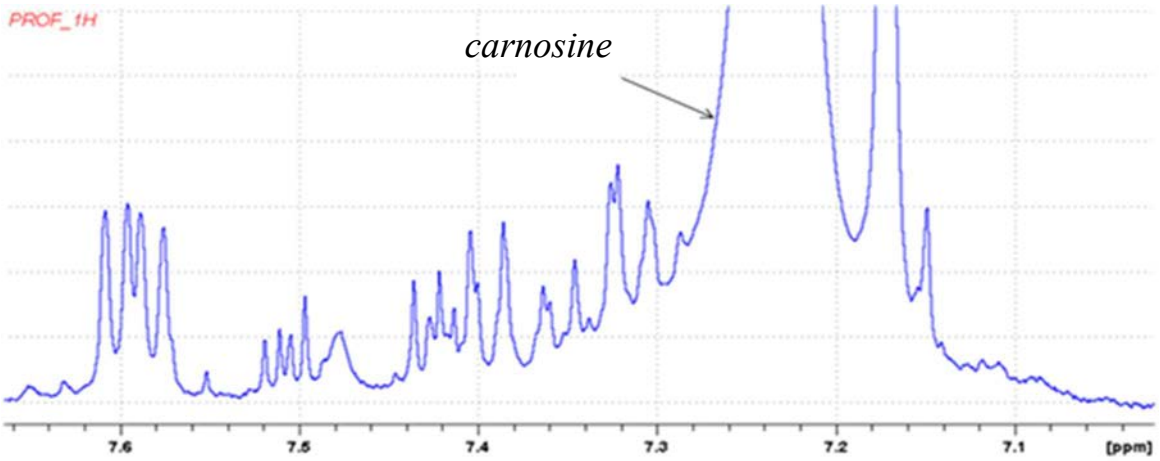

FIG. 8. The [1]H NMR spectra of carnosine.

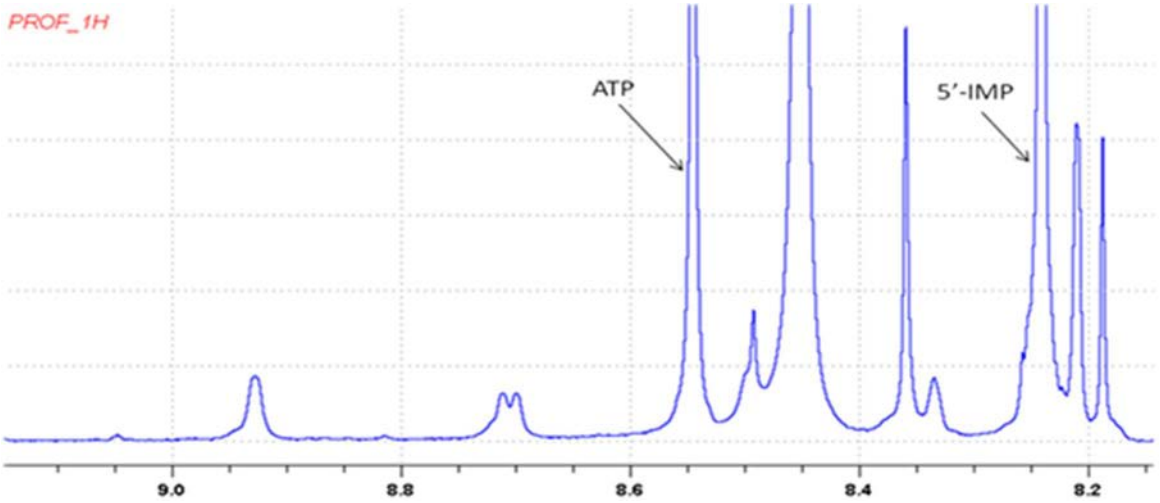

FIG. 9. The [1]H NMR spectra of ATP and 5' IMP.

TABLE 1. THE CHEMICAL SHIFT AND QUANTITATIVE SIGNAL OF 18 POLAR METABOLITES OBSERVED IN THE EXTRACTS OF PORCINE TISSUE

Metabolites	**Chemical shift (multiplicity)**	**Quantitative signal[b]**
ATP	8.57(s),8.24(s),4.60(m),4.24(m)	CH , 8.57(s)
Carnosine	8.55(s),7.23(s),3.11(dd)	CH , 7.23(s)
5' IMP	8.50(s),8.24(s),6.14(d),4.48(m),4.34(m)	CH , 8.24(s)
Inosine	8.36(s),8.24(s),6.10(d),4.30(q)	CH , 6.10(d)
α glucose	5.28(d),3.84(d),3.70(#)	CH , 5.28(d)
β glucose	4.69(d),3.73(dd),3.49(dd),3.26(t)	CH , 4.69(d)
Glycine	3.58(s)	CH_2 , 3.58(s)
Choline	3.21(s)	N $(CH_3)_3$,3.21(s)
Phenylalanine	3.30(dd),3.14(dd),7.33(m),7.45(m),7.40(m)	CH_2 ,3.14(dd)
Creatine	3.05(s),3.95(s)	N CH_3 ,3.05(s)
Methionine	2.14(s),2.62(#)	S CH_3 , 2.14(s)
Acetate	1.95(s)	CH_3 , 1.95(s)
Alanine	3.77(q),1.51(d)	CH_3 , 1.51(d)
Lactate	4.16(q),1.36(d)	CH_3 , 1.36(d)
β hydroxybutyrate	1.20(d)	CH3, 1.20(d)
Valine	3.60(d),1.06(d)	CH_3 ,1.06(d)
Isoleucine	1.99(m),1.42(m),1.25(m),1.03(d)	CH_3 , 1.03(d)
Leucine	1.76(#),1.72(#),1.02(d)	CH_3 , 1.02(d)

2.4. STATISTICAL ANALYSIS

In this study, statistical analysis was performed using SPSS statistical software (Version 22.0). Differences between the means of samples were analysed by t test at a significance level of 0.05.

3. RESULTS

Composition and relative concentration of polar metabolites for differentiating between organic and conventional reared pig muscle tissue (pork) are displayed in Table 2. Single factor variance analysis showed that among the 18 polar metabolites identified, glycine content was significantly different ($p < 0.01$). Moreover, the content of methionine, valine and leucine differed significantly. The number of polar metabolites ranked from high to low were: lactate > carnosine > creatine > ATP > 5' IMP > phenylalanine > inosine > β glucose > α glucose (Table 2).

TABLE 2. POLAR METABOLITE CONTENT OF ORGANIC AND CONVENTIONAL PORK SAMPLE (%)

No.	Polar metabolites	Organic pork	Conventional pork	Significance
1	ATP	0.0976±0.0089	0.0939±0.0030	0.221
2	Carnosine	0.2105±0.0292	0.2090±0.0166	0.890
3	5' IMP	0.0746±0.0075	0.0751±0.0041	0.862
4	Inosine	0.0410±0.0080	0.0366±0.0089	0.267
5	α glucose	0.0188±0.0098	0.0242±0.0108	0.255
6	β glucose	0.0257±0.0108	0.0336±0.0109	0.119
7	Glycine	0.0057±0.0007A	0.0045±0.0009B	0.007
8	Choline	0.0005±0.0001	0.0006±0.0002	0.233
9	Phenylalanine	0.0524±0.0067	0.0505±0.0041	0.453
10	Creatine	0.1100±0.0128	0.1038±0.0086	0.222
11	Methionine	0.0016±0.0003a	0.0013±0.0003b	0.034
12	Acetate	0.0024±0.0005	0.0023±0.0005	0.750
13	Alanine	0.0062±0.0012	0.0056±0.0014	0.312
14	Lactate	0.3439±0.0374	0.3515±0.0153	0.561
15	β hydroxybutyrate	0.0030±0.0004	0.0029±0.0004	0.355
16	Valine	0.0024±0.0006a	0.0019±0.0004b	0.040
17	Isoleucine	0.0015±0.0005	0.0011±0.0003	0.074
18	Leucine	0.0023±0.0007a	0.0017±0.0004b	0.033

Annotation: Data marked different letter (A and B), (a and b) represent highly significant difference ($p < 0.01$) and significant difference ($p < 0.05$), respectively.

In the cumulative contribution to the variance of the first three principal components (Table 3); The first and second principal components accounted for 51.7 % and 14.9 % of the total variance, respectively. In total three principal components accounted for 76.742 % of the sum of variation. This demonstrates that the selected metabolites are able to differentiate organic pork tenderloin from its conventionally reared equivalent. A Scatter plot of the first and second principal components (Figure 10) and first three principal components (Figure 11) showed that conventional pork (red squares) and organic pork (green circles) were well separated using principal component analysis.

TABLE 3. THE CUMULATIVE CONTRIBUTION OF VARIANCE OF FIRST THREE PRINCIPAL COMPONENTS

Principle components	The initial eigenvalue		
	Total	Contribution (%)	Cumulative contribution (%)
1	9.298	51.7	51.7
2	2.688	14.9	66.6
3	1.827	10.1	76.7

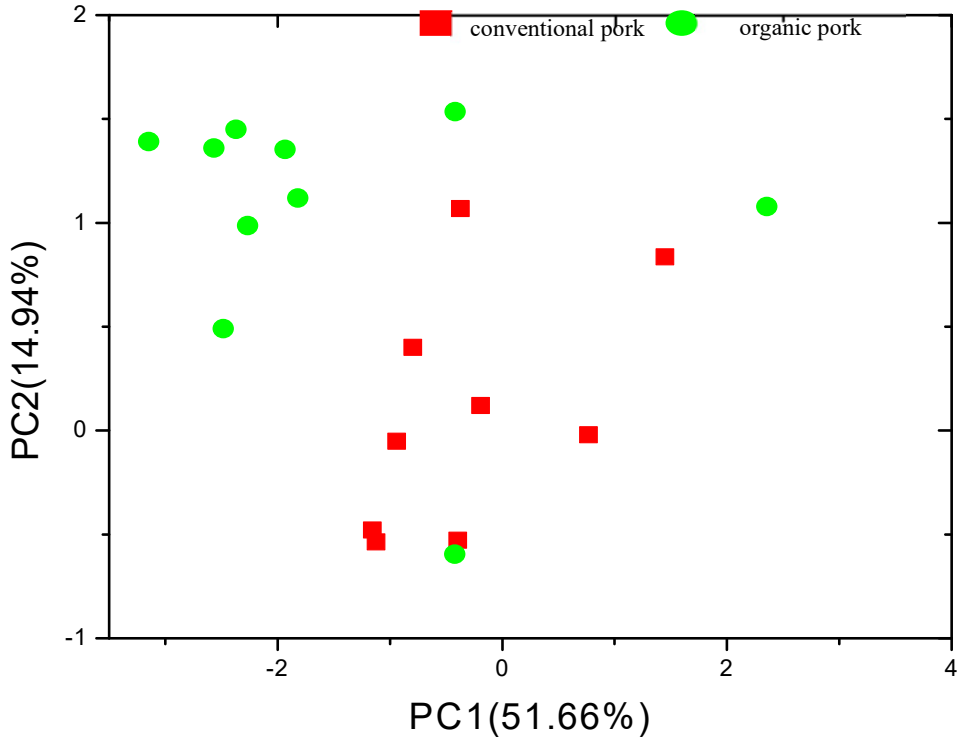

FIG. 10. Scatter plot of the first and second principal components in the unsupervised ^{1}H NMR analysis of pork metabolites extracted from conventionally and organically reared pig muscle tissue.

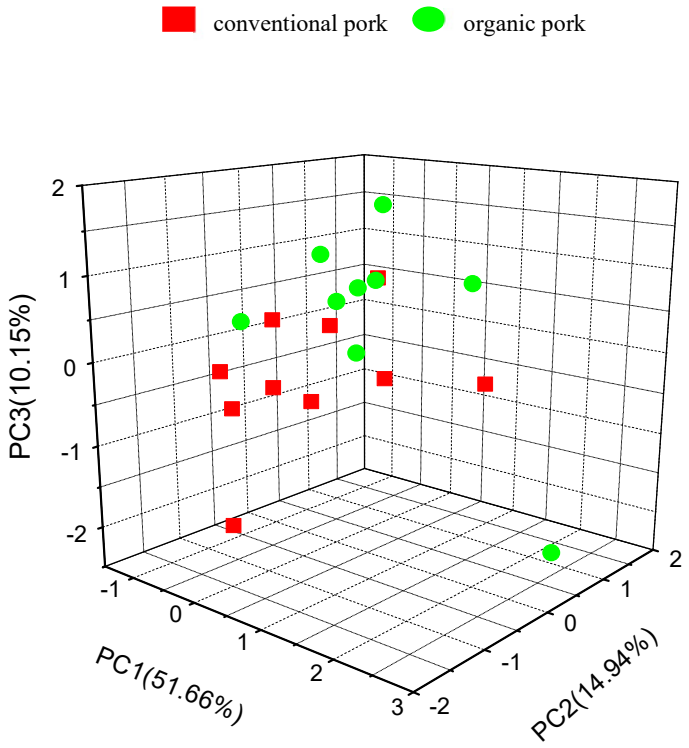

FIG. 11. Scatter plot of the first three principal components in the unsupervised ^{1}H NMR analysis of pork metabolites extracted from conventionally and organically reared pig muscle tissue.

Two discriminant models corresponding to pork tenderloin were generated by SPSS analysis. The discriminant model of conventional pork was described by the discriminant function:

$$Y1 = -15.230 + 6446.438X_1$$

and the discriminant model of organic pork by the discriminant function:

$$Y2 = -23.588 + 8090.209X_1$$

Where X_1 represented the glycine concentration, which was expressed as a percentage content relative to TSP. X_1 was known as the discriminative factor in the model. Application of the model is achieved by taking the measured glycine concentration and calculating the two values of Y1 and Y2. On the basis of Fisher discriminant function principle, when $Y1 > Y2$, an unknown pork was judged to be conventional pork, and the opposite result yielded that it was organic pork.

The overall rate of correct classification for organic and conventional pork was 70 % and 80 % respectively after leave one out cross validation (Table 4).

TABLE 4. RESULTS OF DISCRIMINANT ANALYSIS FOR ORGANIC AND CONVENTIONAL PORK

Discriminant result			Discriminant of pork		Discriminant of pork
			Organic	Conv.	
Initial discriminant result	Count	Organic	7	3	10
		Conv.	1	9	10
	Positive discrimination rate (%)	Organic	70.0	30.0	100.0
		Conv.	10.0	90.0	100.0
Discriminant result after cross validation	Count	Organic	7	3	10
		Conv.	2	8	10
	Positive discrimination rate (%)	Organic	70.0	30.0	100.0
		Conv.	20.0	80.0	100.0

Annotation: where Conv. = the conventionally reared pork.

4. CONCLUSION

Unsupervised principal component analysis of the ^{1}H NMR spectra of polar metabolites extracted from porcine muscle tissue permitted the differentiation of organic pork tenderloins from conventional produced equivalents. In addition, the correct classification rate of organic pork was 70% via discriminant analysis which showed that the discriminant model built in this research was reliable for the relatively small data set studied here. Further work is required to establish the natural variability in the metabolite profiles to include temporal and dietary variations in order for the method to be applied in practical inspections of retail samples.

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SEASONAL VARIATION OF $\delta^{18}O$, $\delta^{13}C$ AND $\delta^{15}N$ IN COW'S MILK

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Abstract

Dairy products are of particular interest as a group of foods that play an important role in feeding the human population. The composition of milk is dependent upon the feed supplied to dairy cows, and as a consequence, especially for grazing cattle, on their particular environment. To better understand the temporal variation of $\delta^{18}O$, $\delta^{13}C$ and $\delta^{15}N$ values in milk from a single environment, measurements of stable isotope ratios in cow's milk water were compared to, artesian water, precipitation and the stable isotope values of the forage and feed. The main water source for the cow was artesian water during the winter, while during the summer grass water influenced oxygen stable isotope values in the milk water. Stable oxygen isotope ratios in milk water were relatively lower in winter/transitional seasons and higher in summer showing the dependence on the main water source, meanwhile $\delta^{13}C$ and $\delta^{15}N$ values reflected the particular season's food source.

1. INTRODUCTION

The stable isotope composition in the milk of cows (and other mammals) is related to food and water intake, as well as other factors such as environmental conditions (e.g., temperature, humidity) and the metabolic status of the animal (e.g., stress). Knowing the isotope distribution of the source food and water it is possible to develop a model to predict the ratio of stable isotopes in milk. Furthermore, the database of collected data allows assessment of the geographical origin of dairy products. This information may be valuable in the fight against trade fraud and may also be relevant for the approval or rejection of the declared origin of production by producers or suppliers.

Previous studies have shown that stable isotope analysis has the potential to verify the geographical origin of a variety of food products and beverages, however, in an environment the distribution of stable isotopes is not always constant and may vary widely according to environmental conditions. Dairy products are particularly important as a food group that plays a significant role in human nutrition. The composition of milk depends essentially on the feeding of cows and on its environment, so for a better understanding of the reasons for the temporal variation in cow‘s milk stable isotope composition $\delta^{13}C$ and $\delta^{15}N$ values were measured in grass, while $\delta^{18}O$ was measured in artesian water and precipitation during several seasons of milk production.

In order to ensure comparability of isotopic data between countries and regions, there is a need not only for a large comparative database, but for fundamental knowledge of the variation in space and time of isotopic values for each region. This work analyzes the stable isotope variation in cows' milk samples from a single location and these studies are relevant to underpin and ground truth further studies of on the wider geographical origin of cow‘s milk.

The main purpose of this work was to determine the seasonal variation of stable isotopes in cow's milk and link it to the feed and geographical origin at a single location.

2. MATERIALS AND METHODS

2.1. SAMPLING OF THE MILK AND WATER

In this study, milk, drinking water, precipitation and grass were collected repeatedly from 2014 to 2016. The study involved a single cow in the Vilnius district in the village of Glitiškės. In winter, the cow was sheltered in a barn, fed with dry hay and with local arteic water. During the summer season it was kept outdoors and grazed on fresh grass. The amount of milk produced by the cow depended on the season and ranged between 20 30 litres per day.

Samples of milk and artesian water were frozen immediately after collection in order to avoid evaporation and possible microbial activity which could affect isotope ratios in the samples. Precipitation samples were collected into a water container and poured into 50 mL tubes. During the summer periods, precipitation samples were collected as soon as possible after the event. All collected samples were labelled and stored in different vials in a freezer at a temperature not exceeding 18 ºC. For the collection and storage of samples, 50 mL centrifuge tubes, suitable for low temperature storage, were used (Figure 1). The samples were thawed immediately before processing for analysis.

FIG. 1. Collection milk samples in 50 mL centrifuge tubes compatible with 18 °C storage.

In total, more than 150 samples of precipitation, water, cow's milk and grass were collected and analysed. The grass samples were collected during spring summer seasons and dried hay in the winter season.

2.2. SAMPLE PREPARATION FOR ISOTOPE ANALYSIS

The thawed milk was centrifuged in 1.5 mL tubes for 30 minutes at a speed of 20 000 rpm. The upper fraction (lipid) was removed. If necessary, the centrifuge step was repeated to remove the entire upper lipid material. The remaining liquid (whey) was poured into a separate vial and the Ph adjusted to 4.6 with 0.1 M hydrochloric acid. The remaining precipitate (casein) was collected. For stable oxygen isotope measurements, the water remaining after whey was used. The flow chart representing the process of preparing milk sample fractions is shown in Figure 2 below.

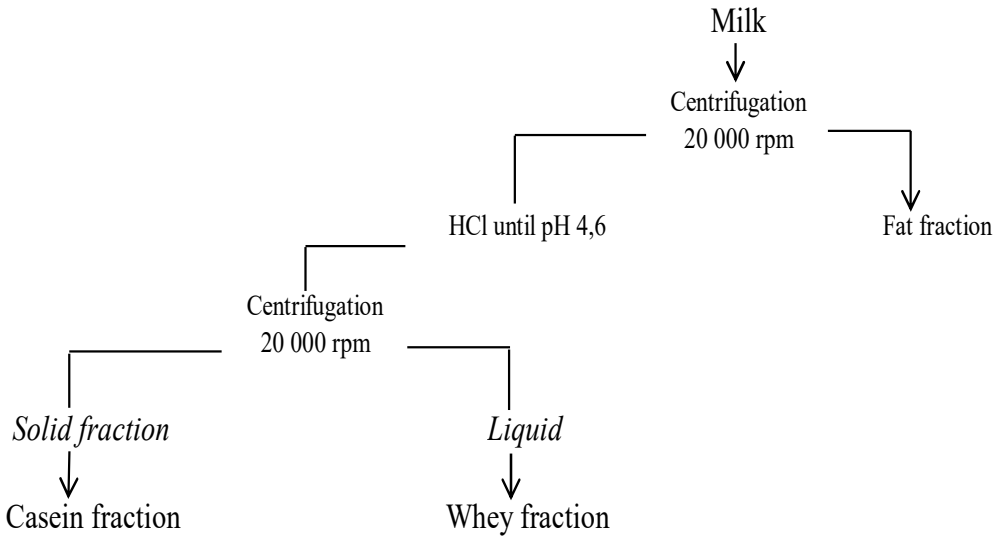

FIG. 2. Flow diagram of preparation of milk fractions prior to isotopic analysis.

For stable carbon and nitrogen isotope measurements, the milk and grass samples were freeze dried to A dry constant weight. For stable oxygen isotope measurements, the separated milk water was measured immediately after sample preparation, otherwise the samples were stored in a freezer at a temperature not greater than -18 °C.

2.3. STABLE ISOTOPE RATIO MASS SPECTORMETRIC MEASURMENTS

The carbon and nitrogen isotope ratios were measured with an Elemental analyser (Flash EA 1112) connected to the stable isotope mass spectrometer (Thermo Delta V Advantage). The dried and homogenised specimens were placed in tin capsules and combusted in the oxidation furnace of the analyser at a temperature of 1020 º C in the presence of an excess of oxygen. The sample carbon was oxidised to CO_2 and the sample nitrogen was oxidised to nitrous oxides and then reduced to N_2 in the reduction column. The CO_2 and N_2 gasses in the mixture were separated by a chromatographic Porapak GC column (2 m) maintained at 50 º C.

For $\delta^{18}O$ measurements, 500 µL of the milk water sample was put into an open vial using a disposable syringe and sealed with a new septum. Residual air was removed from the sample vials following an autosampler assisted flushing procedure, which uses a mixture of 0.2% v/v, up to 1% v/v, CO_2 in He. Measurements were carried out after an equilibration time of 24 h at 24 °C. The sampling loop aliquoted 100 µL samples of the headspace into an isothermal gas chromatograph, where CO_2 was separated from any other gas species using a Porapak column. The use of repetitive loop injection (1–2 min per replicate) allowed the precision to approach comparable levels to that of a dual inlet system. Measurements were performed with a Gas Bench II coupled to a Thermo Delta V Advantage Isotope Ratio Mass Spectrometer. Accuracy of carbon and nitrogen stable isotope measurements was assessed by the repeated measurements of secondary certified reference material (CRM) IAEA 600 (caffeine) and internal laboratory reference material (flour) and VSMOW 2, GISP, or SLAP 2 (all secondary RMs from IAEA) as well as laboratory reference material (tap water) for oxygen stable isotope analysis. Laboratory RMs were calibrated against secondary RMs by repeated measurements. Precision for all isotope ratio analyses was equal or better than 0.15 ‰.

3. RESULTS

The measurement of precipitation in the framework of this project was started in 2014 and is still collected as a long term monitoring programme. The data from the year 2014 to 2016 are presented in this paper. The total arithmetic mean of $\delta^{18}O$ values for all seasons in precipitation was -8.7 ‰ (± 5.1 ‰). In the warm season, the $\delta^{18}O$ values of precipitation were approximately -6 ‰, while in the cold season they were approximately -13 ‰ (Table 1). The lowest oxygen isotope value observed was in sample of precipitation collected on 2nd December 2016 at -19.4 ‰, whilst the highest $\delta^{18}O$ value observed was 2.6 ‰ in a precipitation sample collected on 24th May 2014.

The values of precipitation $\delta^{18}O$ correlate with ambient temperature fluctuations, so the distinction in the seasons is not always very precise when considering the specific months. In the early spring months, the oxygen isotope ratio can be much lower, similar to the winter season, while in the warmer spring months the $\delta^{18}O$ values may be much higher, similar to the average of the summer season. This is illustrated by a relatively large variation of the grouped data (SD > 4 ‰).

TABLE 1. $\delta^{18}O$ VALUES IN PRECIPITATION AND ARTESIAN WATER (‰)

Sample type	Seasons (during years 2014 2016)			
	Spring	Summer	Autumn	Winter
Precipitation	-6.7 ± 4.2	-6.0 ± 3.0	-11.6 ± 6.8	-13.5 ± 4.3
Artesian water	-10.3 ± 0.3			

Data are shown with ± 1 sample standard deviation (σn – 1)

The groundwater measurements show that the isotope ratio of the groundwater is quite constant in the course of the year and had a $\delta^{18}O$ value of 10.3 ‰ (± 0.3). Precipitation contributes to the formation of groundwater; however, precipitation water replenishes with groundwater resources slowly. In our region, the isotopic composition of the groundwater is close to the average annual precipitation isotope ratio.

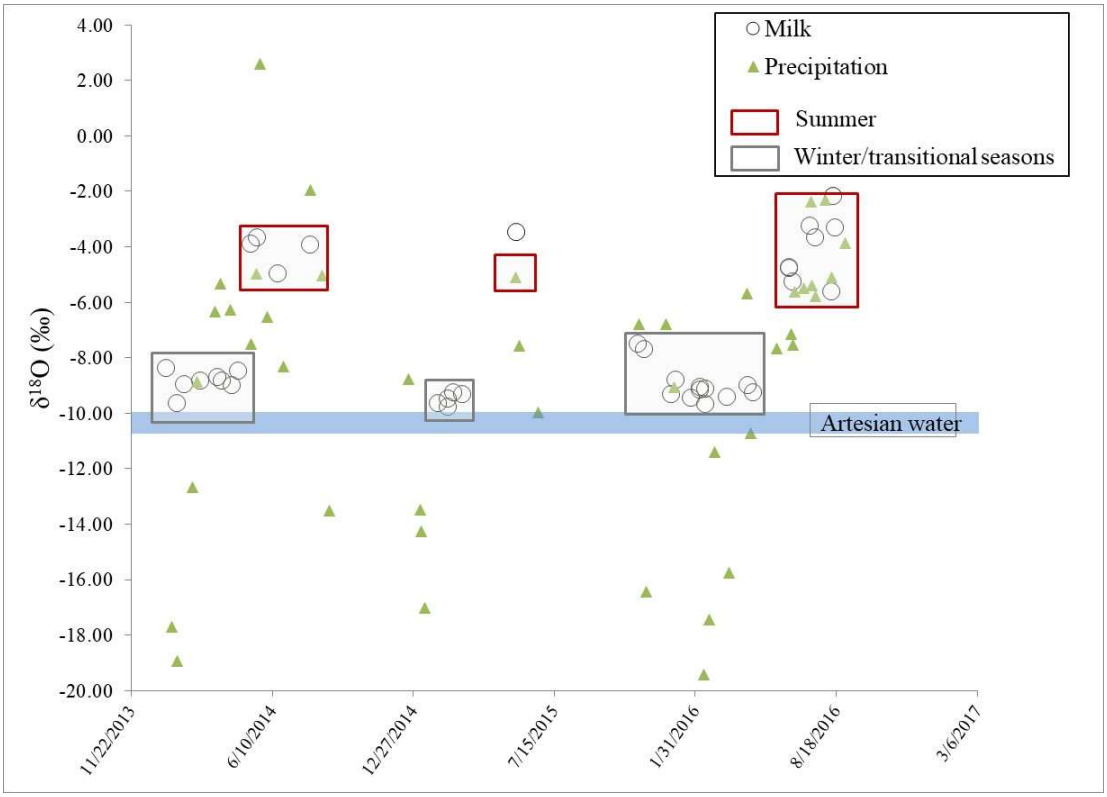

FIG. 3. $\delta^{18}O$ values in cow's milk, precipitation and artesian water (‰).

A comparison of the isotope ratios of oxygen in cow's milk water, drinking water and precipitation water (Fig. 4) showed that the isotope ratio of the milk water is higher than the groundwater oxygen isotope ratio by 1 to 8 ‰ dependent on season. The minimal difference between groundwater and milk $\delta^{18}O$ vales was during winter and transition seasons, while the greatest differences occurred during summer seasons. This correlates with the change of water source during the year. In the coldest seasons, groundwater, with a constant oxygen isotope ratio, is the main source of water for the cow, while in the warm season an additional source of water is consumed through fresh grass and herbage that is enriched in leaf water ^{18}O due to evapotranspiration. The values of milk casein $\delta^{15}N$ varied from 2.9 to 6 ‰ (Figure 4). These relatively high values were correlated with the isotope ratio of the grass. The nitrogen fixing plants have a similar isotope ratio to atmospheric nitrogen (~ 0 ‰), whereas plants that absorb nitrogen in the form of nitrates have a higher $\delta^{15}N$ values and these values correlate with the isotope ratio of the source of nitrate (Figure 5A). It has been found that organic fertilisers, usually animal manures, can have very high values sometimes even going beyond > 20 ‰ due to fractionation of nitrogen isotopes during the preferential loss of the lighter isotopologue of ammonia ($^{14}NH3$ rather than $^{15}NH3$) [1].

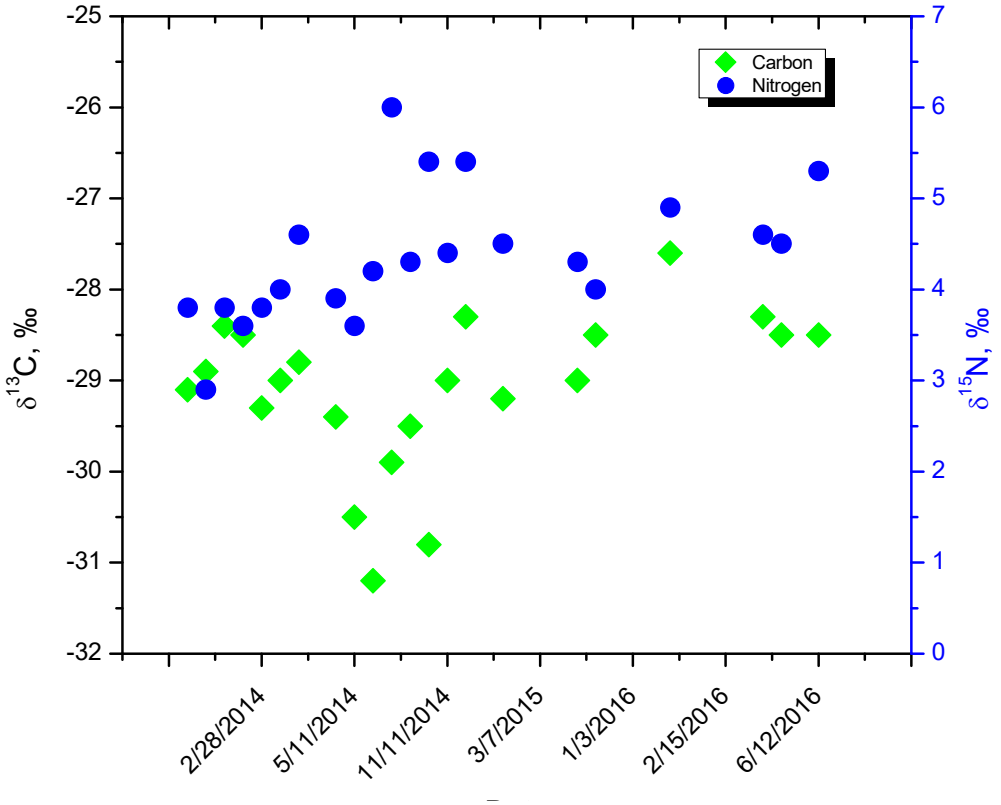

FIG. 4. Carbon and nitrogen stable isotope ratio values of the casein in the cow's milk.

Meanwhile, the carbon isotope ratio varied from -31.2 to -27.6 ‰, and correlated with hay or grass consumption. The isotope ratio of the individual varies according to the contribution of dietary sources until equilibrium is reached, since each new source of material begins to make its own isotope contribution and change the isotope ratio of the final product. The carbon isotope ratio of the grass taken during the sampling period was ~ -30 ‰, while the dried hay was about -26 ‰ (Figure 5B). In general, both higher and lower $\delta^{13}C$ values indicate that these plants fall into the range of C_3 photosynthetic plant ranges with an average of -26 ‰. The lower limit usually includes hernials with higher ^{13}C fractionation. If the nitrogen isotope ratio change in different seasons is minimal, the carbon isotope ratio is more correlated with the isotope ratio of the source of nutrition (fresh grass in summer and dried hay in autumn and winter). The lower $\delta^{13}C$ value measured during the summer season correlated with pasture grazing.

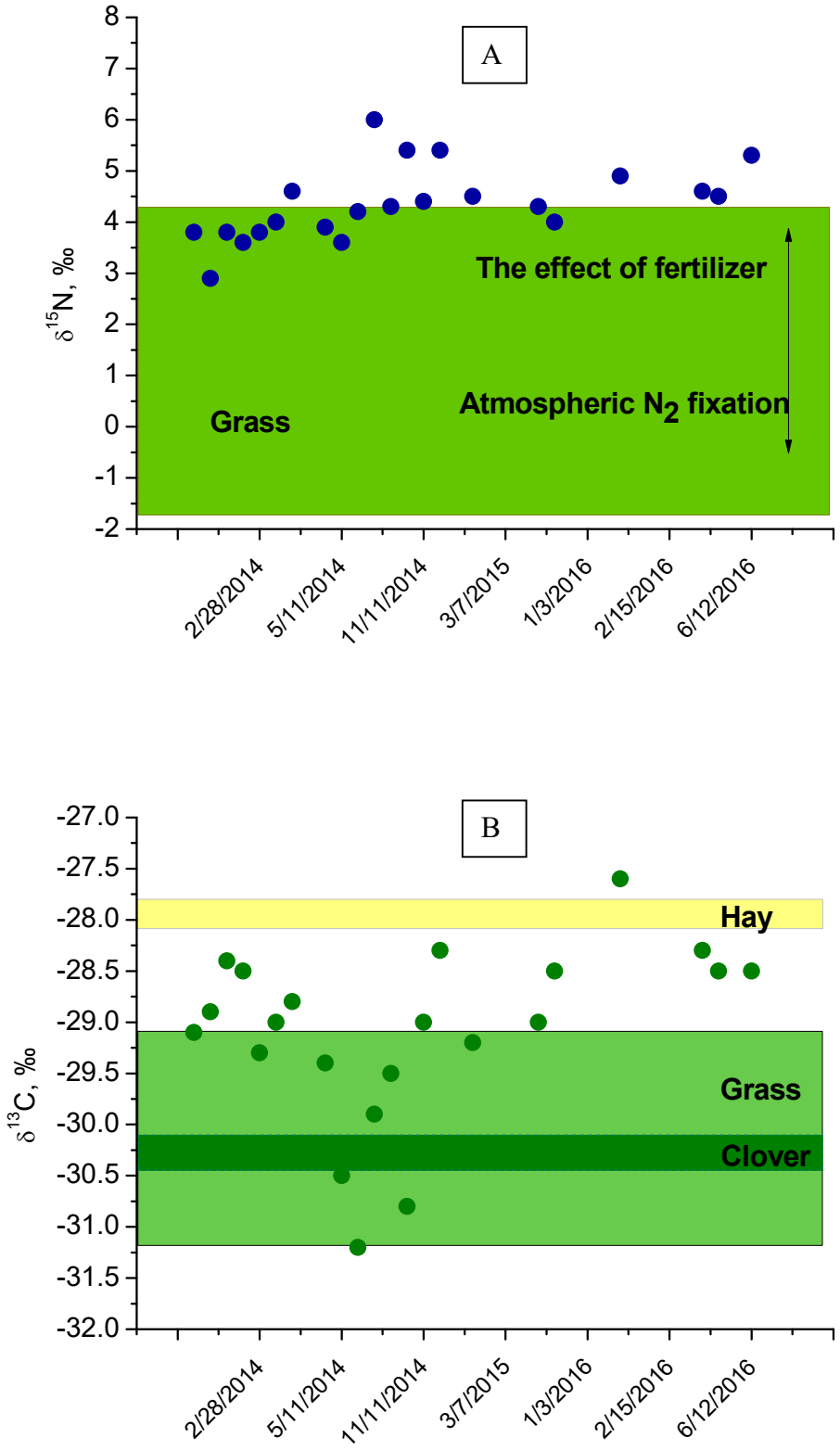

FIG. 5. Nitrogen (A) and carbon (B) stable isotope values in cow's milk, grass and hay during the years.

4. DISCUSSION

The main factors determining the isotopic composition of carbon and nitrogen is the forage, feed and/or fodder consumed by the animal. Camin et al. (2016) [2] demonstrated that the transition from C_3 to C_4 plant nutrition significantly altered the $\delta^{13}C$ value of casein and lipid in milk. Every 10 % increase in the quantity of C_4 plants in diet changes the $\delta^{13}C$ value of casein by approximately 1 ‰. Skipitytė et al. (2017) [3] showed that this increase was different for different broiler tissues (muscle, feathers, skin and blood) of the chickens tested. Cow's milk is also a product of livestock metabolism and reflects the result of the source of nourishment and the processes occurring in the body. During this work, it was found that the diet of the cows tested consisted of C_3 plants with $\delta^{13}C$ values ranging from -26 to -31 ‰.

For terrestrial plants, there are two main nitrogen sources, i.e., atmospheric nitrogen (N_2) and nitrates in the soil [4]. In the nitrogen fixing plants, e.g., in the legumes (peas, beans, clover, lucerne, etc.), the values of $\delta^{15}N$ are similar to atmospheric dinitrogen gas (0 ‰) [5]. The nitrogen isotope values of the plants depend on the isotope values of the nitrogen source; the isotopic ratio of the fertilizer nitrogen depends on the type of fertiliser. Fertilizers of natural (often animal) origin are isotopically enriched (up to + 25 ‰) and synthetic fertilizers have the values $\delta^{15}N$ ranging from -4 to + 4 ‰ as they are derived from atmospheric nitrogen through the Haber process. Areas contaminated with organic fertilisers can have very high values and the isotopic signal of plants is reflected at all higher nutritional levels [1]. The isotope ratio of nitrogen can thus be an indicator of organic farming. After combining $\delta^{13}C$ and $\delta^{15}N$ values, it is possible to distinguish the region of origin of milk. The composition of feeding stuffs is therefore one of the most important factors determining the isotope ratio values of the different locations of farmed animals.

The oxygen isotope ratio in the milk is the result of drinking water, food and metabolic influences [6]. $\delta^{18}O$ in water varies systematically and can often be predicted by geographical location, altitude and distance from the ocean [7]. The isotope ratio of oxygen in plants depends on the intensity of water use. In the meantime, the value of $\delta^{18}O$ in animal fluids is approximately 3 ‰ higher compared to the drinking water [8]. In order to better understand the distribution of isotope ratios in cow's milk, the measurements of the stable isotope ratios of the milk samples were compared with the results of precipitation and arteic water during this work. It was assumed that the value of the milk water $\delta^{18}O$ should reflect the ratio of $^{18}O/^{16}O$ in the area of drinking water. Therefore, the measurement of the milk water $\delta^{18}O$ should provide valuable information on geographical origin. However, the results of the measurements showed that the stable isotope values fluctuate over the years in different seasons. In winter/transitional period, $\delta^{18}O$ values reflect the groundwater signal, and during the summer season a large part of the water is obtained from fresh grass, which in turn usually receives water from precipitation, which shows dependence on different seasons. It may be possible to correlate the time of milk production with the presence of certain chemicals derived from fresh pasture such as higher concentrations of beta carotene in the milk.

The milk water is enriched by ^{18}O compared to ground water. This is explained by the fact that a significant part of the water is absorbed with fresh grass containing more than ^{18}O isotope compared to groundwater due to the evapotranspiration process, during which plants are more likely to remove the lighter isotopes. During the summer period, the animals also lose more water, which leads to a loss of the lighter isotope, while the remaining fluids in the body are more enriched the heavier isotopes.

ACKNOWLEDGEMENTS

The authors would like to offer their gratitude to The International Atomic Energy Agency (IAEA) for their funding. This work was carried out with funding under IAEA/FAO CRP D52038 project as part of the IAEA “Accessible Technologies for the Verification of Origin of Dairy Products as an Example Control System to Enhance Global Trade and Food Safety”.

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STABLE ISOTOPE RATIOS IN DAIRY PRODUCTS (MILK) AS NEW TOOL TO DETERMINE THEIR DIFFERENT ORIGINS IN MOROCCO

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Abstract

The research described in this paper evaluated the use of stable isotope ratio analysis for tracing the geographical origin of the milk dairy products produced within Morocco, with a view to extending the methodology to other Moroccan agricultural products. Twelve agro-ecological zones were selected characterized by different climates (Gharb region, Middle atlas and Meknes region, Loukouss, North, South (Agadir and Taroudant), Settat and Casablanca). During the sampling missions milk was taken directly from both modern and traditional bovine cattle dairies along with samples of cattle drinking water. For the isotope analysis of the milk and cattle drinking water, various techniques have been used such as Isotopic Ratio Mass Spectrometry (IRMS) and Cavity Ring Down Spectroscopy (CRDS). The analytical data obtained from these methods were then analysed using various chemometric methods such as principal component analysis (PCA) and hierarchal cluster analysis (HCA). The results demonstrated that stable isotope ratios analysis (SIRA) of hydrogen, carbon, nitrogen and oxygen of milk were linked to the territory, particularly the type of vegetation and the environment. From the oxygen and hydrogen SIRA we can observe three groups depending on altitude and distance from sea of the production zone. Regarding the $\delta^{18}O$ of the milk we observed three groups; the first one from the north, the second from the region with low altitude and close to Atlantic Ocean and the third for milk coming from the regions with high altitude and furthest from the Atlantic Ocean. The relationship between the $\delta^{18}O$ of liquid milk and cattle drinking water of dairies shows that isotopic composition of milk is influenced by drinking water. Regarding the $\delta^{13}C$ composition of milk we observed two separate groups of milk; milk coming from dairies that use only feed derived from C_3 plants, and the milk coming from the dairies that use feed with mixture of C_3 and C_4 plant material. The $\delta^{15}N$ values of the milk permitted separation between two groups; the first one for milk produced in dairies that utilise synthetic fertilizer (intensive agriculture) with relatively low $\delta^{15}N$ values, and secondly from dairies using less intensive agriculture, with higher $\delta^{15}N$ values.

1. INTRODUCTION

Located in western North Africa, Morocco is characterized by a predominantly semi-arid climate and a rapidly growing human population. Faced with this demographic expansion and changing nutritional habits, in the early 1970s, the Moroccan authorities launched ambitious plans to satisfy the demand for food. Revised livestock policies sought to establish intensive cattle production, based on importing heifers of dairy breeds and extending forage areas. At the same time, State authorities implemented a milk collection policy. Dairy cattle production has major social and economic roles in Morocco. In Morocco, milk and calves are produced on about 790 000 farms, of which only about 5 percent can be considered specialized dairy farms [1]. Milk and its derivatives have always been considered important but expensive components in the Moroccan diet, which is based mainly on cereals (bread) and vegetables. Milk has a strongly symbolic value in local traditions, as it is used with date fruits to welcome guests. Milk originates

principally from bovine cattle (2.7 million animals producing more than 96 % of annual dairy output) [1].

The globalization of food markets, and the facility of transportation of products through and between countries, generates worries for consumers about the origin of the food which they purchase. In recent years, geographical indications and appellation of origin have gained more importance in Morocco, which led to the establishment of the law #25 06 that was published in the Official Bulletin # 5640 on June the 19, 2008. This law regulates the use of distinctive signs of origin as they relate to the quality of food and agricultural products, including labelling of geographical indication (GI). To apply for labelling, geographical indication and origin appellation, producers' associations and food processors have to file a request (cahier des charges) to a national commission created for this purpose (article 17 of the law). Food and agricultural products that have been approved by this commission are registered and published in the Official Bulletin. To date, there are ten products that have been approved by The Moroccan National Commission for Geographical Indications and Appellation of Origin among them Chefchaouan Goat cheese (Minister of Agriculture decision that was published in the Official Bulletin # 5976 on September 8, 2011).

Many analytical techniques may be used to verify GI labelling claims, but stable isotope ratio and trace element characterisation is widely accepted as one of the best scientific approaches to determine the geographic origin of food products. The $^{18}O/^{16}O$ ratio of liquid milk depends on the water ingested and the proportion of fresh versus dry fodder. In turn, isotope ratios of precipitation and groundwater vary systematically dependent largely upon temperature, latitude, altitude and distance from the sea [2]. The$^{13}C/^{12}C$ ratio for both milk fat and cheese protein gives information on the type of forage fed to the cows. The carbon isotope value of plants depends on their photosynthetic cycles for CO_2 fixation [3]. C_4 plants such as maize exhibit relatively higher $\delta^{13}C$ values than C_3 plants, which constitute a major part of a cow's fodder in Morocco. Differences in the $^{15}N/^{14}N$ ratio also result essentially from forage. The nitrogen isotopic compositions of animal manures, composts, and other fertilizers permitted in organic production systems are significantly enriched compared to synthetic nitrogen fertilizers [4], [5]. The mean $\delta^{15}N$ values of organic fertilizers cluster around +8‰ and some fertilizers (e.g., animal manures) can have values higher than +35‰, due to the preferential volatilization of ^{15}N depleted ammonia in the field or during storage [5]. The $^{87}Sr/^{86}Sr$ ratio can also be useful for origin assignments as it is dependent only on the types of rocks and soils, and not on human activity, climate or season of production and is unfractionated by biological systems [6]. In this CRP the variability of $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{2}H/^{1}H$ isotope ratios and trace elements in milk were studied in order to determine the geographic origin of dairy products in Morocco. Consequently, the sampling strategy was based on the nature of the rock and soil, climate of the region and irrigated areas of rainfall and agriculture practices in the dairy farm locations.

2. MATERIALS AND METHODS

2.1. SAMPLING

The following ten physiographic units, described for soils, could be considered as major agro ecological zones as they represent some homogeneity in terms of landform / substrate soil, rainfall and the growing season (bioclimates and their thermal subdivisions).

The Middle Atlas
The Rif
The Loukkos area

The Rharb area
The Sais plateau
The Mamora and Central Plateau
The Chaouia plain and Casablanca
The plains and plateau north of the Atlas
The Argan zone
The High Atlas

The cow's milk samples were collected from various farms early in the morning. In total, 49 authentic samples of cow's milk from various geographical regions were obtained. At the same time farm water that was fed to the cow's was also collected. Table 1 shows where milk samples were collected e.g., the geographical information (longitude and latitude) and some of the farm's characteristics. Sampling was performed from the mixing tank where milk provided by the entire group of cows from a particular farm collected. Milk samples were transported refrigerated to the laboratory and stored at −18 °C until freeze drying.

TABLE 1. BACKGROUND INFORMATION FOR MOROCCAN MILK SAMPLES

Region	Coordoné X	Coordoné Y	Elevation	Distance from sea (km)	water sources	main product	Dominat breed	Stok lifestyle
KHM	33.79419	-6.11152	474.00	67.00	GW	Milk	straw, and local herb	Pasture
KHM	33.76108	-6.117877	453.00	73.00	GW	Milk	straw, and local herb	Pasture
MEK	33.78849	-5.50289	668.00	120.00	GW	Milk	straw, and local herb	Mixte
KHEN	33.61827	-5.43091	973.00	135.00	Surface water	Milk	straw, and local herb	Pasture
KHEN	33.3638	-5.553877	824.00	142.00	GW	Milk	straw, and local herb	Pasture
KHEN	33.110262	-5.58392	1155.00	155.00	GW	Milk	straw, and local herb	Pasture
KHEN	33.02809	-5.61646	908.00	163.00	GW	Milk	straw, and local herb	Pasture
KHEN	32.866758	-5.626805	862.00	172.00	GW	Milk	straw, and local herb	Pasture
KHEN	32.77009	-5.66733	870.00	178.00	GW	Milk	straw, and local herb	Pasture
KHEN	32.74449	-5.68693	853.00	179.00	GW	Milk	straw, and local herb	Pasture
KHEN	32.6301	-5.95201	713.00	170.00	GW	Milk	straw, and local herb	Pasture
BENI	32.2561	-6.56871	438.00	180.00	GW	Milk and derived	straw, and local herb	without Pasture
BENI	32.253567	-6.589554	443.00	181.00	Tap water	Milk and derived	straw, and local herb	without Pasture
BENI	32.208757	-6.824253	433.00	175.00	Tap water	Milk and derived	Straw and composite	without Pasture
MARA	31.729758	-7.643462	535.00	160.00	GW	Milk and derived	Straw and composite	without Pasture
MARA	31.729758	-7.643462	535.00	162.00	GW	Milk and derived	Straw and composite	without Pasture
MARA	31.694982	-7.703827	512.00	158.00	Tap water	Milk and derived	Straw and composite	Mixte
ESSA	31.574807	-9.270985	425.00	38.00	GW	Milk	Straw and composite	without Pasture
ESSA	31.537647	-9.54612	220.00	15.00	GW	Milk	Straw and composite	Pasture
ESSA	31.537647	-9.54612	220.00	15.00	GW	Milk	Straw and composite	Pasture
LARA	35.18184	-6.128093	3.00	3.00	GW	Milk and derived	local herb	Pasture
LARA	35.201349	-6.085113	3.00	5.00	Surface water	Milk and derived	local herb	Pasture
LARA	35.201042	-6.068602	3.00	7.00	Surface water	Milk and derived	local herb	Pasture
AROUS	35.264232	-5.617586	196.00	44.00	GW	Milk	local herb	Pasture
AROUS	35.305539	-5.631101	196.00	42.00	Surface water	Milk	local herb	Pasture
AROUS	35.309041	-5.644547	196.00	41.00	Surface water	Milk	local herb	Pasture
AROUS	35.37188	-5.778763	196.00	26.00	GW	Milk	local herb	Pasture
TROU	30.481339	-9.254076	132.00	45.00	GW	Milk and derived	composite and local herb	without Pasture
TROU	30.492297	-9.223522	134.00	39.00	GW	Milk and derived	composite and local herb	without Pasture
TROU	30.468816	-9.153006	133.00	43.00	Tap water	Milk and derived	composite and local herb	without Pasture
AGAD	30.270904	-9.504519	54.00	12.00	GW	Milk and derived	composite and local herb	without Pasture
AGAD	30.228748	-9.562233	50.00	6.00	GW	Milk and derived	composite and local herb	without Pasture
SETT	30.170873	-9.511819	83.00	12.00	GW	Milk and derived	composite and local herb	without Pasture
SETT	32.960727	-7.601045	495.00	63.00	GW	Milk and derived	Straw and composite	Pasture
SETT	32.898679	-7.573739	495.00	71.00	GW	Milk and derived	Straw and local herb	Pasture
SETT	33.080422	-7.625909	255.00	52.00	GW	Milk and derived	Straw and local herb	Pasture
SETT	33.163701	-7.618816	231.00	44.00	GW	Milk and derived	Straw and local herb	Pasture
CASA	33.295244	-7.577252	212.00	32.00	GW	Milk and derived	Straw and local herb	Pasture
CASA	33.421881	-7.520807	177.00	23.00	GW	Milk and derived	Straw and local herb	Pasture
Kenitra	34.478842	-6.385339	17.00	11.00	GW	Milk and derived	Straw, and local herb	Pasture
Kenitra	34.525358	-6.31043	10.00	16.00	GW	Milk and derived	Straw, and local herb	Pasture
Kenitra	34.603329	-6.170604	18.00	20.00	GW	Milk and derived	Straw, Maize, sugarcane, and local herb	Pasture

2.2. ANALYSIS OF MILK SAMPLES

In the laboratory the all the milk samples were stored frozen until the analyses were performed. The ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, were measured by an isotope ratio mass spectrometer coupled to an element analyser (EA IRMS).

Stable isotope data are expressed as δ values according to :

$\delta = (R_{sample}/R_{standard}) - 1$

Where R is the ratio of the heavy to light stable isotope of the element (e.g., $^{2}H/^{1}H$).

Raw milk samples were directly lyophilized using freeze drying in laboratory for preparation of milk powder.

Statistical data analyses were carried out on the data obtained from the various environmental and chemical and isotopic data. The data were processed using the statistical software package XLstat using principal component analysis and an analytical hierarchy process.

3. RESULTS AND DISCUSSION

3.1. STABLE ISOTOPE ANALYSIS OF CATTLE FEED WATER FROM FARMS

The first parameters that were analysed were the stable isotopes of samples taken from the groundwater used for cattle drinking water. The $\delta^{18}O$ and $\delta^{2}H$ values of cattle drinking water samples fell below the Local Meteoric Water Line (GMWL) [7], along a trend line shown in Figure. 1 below, indicating that they have been affected by evaporation [8]. By consulting the graph on water collected, we can observe some distinction between one region of production from another and correlation with the Local Meteoric Water Line (LMWL).

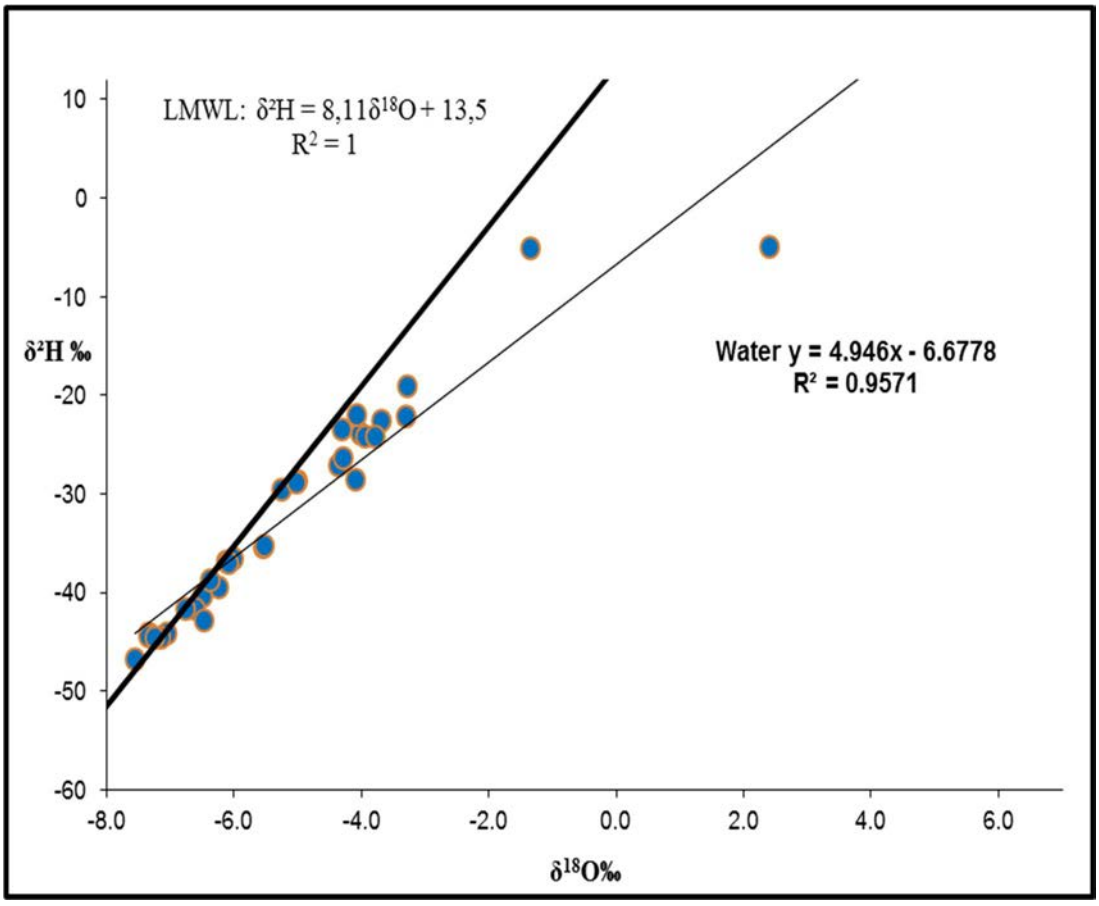

FIG. 1. A X-Y scatter plot of $\delta^{2}H$ versus $\delta^{18}O$ values of cattle feeding water from farms.

3.2. OXYGEN AND HYDROGEN STABLE ISOTOPE COMPOSITION OF FRESH MILK

Visual inspection of the diagram of the Dairy waters compared to the diagram of the water coming from milk, exhibits enrichment in the milk (Figure 2). The δ^2H and δ^{18}O values of water extracted from dairy milk samples were isotopically enriched relative to cow drinking water [9]. This phenomenon can be explained by the presence of internal metabolic (oxidation) water present in the milk. All milk samples exhibit enrichment with respect to water samples presenting a slope that is reminiscent of waters that have undergone evaporation process.

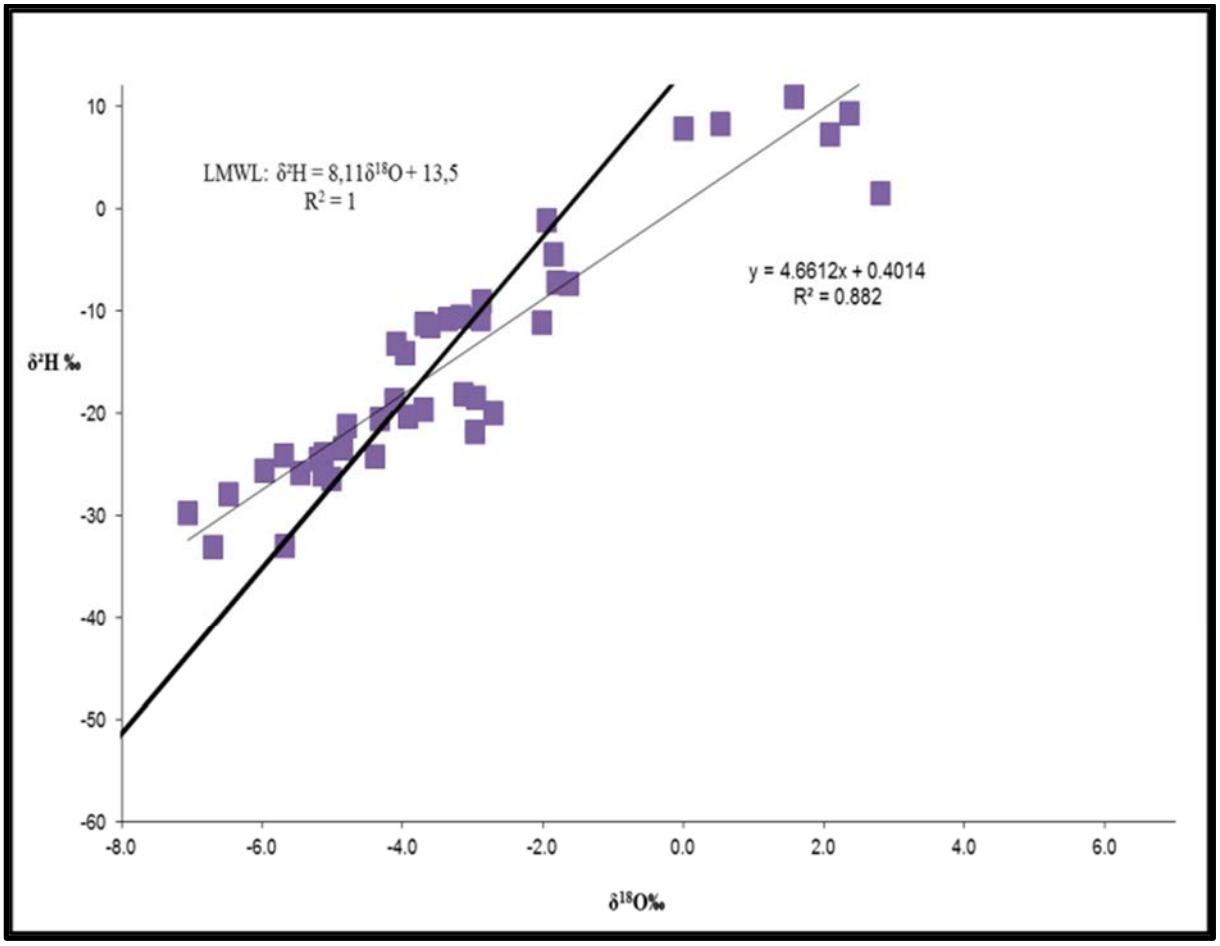

FIG. 2. A X-Y scatter plot of δ^2H vs. δ^{18}O values of water present in liquid Milk.

There was a significant linear relationship between δ^{18}O of farm water and δ^{18}O of milk water demonstrating that the isotopic composition if milk is influenced by the cattle's drinking water ($r^2 = 0.5$; $p < 0.05$) (Figure 3). A clear relationship between the oxygen isotope ratios in the milk samples and water from the same farm was also evident and aligned with anticipated effects of climate on $^{18}O/^{16}O$ fractionation [10]. However, clear correlations between geography and isotopic fractionation of the other elements examined were difficult to discern, due to limited availability of metadata and knowledge of the main factors influencing isotope abundance for these elements at the various dairy farm locations.

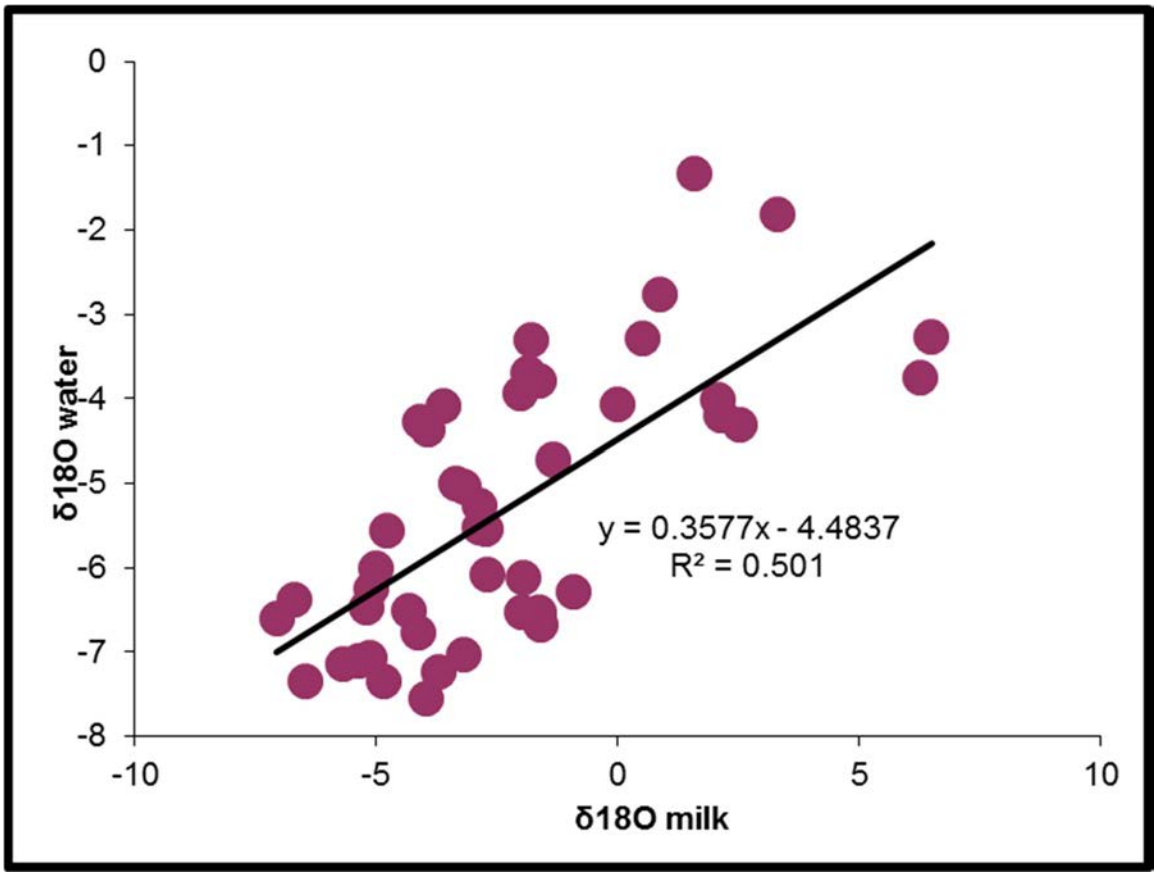

FIG. 3. A X-Y scatter plot showing the relationship between $\delta^{18}O$ of the water in fresh milk and $\delta^{18}O$ of the dairy cattle's drinking water obtained from the corresponding dairy farm.

In the same way as the isotope data for groundwater the water present in liquid milk showed a significant negative correlation ($r^2 = 0.5$ $p < 0.05$) between the $\delta^{18}O$ value and Altitude (Figure 4A). In the addition a significant negative correlation ($r^2 = 0.5$ $p < 0.05$) between isotopic value and distance from the Atlantic Ocean (Figure 4B). This can be attributed to progressive 'rainout' or 'continental effect' of the heavier isotopologues of water leaving the vapour phase as precipitation, in preference to the isotopically lighter isotopologues, as clouds move in land and to higher altitudes [11].

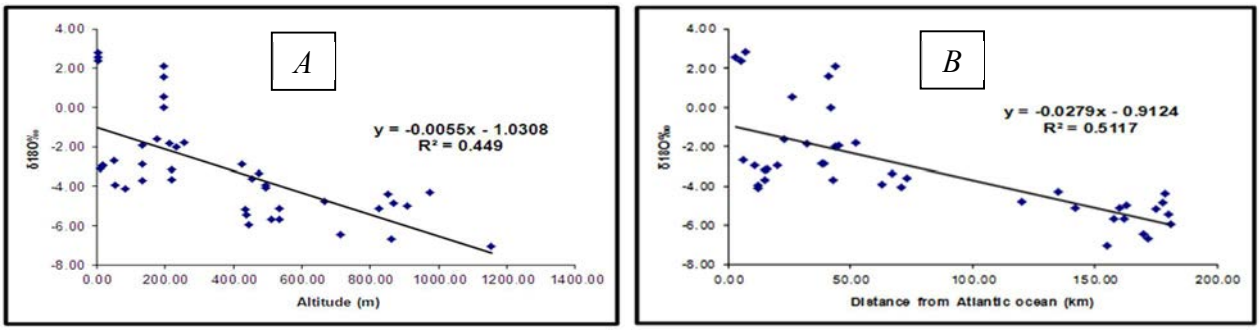

FIG. 4. Relationship between $\delta^{18}O$ of water in fresh milk, Altitude (A) and distance from the Atlantic Ocean (B) for the studied dairy farms.

Regarding the stable isotope composition of the water in milk (Figure 5 and Figure 6), three clearly distinguishable milk production areas were observed: The first from milk production at dairy farms located in the north influenced by Mediterranean Sea; the second one coming from the continental region with low altitude and the furthest distance from the Atlantic Ocean; and last one from the region close to Atlantic Ocean and at low altitude.

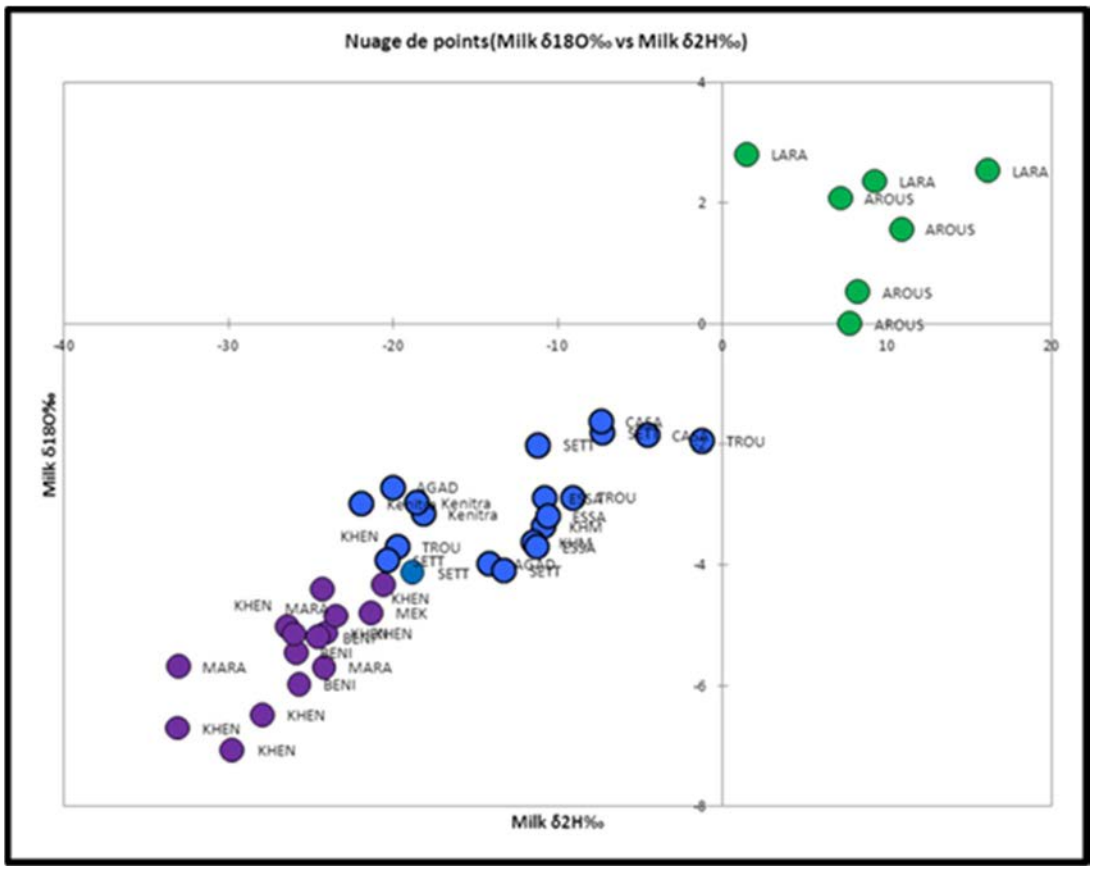

FIG. 5. A X Y scatter plot of $\delta^{18}O$ versus $\delta^{2}H$ in the water from liquid milk produced in different regions of Morocco.

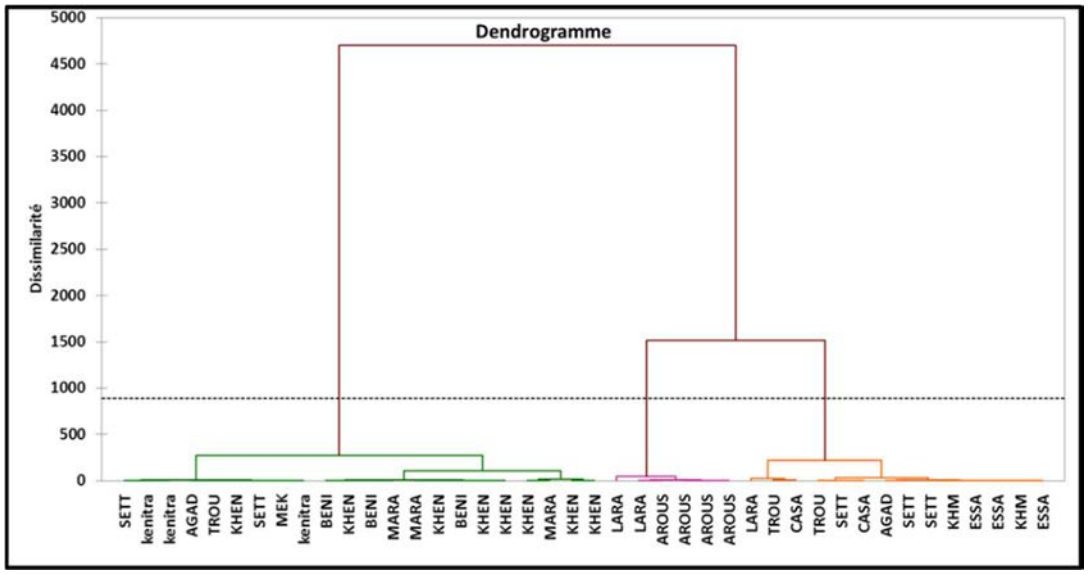

FIG. 6. Dendrogram for the origin of fresh milk generated from hierarchical cluster analysis. The first branch in brown illustrates the 3 main groupings of milk production.

3.3. CARBON AND NITROGEN STABLE ISOTOPES OF POWDERED MILK

The dominant geographical phenomena influencing isotopic fractionation varies for each of the light bio elements found in the nutritive components of our food (H, C, N, O and S). In the case of carbon, the differing photosynthetic CO_2 fixation pathways used by plants, Calvin cycle (C_3) or Hatch Slack cycle (C_4) leads to the predominant and characteristic $\delta^{13}C$ fractionation in plant tissues, which have the relatively minor fractionating effects such as temperature, humidity hours of sunlight, wind speed etc superimposed upon them. The net effect of these fractionations is diet related and then rapidly reflected in the milk of cattle grazing on pasture or eating plant fodder material [12]. Carbon isotope tissue ratios vary from -16 ‰ to -7 ‰ in C_4 plants and from -35 ‰ to -20 ‰ in C_3 plants [13]. The proportion of C_4 plant material in cattle diets is often related to the use of maize fodder over winter months in dry rations, but in some countries such as Brazil and the USA this can be due to grazing on C_4 grasslands [14].

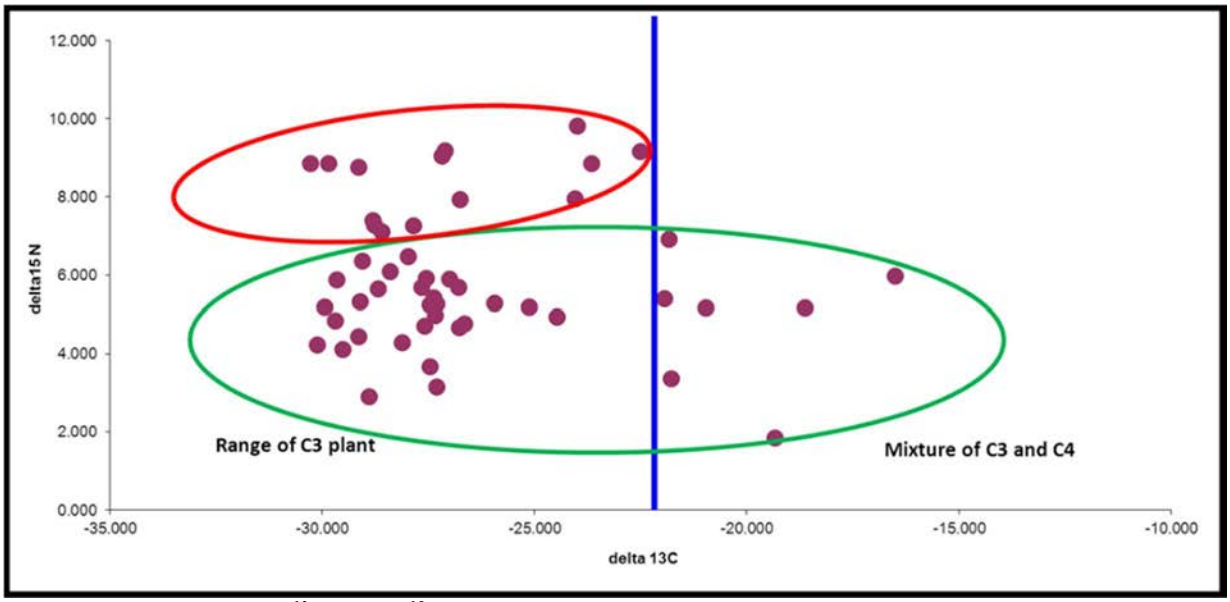

FIG. 7. A X Y scatter plot of $\delta^{13}C$ versus $\delta^{15}N$ in powdered Moroccan milk from different regions of production.

Based on $\delta^{13}C$ analysis of powdered milk solids produced from the liquid milk samples (Figure 7) there are, broadly speaking, two groups; the first one ranged from -23,98 to -30,27 ‰ corresponding to C_3 plant intake and second one from -16,5 to -21,9 ‰ corresponding to a mixture of C_3 and C_4 plant fodder. However, there is an understanding of at least some of the factors that influence the fractionation of ^{15}N in soils and in plants. The intensity of agricultural practices, fertilisation regimes, and the proportion of legumes and grain in the diet of animals impacts on the $\delta^{15}N$ values of cattle tissues [15]. Broadly speaking the $\delta^{15}N$ values of powdered milk solids shown in Figure 7 fall into two groups: the first one ranging from 7.2 to 9.8 ‰ corresponding to region with less intensive agriculture; and the second one ranging from 1.8 to 6.4 ‰ corresponding to intensive agriculture.

4. CONCLUSIONS

The results demonstrated that stable isotope ratios of H, O and C, N of milk water and solids were linked to the territory, particularly the type of climate/environment and vegetation/fodder respectively. From the $\delta^{18}O$ and $\delta^{2}H$ three regions of production could be observed depending on the dairy farms altitude and distance from sea; the first one from the north of Morocco; the second from the dairies in the region with low altitude and close to Atlantic Ocean; and lastly those dairies located in continental regions with high altitude and a significant distance from the Atlantic Ocean. The relationship between the $\delta^{18}O$ of liquid milk and drinking water of dairy

cattle showed that the isotopic composition of milk was clearly influenced by drinking water. The $\delta^{13}C$ values allow the milk production to be separated into two groups coming from dairies that use only feed with C_3 plant materials, and milk coming from the dairies that use feed with mixture of C_3 and C_4 rations The stable nitrogen isotope values exhibited a separation between two groups; the first one for the milk coming from dairies with utilization of fertilizer (intensive agriculture) characterised by relatively low value of $\delta^{15}N$ values and the second one from les intensive agriculture, with relatively high values of milk solid $\delta^{15}N$).

As a consequence of this study on Moroccan bovine milk we have demonstrated that the geographical, climatic, pedological, geological, botanical, and agricultural factors affect the stable isotope ratios of bio elements in nature, and isotopic variations are ultimately incorporated into animal tissue and products such as milk throughout eating, drinking, breathing, and exchange with the local environment, being incorporated into the resulting foods. As a consequence, the stable isotope ratios analysis of H, C, N, O, have shown high potential for determining geographical origin of milk.

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APPLICATION OF STABLE ISOTOPE TECHNIQUES AND ELEMENTAL ANALYSIS TO CONFIRM GEOGRAPHICAL ORIGIN OF MILK PRODUCED IN THE RUSSIAN FEDERATION

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Abstract

A method to determine the provenance of milk using isotope ratio mass spectrometry combined with inductively coupled mass spectrometry has been developed by measuring the isotope ratios (C, N, O, H) and the concentrations of 28 elements in milk, respectively. The applicability of the method was evaluated on 400 authentic milk samples collected from different geographical locations in the Russian Federation. A quantitative assessment was carried out by performing several classification tests, such as linear discriminant analysis, random forest, support vector machines, artificial neural network and stochastic gradient busting. Nonlinear models were optimized by repeated k fold cross validation. The highest classification accuracy obtained on an independent test set of 100 milk samples was 96%, with overall precision within 93 96 % for all nonlinear models.

1. INTRODUCTION

This work was carried out under Coordinated Research Project (CRP) D52038 with the aim to facilitate developing and creating a control system to trace the geographical origin of dairy products. Through cooperation with the participants of the CRP, the key research steps as well as the main methodology have been established. Based on the participants' previous experience and overall scientific evidence, it was recognized that isotope and elemental analysis would be the main instrumental techniques of the CRP. It is expected that the experience gained in this project might be applied in the future to other food commodities. Stable isotope analysis, including its combination with other complementary analytical techniques, has been used previously to trace the origin of food [1]. The complementary analytical methods for checking the origin and authenticity of products are diverse [2, 3]; such as elemental analysis (ICP-MS, ICP AES) [4, 5]; methods based on spectroscopic 'fingerprinting' techniques (^{1}H NMR, ^{13}C NMR, SNIF NMR) [6, 7, 8], infrared (near, MIR) spectroscopy [9] and fluorescence spectroscopy [10, 11]; and chromatographic/metabolic profiling (HPLC-MS, GC-MS) [12, 13]. The listed complementary methods are often powerful tools in their own related fields of application, and they may also have their limitations.

In addition to developing the method itself, an important aspect of this project was to establish a database from the authentic cows' milk samples. This would permit unknown test samples to be compared with the database and assigned to a geographical area, or milk samples with a claimed origin to be verified, with some degree of probability. However, collecting authenticate milk samples directly from farms in different countries is an expensive and time consuming step. Sharing National data bases and harmonizing methods, with other scientific groups, in other countries is fundamentally more rational approach. In order to achieve that concept, it requires well established, equivalent standard operating procedures; that is why under this project many international laboratories were united in the collaborative CRP D52038. The objective of this particular study was to establish a method based on stable isotope and elemental analysis, and to assess its applicability for confirmation of geographical origin of milk produced in the Russian Federation.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

Ultratrace grade nitric acid (67 69 %) and hydrogen peroxide (30 %) were obtained from Fisher Chemicals (UK), ultrapure water (18.2 MΩ.cm) was generated by a WaterPro PS water–purification system (Labconco, USA), analytical grade methanol and chloroform were obtained from Sigma Aldrich (Germany). For the ICP MS instrument calibration a multi element standard solution (100 mg/L) containing Ag, Al, As, Be, Ba, Cd, Co, Cu, Cr, Mn, Ni, Se, Tl, Pb, V, U, Zn (Minor elements, Agilent, USA), a multi element standard solution (500 mg/L) containing Ca, Na, Mg, K, Fe (Major elements, Agilent, USA), an internal standard mix (10 mg/L) containing Li^6, Sc, Y, Ge, In, Tb, Bi (Agilent, USA), calibration solutions (each 10 g/L) containing Rb, Sr, Mo (Panreac Quimica S.A.U., Spain) were used. A tuning solution (10 mg/L) consisting of Mg, In, Ba, Cs, Pb, U was supplied by PerkinElmer (USA). Working (or monitoring) gases for IRMS e.g., liquid carbon dioxide (>99.8), nitrogen (99.995%), helium (>99.995%), oxygen (99.999), hydrogen (99.999%), carbon monoxide (>99.99%) were supplied by LCC «NII KM» (Russia). Silver and tin capsules were obtained from ThermoFisher Scientific (Germany). The following reference materials and standards: IAEA 153 Milk powder, IAEA 155 Whey powder, IAEA 600 Caffeine, IAEA 601 Benzoic acid, IAEA CH 7 Polyethylene foil, were provided by IAEA (Vienna / Seibersdorf, Austria).

2.2. STANDARD SOLUTIONS

The working internal standard (IS) solution of 500 µg/L was prepared by dilution of stock internal standard mix in 5% nitric acid. The IS was stored in a tightly sealed plastic bottle under room temperature and pressure (RTP) conditions and remained stable for up to 6 months. The working solution A (WS A) of concentration 10 mg/L was prepared by transferring into a 50 ml volumetric flask, containing approximately 40 ml of 2 % nitric acid, 50 µL of stock solutions (Rb, Sr, Mo), adding 5.00 ml of the minor elements stock solution and diluting with ultrapure water. WS A was stored in a polypropylene tube at RTP conditions for up to 2 weeks. The working solution B (WS B) of concentration 500 µg/L was prepared in a 10 ml volumetric flask filled with 5 ml of 5 % nitric acid by pipetting 500 µL of WS A, 100 µL of the major elements stock solution and diluted with ultrapure water. WS B was stored in a plastic tube at 4 8°C and remained stable for up to 1 week. A set of 7 calibration standards was used to create multilevel calibration curves covering all concentration ranges of interest for 24 elements. The preparation scheme is summarized in Table 1 below.

TABLE 1. PREPARATION OF MULTI ELEMENTCALIBRATION STANDARDS (VALUES are µL)

Standard	WS A	WS B	Major mix	IS
Blank				250
STD#1		50		250
STD#2		100		250
STD#3		500		250
STD#4	50		50	250
STD#5	250		100	250
STD#6	500		500	250
STD#7	2500		1000	250

All standards including the calibration blank were prepared daily in 50 ml volumetric flasks by diluting corresponding amounts of the working solutions and standards using 2% nitric acid.

2.3. STATISTICAL SOFTWARE

The raw IRMS and ICP MS data were acquired using the ISODAT 3.0 (Thermo Electron, Germany) and ELAN 3.0 (PerkinElmer, USA), respectively. Statistical data processing was performed using a free programming language for statistical calculations R version 3.4.1 [14] launched though open source integrated development environment RStudio Desktop version 1.0.153., with the following software packages installed: caret 6.0 76 [15], e1071 1.6 8 [16], gbm 2.1.3 [17], ggplot2 2.2.1 [18], kernlab 0.9 25 [19], MASS 7.3 47 [20], nnet 7.3 12 [20], pROC 1.10.0. [21], randomForest 4.6 12 [22], tmap 2.1 1. [23], tidyverse 1.2.1[24].

2.4. SAMPLES

2.4.1. Sampling

Raw milk samples were collected twice. Initial sampling was undertaken in 2013–2014 and then repeated in 2017. The sampling strategy had been developed with the aim of taking representative samples from various geographical areas and at the same time including regions with the largest volume of milk production. As a result, the sampling on the territory of the Russian Federation was carried out within the extreme points summarized in Table 2 below.

TABLE 2. EXTREME POINTS OF THE SAMPLING AREA IN THE RUSSIAN FEDERATION

Direction	Latitude	Longitude
Northernmost	60.6686	46.3289
Easternmost	50.3587	127.9991
Southernmost	43.5667	43.5833
Westernmost	56.2179	35.6480

Overall, 251 milk samples were collected in 2014 and 396 in 2017. The number of samples provided by 65 administrative units of the Russian Federation are shown on the circular bar plots (Figure 1). All milk was delivered directly to the laboratory from remote regions after being deep frozen prior to transport. All samples were stored at 35 °C prior to analysis.

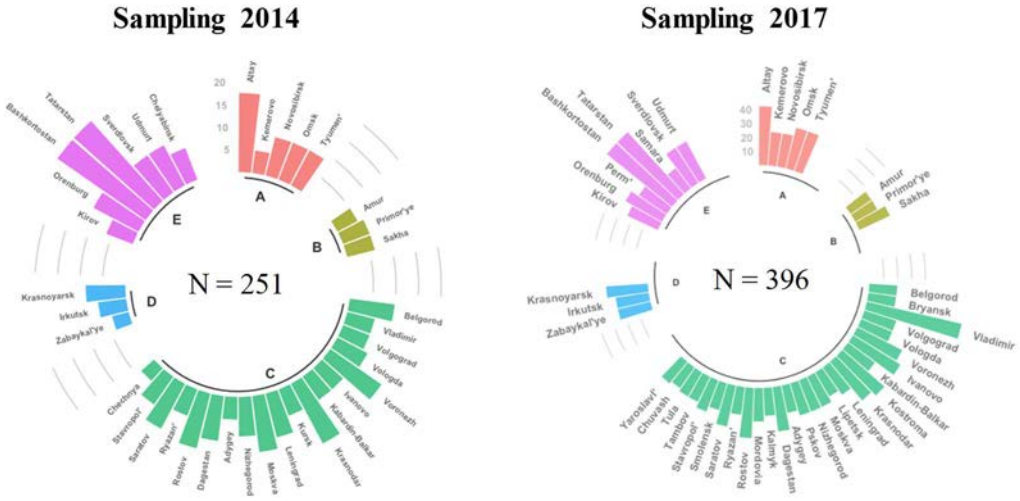

FIG.1. Detailed distribution of milk samples by regions. Labels A – E represent the 5 major groups formed by geographical proximity of the regions.

2.5. SAMPLE PREPARATION

2.5.1. Freeze drying

Frozen milk samples were thawed at RTP for 2 3 ours. The thawed milk (approximately 100 mL was homogenized for 10 min at 25 000 rpm using a dispersing tool T25 basic ULTRA TURRAX® (IKA, Germany). A portion of the liquid (thawed or fresh) milk (approximately 20 25 mL was placed into a 100 mL bottle, closed with a lid and frozen at 70 °C. After cooling samples below 30 °C, the bottles were attached to a Freeze Drier Virtis BenchTopK2 (SP Industries, USA) and left under low pressure (65 120 mTorr) overnight. When sublimation was complete, the resulting dry matter was transferred into a 15 mL plastic tube, tightly capped, placed in a desiccator, and stored in the dark until further processing and analysis.

2.5.2. Fat extraction

The procedure for defatting of milk powder was adapted from a protocol provided by Isotrace Research laboratory[1], and implemented with some minor modifications. Briefly, approximately 2 g of milk powder (lyophilised milk) was weighed into 15 ml polypropylene centrifuge tube. Then 10 mL of chloroform methanol solution (2:1 v/v) was added, and the lid closed tightly. The mixture was homogenized thoroughly in an ultrasonic bath PSB GALS (Russia) for 10 minutes and placed in a centrifuge MPW 260R (MPW. Med Instruments, Poland) operated at 5000 rpm for 5 minutes (with the temperature at 12 °C). The supernatant was removed with a Pasteur pipette and the extraction centrifugation steps were repeated two more times. To remove residues of organic solvents, the defatted milk powder was left in a desiccator attached to freeze drier overnight. The defatted dry matter (DDM) was stored in closed tubes in the dark at 5–8°C until it was required for isotopic and elemental analysis.

2.5.3. Acid digestion

A portion of DDM in the range of 400 450 mg was accurately weighed and placed in polytetrafluoroethylene (PTFE) vessels (70 mL) for microwave assisted decomposition. Three mL of ultrapure water, 5 mL of 65% nitric acid, and 250 µL of IS solution were added. The vessels were tightly closed and loaded into a microwave digestion system (Mars 5 Xpress, CEM Corp., USA). The following microwave program was used; ramping time 15 minutes, target temperature 200 °C, hold time 10 min, cooling 10 min. After cooling the vessels were opened and the digest was transferred into 50 mL polypropylene tubes and made up to the mark with ultrapure water.

2.6. INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP MS) ANALYSIS

2.6.1. ICP MS conditions

Element analysis was performed using an Aurora M90 (Bruker, Germany) ICP MS system equipped with a single quadrupole mass filter, a peristaltic pump operated at 7 rpm, a low flow concentric nebulizer Micromist (quartz), an autosampler ASX 520 (CETAC, USA), a double pass Scott spray chamber (quartz) whose internal temperature was maintained at +3°C by Peltier Element. The main ICP MS parameters used are summarized in Table 3.

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TABLE 3. THE VALUES OF ICP MS OPERATIONAL PARAMETERS

Parameter	Value/description
RF Power (W)	1370
Plasma Flow (L/min)	13.5
Nebulizer flow (L/min)	0.85 0.94
Auxiliary flow (L/min)	1.05
Sheath Gas (L/min)	0.24
Torch alignment (mm)	15.0 (Sampling Depth)
Sampler cone	Ni
Skimmer cone	Ni
Dwell time (ms)	35 80
Integration Time (s)	3
Data acquisition mode	‘ Peak Hopping ‘
Scans per Replicate	20
Replicates per Sample	3
Isotopes, m/z	^{6}Li, ^{9}Be, ^{23}Na, ^{24}Mg, ^{27}Al, ^{39}K,^{44}Ca, ^{45}Sc,^{52}Cr, ^{55}Mn, ^{57}Fe, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{87}Rb, ^{88}Sr, ^{89}Y, ^{98}Mo, ^{111}Cd, ^{115}In, ^{133}Cs, ^{137}Ba, ^{159}Tb, ^{205}Tl, ^{208}Pb, ^{209}Bi, ^{232}Th, ^{238}U

2.6.3. Calibration

To compensate for instrumental drift, sample loss, and matrix effects, an internal standardization approach was applied. To all calibration standards, blank and samples 2.5 µg/L of internal standard solution was added. Table 4 shows correspondence between analytes and normalization elements.

TABLE 4. ANALYTE – INTERNAL STANDARD CORRESPONDENCE

Internal Standard	Analyte
^{6}Li	Be
^{45}Sc	Cr, Co
^{74}Ge	Cu, As, Se
^{89}Y	Ni, Rb,
^{115}In	Mg, Al, Mn, Cu, Sr, Mo, Cd
^{159}Tb	Ba, Zn
^{209}Bi	Cs, Pb, Th, U,
NONE	K, Ca, Fe, Na

The working concentration ranges covered by calibration standards are summarized in Table 5. After every 15 milk digest sample analyses the ICP MS instrument was recalibrated.

TABLE 5. THE CALIBRATION RANGES AND CORRESPONDING STANDARDS USED

Analyte	Range	Units	Standards
Na, Mg, K, Ca	0.5 10	mg/L	STD#4 – STD#7
Fe	10 – 500	µg/L	STD#1 – STD#4
Al, Sr	5.0 – 50	µg/L	STD#3 – STD#5
Zn, Rb	10 – 500	µg/L	STD#4 – STD#7
Cu, Ba	1.0 – 10	µg/L	STD#2 – STD#4
Be, Cr, Mn, Co, Ni, As, Se, Mo, Cd, Cs, Pb, Th, U	0.5 – 5.0	µg/L	STD#1 – STD#3

2.7. ELEMENTAL ANALYSER – ISOTOPE RATIO MASS SPECTROMETRY (EA IRMS)

Determination of carbon, nitrogen, hydrogen, and oxygen isotope ratios ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{2}H$) was performed using a Delta V Advantage isotope ratio mass spectrometer (ThermoFisher Scientific, Germany) coupled to a Flash 2000 elemental analyser (ThermoFisher, Germany). The EA was configured with oxidative and high temperature conversion reactors and equipped with a 'zero blank' autosampler. Automatic dilution of working gases as well as gaseous products generated by conversion of the sample inside the EA, was provided by the EA IRMS interface device Conflo IV (ThermoFisher Scientific, Germany). To measure $\delta^{13}C$ and $\delta^{15}N$, 400 µg of milk DDM was weighed on the micro balances Mettler Toledo XP6 (Switzerland), wrapped in a 9 × 5 mm tin foil capsule, and then introduced into an oxidizing reactor preheated to 1020 °C. In the case of $\delta^{2}H$ and $\delta^{18}O$, a sample of 170 µg was placed in a 6 × 4 mm foil capsule made of silver alloy and introduced into a pyrolytic reactor maintained at 1350 ° C. The following certified materials were used for oxygen, hydrogen and carbon/nitrogen δ scale calibration; IAEA 601 (180 µg), IAEA CH 7 (50 µg), and IAEA 600 respectively. The procedure is fully implemented in accordance with the Good Practice Guide for IRMS [25].

3. RESULTS AND DISCUSSION

Milk samples collected during 2014 and 2017 were subjected to isotopic and elemental analysis. Data sets acquired during this study were then merged resulting in a new data table that consisted of 400 observations (rows) and 28 variables (columns). The combined data were processed using a multivariate analysis (MVA). The main idea behind statistical treatment in this study is to apply a set of supervised learning algorithms to the experimental data and assess the performance scores of the proposed methods to separate milk samples on the basis of geographical production origin.

In general, any learning algorithm [26] seeks a function $f: \mathbf{X} \rightarrow \mathbf{Y}$, where $\mathbf{X}$ is the input space and $\mathbf{Y}$ is the output response. Dependent variable $\mathbf{Y}$ may be either continuous or discrete and hence can be applied in regression or classification respectively. Since each geographical coordinate pairs, i.e., longitude and latitude are known a priori, they were assigned to every single milk sample. As shown in figure 1, the sampling pattern is inhomogeneous, sparse, and clearly clustered. For a reliable and consistent regression model, given data are ill defined and unsuitable, instead of the coordinates, the dependent variables $\mathbf{Y}$ were recorded as class labels,

so that a learning algorithm would solve the classification task while a desired function became a classifier. In this particular study, all observations (milk samples) were assigned to one of the 5 groups: A, B, C, D or E. Grouping was performed by two clustering algorithms. Both k means and hierarchical clustering analysis (HCA) converged to the equal number of groups with nearly the same composition in them. The aggregation of the objects (single sampling points) into groups was based on proximity of sampling points, i.e., on their geographical coordinates. HCA was derived from a Euclidean distance matrix with Ward's method as an agglomeration algorithm. K means was run with 200 iterations and 25 random starting points.

Once the **Y** response had been obtained, the five supervised classification models were built: stochastic gradient boosting [27], random forest [28], support vector machines (SVM)[29], artificial neural network (ANN)[30], and linear discriminant analysis (LDA)[26]. The further data processing included several steps, such as splitting, pre-processing, training and tuning, and cross validation to make an initial assessment of the successfulness of the models to assign milk to its geographical production area based on stable isotope and multi element analysis. In splitting, the data was divided into training and test sets, where the fraction of the test set is 25% (100 samples). The test set was used exclusively at the final cross validation step. Followed by Yeo–Johnson transformation [30, 31], centering and scaling were applied to all variables. For LDA, ANN, and SVM an additional feature extraction based on the principal component analysis was performed. The optimal combination of model parameters was found by using 10 times repeated 5 fold cross validation [26] procedure. Where the accuracy (a fraction of correctly predicted milk sample origins) were chosen as a performance metric. The classification results and the tuning parameters of the models used are summarized in Table 6.

TABLE 6. CLASSIFICATION RESULTS AND TUNING PARAMETERS OF MODELS

ALGORITHM	**ACCURACY [% correct]**	**PARAMETRES**
Stochastic gradient boosting	96	Number of trees, interaction depth, shrinkage, observation in node
Random Forest	93	none
SVM (Support vector machines with radial basis function kernel)	94	sigma, C
ANN (Artificial Neural Network, with one hidden layer)	94	size, decay
LDA (Linear discriminant analysis)	84	none

The best method performance score (96 %) on the validation set has been achieved for the stochastic gradient boosting model. In general, the results of all nonlinear models are very close, while LDA performance is 10 % lower on average. It suggests that even in the given multidimensional space decision boundaries are quite nonlinear. The obtained confusion matrices are presented in Table 7.

The off diagonal entries (those are not the main diagonal) represent number of misclassified objects between any possible pairs out of the defined groups. Since Group B and E are well isolated, they were classified very accurately even by linear algorithm. Nevertheless, there is a persistent mutual confusion between groups A, C, D, applying more sophisticated algorithms has improved overall accuracy, significantly decreasing misclassification between milk production origins A versus D and C versus D.

TABLE 7. CONFUSION MATRICIES FOR DIFFERENT MVA MODELS APPLIED TO THE DETERMINATION OF THE ORIGIN OF AUTHENTIC RUSSIAN MILK SAMPLES

LDA (SCORE 84%)					
	A	**B**	**C**	**D**	**E**
A	18	0	0	**3**	0
B	0	14	0	0	0
C	0	0	16	0	0
D	**8**	0	**5**	19	0
E	0	0	0	0	17
Accuracy	**69%**	**100%**	**76%**	**86%**	**100%**

Stochastic gradient boosting (SCORE 96%)					
	A	**B**	**C**	**D**	**E**
A	24	0	0	**1**	0
B	0	14	0	0	0
C	0	0	20	0	0
D	**2**	0	**1**	21	0
E	0	0	0	0	17
Accuracy	**92%**	**100%**	**95%**	**95%**	**100%**

The most important variables were extracted from the final stochastic gradient boosting model. In Figure 2, a subset of the top ten variables ranked according to their relative importance [30] are shown. It is established that on the given data major elements, such as Na, K, Fe, Ca, Mg, do not have significant impact on the group discrimination because their concentrations are most likely controlled by the cows' metabolic homeostasis. In contrast, trace elements, e.g., Sr, Co, Co, Cu, Ba, Mn turned out to be very essential distinctive features of the models. It is also remarkable that all stable isotopes of the light bio elements (H, C, N and O) are on the top ten list which reemphasizes the importance of the IRMS technique in geographical origin assignment of foods.

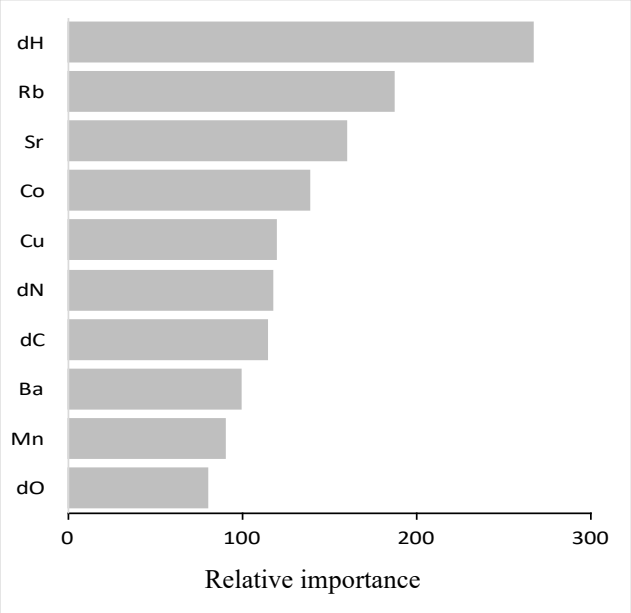

FIG. 2. Top ten most important variables for the assignment of authentic Russian milk samples to their production geographical origin.

4. CONCLUSIONS

The results of the study demonstrated that the isotopic ratios of light bio elements (HCNO) in combination with the concentration of selected elements can effectively discriminate milk samples on a scale as large as the territory of The Russian Federation with acceptable accuracy. An essential role in that result is addressed by the isotopic composition and trace elements. On the other hand, to further increase accuracy of the method and hence to distinguish more closely located points, some improvements need to be achieved. Involving extra factors (variables), for instance obtained from compound specific isotope analysis, or heavy element stable isotope ratios (e.g., $^{87}Sr/^{86}Sr$) might extend the capabilities of the method so that more precise and reliable results can be achieved. All in all, this opens up new opportunities for determination origin of milk.

FURTHER RESEARCH

As an extension of the proposed method, it is intended to develop compound specific isotopic analysis (CSIA) for determination of $\delta^{13}C$, $\delta^{2}H$ in fatty acid methyl ethers derived from milk fat $\delta^{13}C$, $\delta^{15}N$ in amino acids of milk proteins. Anonymized challenge samples tested against the models would also provide an independent means of verifying the reliability of the technique for enforcement work and the temporal stability of the models with respect to changes in factors such as feeding regime of the dairy cattle.

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STABLE ISOTOPE AND MULTI ELEMENTAL PROFILING OF MILK AND DAIRY PRODUCTS IN SLOVENIA

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Abstract

The development and application of analytical tools for the verification of geographical, production and species origin of food products are two of the main topics in food science. The research presented here uses stable isotope ratios and elemental composition to determine the regional provenance of milk and dairy products and to detect possible adulteration. The results of this work include the first database of authentic Slovenian cow, sheep and goat milk and cheese, and includes the isotopic composition of oxygen (δ^{18}O values) in milk; the isotopic composition of hydrogen, carbon, nitrogen, oxygen and sulfur in casein (δ^{2}H, δ^{13}C, δ^{15}N, δ^{18}O and δ^{34}S values); the content of fatty acids, and elemental composition. The data also includes Sr isotope ratios (^{87}Sr/^{86}Sr) in milk that can improve the verification of the origin of milk. The developed and validated methods were further used to verify the situation in the Slovenian retail market. In addition, the use of the δ^{18}O values of lactose as an internal standard was found to be a promising method to detect dilution of milk with exogenous water.

1. INTRODUCTION

Identifying the origin and authenticity of food has become increasingly complex due to changes in modern food markets, including the globalization of food sources and their distribution. Fortunately, stable isotope fingerprinting has proven to be a reliable approach for characterizing food commodities according to their origin and authenticity since it is possible to record aspects of various environmental, chemical and biological processes linked to food origin and production techniques. The application of isotopes can be even more effective when combined with elemental composition [1]. However, stable isotopes and elemental fingerprinting relies on having access to a reference dataset derived from analysis of authentic products, which can be both time consuming and costly to create. Once created, authenticity can then be evaluated by comparing the isotopic and elemental values of commercial samples with the limits computed based on the contents of the authentic reference dataset, which are evaluated in terms of best fit using a suitable statistical model [2]. Overall, taking an integrated approach using appropriate analytical techniques, databases and statistical models, it is possible to objectively and scientifically verify the authenticity and origin of the food, which allows traditional products that have high added-value labelling claims to be protected and promoted.

This approach was the subject of the project titled "*The use of stable isotopes and elemental composition for determination of authenticity and geographical origin of milk and dairy products*" as a part of CRP D52038: "*Accessible technologies for the verification of origin of dairy products as an example control system to enhance global trade and food safety*" using milk and dairy products as an example commodity. Due to their high nutrient content milk and dairy products represent an essential part of a healthy balanced diet. They are also in considerable demand and relatively expensive, which makes them vulnerable to economically motivated adulteration.

This publication summarizes the results of the project and includes:

- guidelines on sampling milk and dairy products
- analytical standard operating procedures (SOPs) for the determination of elemental composition and stable isotope ratios of light (H, C, N, O and S) and heavy (Sr) elements in milk and dairy products
- details of the establishment of a database of the elemental and isotopic composition of Slovenian milk and dairy products
- a statistical evaluation of results and the possibility of differentiating between different species and geographical origin of milk and dairy products
- establishing a model for controlling Slovenian milk in a real world application
- the investigation of the use of the oxygen isotope value of lactose, as an internal isotopic standard, to detect milk dilution with exogenous water.

2. MATERIALS AND METHODS

2.1. SAMPLE COLLECTION

In the first phase of sampling, 122 samples of milk were systematically collected during May, June and July in 2012 and 2013 from several geographical regions in Slovenia. This included cow (76 samples), goat (11 samples) and sheep (35 samples) milk. The cows' milk was from Alpine, Dinaric, Pannonian and Mediterranean regions. Goat and sheep milk production are limited to a few regions in Slovenia namely Bovec (Alpine), Karst, Vipava, Brkini (Mediterranean), central Slovenian region (Dinaric). In August 2012, 30 cheese samples were collected, including sheep (15 samples), goat (6 samples) and cow (9 samples) produced from the milk collected in May from the same producers. Two types of cheese have EU Protected Denomination of Origin (PDO) status: Bovški sheep cheese (Bovški ovčji sir) and cow cheese (Tolminc) and one local PDO status Kraški sheep cheese (Kraški ovčji sir). Authentic Tolminc cow cheeses (9 samples) were collected in May, June, September and December in order to include the maximum variability of production and changes in animal diet. The second part of the project included only cows' milk samples. During the 3 year study, from 2012 to 2014, 277 raw cow milk samples were obtained two times per year (in winter and summer) from four geographical regions: Mediterranean, Pannonian, Dinaric and Alpine in Slovenia. The data represents a database of authentic Slovenian milk samples. Also, eleven commercial milk samples declared as Slovenian milk, and 13 samples of milk from five other European countries: Croatia, Hungary, Germany, Austria and Italy, were collected to verify their authenticity and for evaluating and demonstrating the robustness of the model. All milk samples were stored at −20 ^{o}C prior to analysis.

2.2. MEASUREMENTS AND ANALYSIS

One of the main issues in food authenticity is to ensure high quality and inter laboratory comparability of elemental and isotopic data. The focus of the first phase of the CRP project was to ensure method harmonization and data quality. It also included establishing Standard operational procedures (SOPs) for stable isotope composition measurements of O, H, C, N and

S in water and casein and elemental composition in milk. In this case, the isotopic composition of O in water and C, N and S in milk and cheese casein were determined at the Jožef Stefan Institute (JSI), while stable isotope ratios of H and O in milk casein were measured at the Fondazione Edmund Mach (FEM) in Italy. For elemental analysis, two different methods were applied: energy dispersive X ray fluorescence spectrometry (EDXRF) and the inductively coupled plasma mass spectrometry (ICP MS). Sample preparation and the analytical procedure for both analytical techniques were critically tested and evaluated according to uncertainty, accuracy, limits of detection (LOD) reported in a previous investigation [3]. A SOP for the determination of Sr isotopes using multi collector (MC) ICP MS was also developed [4].

2.2.1. Quality Assurance and Quality Control (QA and QC)

Certified reference materials (CRM), international standards and laboratory prepared standards were used for quality control of analyses. In addition, the Jožef Stefan Institute is involved in the inter comparison scheme: *Food analysis using Isotopic Techniques Proficiency Testing Scheme (FIT PTS)* organized by EUROFINS three times per year, which also includes casein and grated cheese. An inter laboratory exercise for stable isotope determination of C, N and S in rice flour, was also performed under the auspices of CRP D52038. The results are reported as a separate contribution in this publication.

For elemental analysis, the accuracy of the results was checked as follows:

- using certified reference materials: Whole milk powder NIST 8435, Non-fat Milk Powder NIST 1549 (both National Institute of Standard and Technology) and Skim Milk Powder BCR 150 (EC JRC IRMM), ERM BD150 and ERM BD151 (EC JRC IRMM);
- participation in proficiency testing scheme FAPAS (Food and Environmental Research Agency, Sand Hutton, York, UK).

2.3. STATISTICAL EVALUATION

Statistical calculations and multivariate analysis were carried out using the XLSTAT software package (Addinsoft, New York, USA). The basic statistics included mean values (median and arithmetic mean), standard deviation (S.D.), minimum and maximum. The multivariate analysis involved the use of discriminant analysis (DA).

3. RESULTS AND DISCUSSION

The first database of authentic Slovenian cow, sheep and goat milk and cheese was established. The interpretation of results to establish geographical origin was conducted using appropriate chemometric methods such as discriminant analysis (DA).

3.1. COMPOSITION OF COW, GOAT AND SHEEP MILK SAMPLES

A review of the literature revealed that the average amounts of Ca, P, K, Cl and Zn were higher in goats' and sheep milk than in cows' milk, while the concentration of S was slightly higher in cows' milk than in sheep and goats' milk. Our results agreed with the literature data regarding the content of Ca, P and Zn, whereas K and Cl, were higher in goats' and cows' milk than in sheep milk. The average amount of S and Br in the samples was higher in sheep and goats' milk than in cows' milk.

The $\delta^{18}O$ values of water in Slovenian cows' milk samples are comparable with reported values for milk produced in northern and central Europe ($\delta^{18}O$ of −11 ‰ to −2 ‰). Higher values were observed in the Mediterranean region. This reflects the comparatively warm and dry climate. The range in $\delta^{18}O$ values (−5.6 to 1.2 ‰) for sheep and goats' milk was similar. Higher minimum $\delta^{18}O$ values obtained in sheep and goats' milk when compared to cows' milk were predominantly related to water intake. sheep and goats mostly ingest water through grazing on fresh pasture and plants, whereas water intake for cows, in intensive systems, is from groundwater. The $\delta^{18}O_{cas}$ and $\delta^{2}H_{cas}$ values were determined only in cows' milk in 2013 and 2014. the $\delta^{18}O_{cas}$ values ranged from 9.0 ‰ to 14.6 ‰, and the $\delta^{2}H_{cas}$ values were from −149 ‰ to −93 ‰. $\delta^{18}O_{cas}$ values agree with literature values, whereas the $\delta^{2}H_{cas}$ values are lower than those reported in the literature. No correlation was observed between $\delta^{18}O_{cas}$ and $\delta^{2}H_{cas}$ values, which supports the finding of a previous study where 30% of the H and 70 % of the O in milk protein derives from local water [5], while the remaining fraction originates from dietary oxygen e.g., carbohydrate.

The results also reveal a broad range of $\delta^{13}C$ and $\delta^{15}N$ values in casein of cows', sheep and goats' milk. The $\delta^{13}C$ values vary from −28.2 to −17.8 ‰ for cows' milk, from −29.3 to −21.8 ‰ for sheep milk and from −29.2 to −23.7 ‰ for goats' milk. Variations in $\delta^{15}N$ values were similar for cows' and sheep milk (2.5 to 6.5‰), while for the goats' milk, the variation in $\delta^{15}N$ values was higher (−1.8 to 7.5 ‰). Figure 1 shows a plot of $\delta^{13}C$ versus $\delta^{15}N$ values for all milk samples. More than 80% of the cows' milk samples have $\delta^{13}C$ values above −23.5 ‰, indicating the presence of maize in the diet [6]. Most of these samples are from the Pannonian region with intensive milk production by barn fed cows. The lowest $\delta^{13}C$ values were observed in the Alpine and Mediterranean regions where the animals graze on grass pasture and hay. With the exception of three samples of sheep and goat milk, all the other milk samples were found to have $\delta^{13}C$ values < −23.5 ‰ (Figure 1).

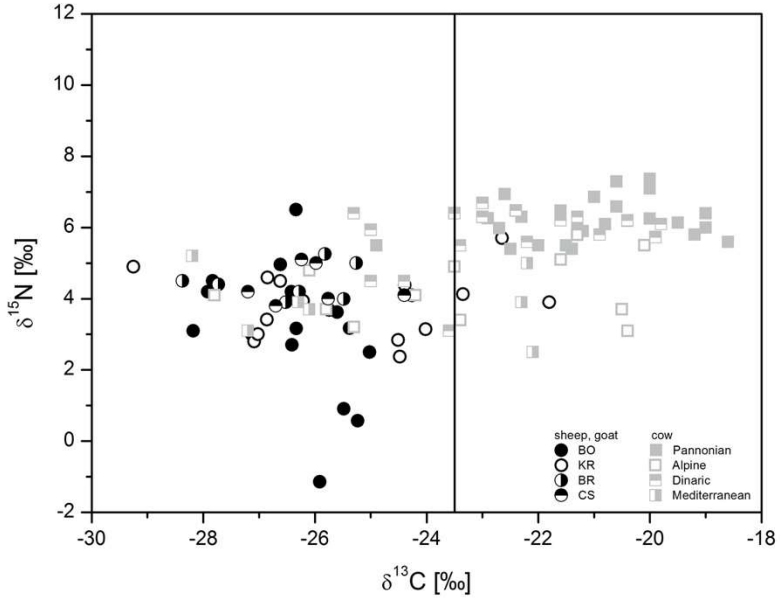

FIG. 1. Distribution of $\delta^{15}N$ versus $\delta^{13}C$ values in cows', sheep and goats' milk. The vertical line at $\delta^{13}C$ = −23.5 ‰ indicates the limit values above which the presence of maize in the diet can be detected [6].

The higher $\delta^{13}C$ values in these three samples suggest a maize contribution in the feed of about 30 to 40%. Additionally, grasses tend to be depleted in ^{15}N compared with maize and rape [7], which means that $\delta^{15}N$ values are useful in distinguishing between dairy products from animals raised on pastures or in intensive agriculture. Indeed, higher $\delta^{13}C$ values were accompanied by higher $\delta^{15}N$ values showing a higher content of maize in the feed. Maize is usually harvested from intensively fertilized fields. The lowest $\delta^{15}N$ values observed could be due to the presence of leguminous plants and the absence of intensive agricultural practices. The combination of $\delta^{13}C$ and $\delta^{15}N$ values also provide useful information concerning the geographical and agricultural origin of the milk (extensive production based on pasture based feed, or more intensive farming with a higher degree of corn) and thus on its quality. The $\delta^{34}S$ values are from 2.3 to 6.7 ‰ for cows' milk, 3.5 to 5.6 ‰ for sheep milk, and 3.1 to 6.7 ‰ for goats' milk. These values are typical for European dairy products, which are from 3 to 8 ‰ but differ significantly from the values determined in dairy products from various regions within Australasia, which are typically 9 to 15 ‰ [8].

Despite milk being a relatively complex food matrix with a high fat content and a low Sr content, the method proved to be effective at extracting Sr from milk samples. The measured $^{87}Sr/^{86}Sr$ ratios in milk from different locations reveal a complex underlying lithology and how the $^{87}Sr/^{86}Sr$ content of the milk differs within a specific region. For example, milk samples from two locations 16 km apart, namely Gornja Radgona ($^{87}Sr/^{86}Sr = 0.71199 \pm 0.00001$) and Radenci ($^{87}Sr/^{86}Sr = 0.72481 \pm 0.00001$), have significantly different values. However, milk samples from three different regions have a similar Sr isotopic composition (e.g., Sevnica:$^{87}Sr/^{86}Sr = 0.70925 \pm 0.00001$, Vinica: $^{87}Sr/^{86}Sr = 0.70948 \pm 0.00001$ and Šmarje pri Jelšah:$^{87}Sr/^{86}Sr = 0.70986 \pm 0.00001$). The reason for these differences is likely to be the diverse geology as well as the source of the feed, which is not necessarily from the same region where the cows graze. Consequently, determining the provenance of Slovenian milk from parameters such as $^{87}Sr/^{86}Sr$ is a challenging subject and requires further investigation in order to characterize the [9].

3.2. SEASONAL VARIATION AND GEOGRAPHICAL ORIGIN DETERMINATION

All results were statistically evaluated in order to determine the differentiation of the samples according to the season and origin of production. The evaluation of sheep, goats' and cows' milk and cheese is the subject of a paper by Nečemer et al. [10]. This study highlights the critical parameters, which in discriminant analysis (DA) classification, can differentiate milk and cheese samples according to geographical origin and can contribute towards supporting a Protected Designation of Origin (PDO). The results showed how it was possible to refuse possible imitations with a correct classification rate of 97 % and 100 % in cases relating to milk and cheese, respectively. Furthermore, results indicated that DA classification could be a useful tool for inspection and identification of the quality of milk and cheese according to its declaration since it is possible to differentiate between the different species. Based on the data obtained, a model to support traceability systems can be created, which may then be used to protect Slovenian PDO cheeses from mislabelling and can be used to independently verify the authenticity of commercial products.

The milk results were also evaluated statistically, including all sampling periods from 2012 to 2014. The study demonstrated that the database must be maintained on an annual basis, using a minimum of at least six samples obtained during summer and winter from each region. Differentiating the samples in terms of the geographical region was only possible if the same season and year of production were included. The current database, therefore, represents an excellent foundation for establishing a model for checking the origin of milk sold on the Slovenian market. The database contains twenty five test samples; eleven declared as Slovenian

and fourteen from different European countries (Croatia, Hungary, Germany, Austria and Italy). Five variables: $\delta^{18}O_{casein}$, $\delta^{2}H_{casein}$, $\delta^{15}N_{casein}$, Fe and Sr, were identified by canonical discriminant analysis as providing the optimum differentiation between Slovenian milk and milk produced elsewhere in the EU. In this study all milk labelled as Slovenian is within the authentic Slovenian group and leave one out cross validation correctly classified 86.8% of the samples.

3.3. LACTOSE AS AN INTERNAL STANDARD FOR DETECTING ADULTERATION OF MILK WITH WATER

This procedure utilizes the $\delta^{18}O$ value of the lactose extracted from milk in comparison to the water $\delta^{18}O$ value of the same milk sample. Several experiments were prepared, where the milk was diluted with water (1 to 30 % v/v) and the lactose isolated according to the standard procedure. The isotopic composition of oxygen in the lactose was determined using a DELTA IRMS connected to a TC/EA pyrolizer (Thermo Scientific) at Fondazione Edmund Mach (Italy). The $\delta^{18}O$ values in milk were determined directly in milk samples after equilibration with reference CO_2. Measurements were performed on an IRMS (GV Instruments) with an IsoPrime MultiFlow Bio equilibration unit. The $\delta^{18}O$ values in lactose were 20.3‰. The $\delta^{18}O$ value of lactose is correlated to that of the $\delta^{18}O$ of the milk water and can be considered as a reliable internal reference. The results show that by using this method, it is possible to detect 10% of added ground water. However, the detection limit is dependent upon the $\delta^{18}O$ of the added exogenous water source.

4. CONCLUSION

This study included a combination of different nuclear and complementary analytical techniques and approaches that can contribute to establishing and updating largescale databases and provides a scientific basis for the development of an appropriate system to support milk and dairy products traceability. A statistical model was developed and applied to control Slovenian milk in a ‘real world’ application. Further, the established system has broader applicability and can be readily transferred to other countries and food commodities.

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STABLE ISOTOPE INTERLABORATORY MEASUREMENT EXERCISE ON RICE FLOUR

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Abstract

This paper describes the interlaboratory study for the determination of stable isotope ratios of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$) and sulfur ($^{34}S/^{32}S$) in rice flour to demonstrate core measurement capabilities in this area for participants in CRP D52038. The characterization was performed on rice flour reference material (RM) prepared by the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA). The development of a new rice flour RM has been selected in order to promote stable isotopes for the use as markers of origin/authenticity. The performance of the nine participants was very good, illustrating their ability to obtain accurate results for carbon, nitrogen and sulfur isotope ratios within the calibration range afforded by internationally agreed reference materials. This was despite the fact that no two participants used exactly the same approach in terms of instrumentation or data treatment.

1. INTRODUCTION

Metrological support represents an essential part of stable isotope measurements since many analytical methods and approaches are not yet standardised and/or properly validated. One of the most important factors for improving method performance and lowering measurement uncertainty is the availability of suitable Reference Materials (RMs). Despite an increase in the production of new RMs in recent years, there is still a lack of fit for purpose RMs especially for stable isotope analyses in food science and there is a continuous need to develop new RMs with different matrix/analyte combinations to cover analytical requirements. This is especially the case for stable isotopes. The accurate measurements of stable isotopes in different matrices is also challenging for the following three reasons:

(1) the analytical methods available cannot not be properly validated given the lack of suitable CRMs and/or reference methods;

(2) isotope fractionation during the conversion of components to a gas for analysis remains poorly understood especially for complex multicomponent matrices; and

(3) components in the column separation combustion cycle often overlap.

The purpose of this interlaboratory exercise was to characterize a candidate rice flour RM prepared by the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA). The characterization included the determination of the stable isotope ratios of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$) and sulfur ($^{34}S/^{32}S$) in the bulk rice flour sample. The development of the new rice RM has initiated in order to promote, besides the characterization of 'conventional' parameters, stable isotopes for use as markers of origin/authenticity. Furthermore, this exercise was performed with the intention of providing a means of self-assessment regarding the quality of measurements (competence) as performed by a selection of stable isotope laboratories within CRP D52038 and a selection of recognised stable isotope food control laboratories to further improve the consistency of data reported. This paper describes the design and outcome of that interlaboratory comparison exercise for laboratories engaged in routine analysis of carbon, nitrogen and sulphur stable isotope composition in food samples organized by Jožef Stefan Institute (JSI).

2. LIST OF PARTICIPANTS/ORGANISATIONS

In total, nine institutions from nine different countries agreed to participate in the inter laboratory exercise. The participant laboratories and shown in Table 1.

2.1. SCHEDULE

Call for participation	April 2017
Registration	Deadline 31st May 2017
Sample Distribution	By end of June 2017
Deadline for Reporting Results	30th October 2017

TABLE 1. PARTICIPANTS IN THE INTERLABORATORY EXERCISE

No.	Institution	Abbrev.	Country	Contact Person
1	Isolab GmbH Laboratorium für Stabile Isotope	Isolab	Germany	Mr Andreas Rossmann
2	Foundazione Edmund Mach	FEM	Italy	Ms Federica Camin
3	Center for Physical Sciences and Technology	CPST	Lithuania	Mr Andrius Garbaras
4	Centre national de l'énergie, des sciences et des techniques nucléaires	CNESTEN	Morocco	Mr Noureddine Amenzou
5	Institute of Nuclear Chemistry and Technology	INCT	Poland	Mr Ryszard Wierzchnicki
6	Agri Food & Veterinary Authority	AF&VA	Singapore	Mr Lian Jie Bay
7	Jožef Stefan Institute	JSI	Slovenia	Ms Nives Ogrinc
8	Federal Centre for Animal Health	FCAH	Russian Federation	Mr Ivan Podkolzin
9	The James Hutton Institute	JHI	United Kingdom	Mr Barry Thornton

3. SAMPLES AND INSTRUCTION TO PARTICIPANTS

3.1. SAMPLE MATERIAL

Two batches of rice flour RM were prepared from 80 kg lots of super fine Carnaroli white rice flour (same variety and same origin) provided by Ente Nazionale Risi (IT). RM preparation was performed initially at the ENEA Agrofood RM Plant c/o Trisaia Research Centre (industrial scale production Basilicata Region, IT) and then completed at the RM Plant c/o Casaccia Research Centre (small scale production Lazio Region, IT). Raw materials were first homogenized in a Vrieco Nauta® Conical Mixer (V = 240 L) equipped with a dosing valve. After homogenization of the flour, the dust remaining on the Nauta inner walls was removed using N_2. The Nauta mixer was first rinsed with an aliquot (5 kg) of rice flour by rotating the mixer for 15 minutes and after rinsing the rice flour was discarded. This mixer was then reloaded and the process repeated multiple times using the same material. After rinsing the Nauta mixer was then loaded with the rice flour (75 kg) and allowed to rotate for 2 hours. Aliquots (3.2 kg) were then collected in plastic bags using the dosing valve, while maintaining rotation. The bags were sealed under vacuum and then shipped to the Casaccia Research Centre. On arrival, the material contained in each bag was re homogenised and partitioned using a Retsch sample divider (8 sub aliquots) by applying a two-step procedure. Finally, 50 g aliquots of rice flour where collected, labelled and shipped to the waiting testing laboratories for characterization. Each participant received at least two portions of the RM. Confirmation of receipt of the samples was requested from each participant by regular mail or e mail using the sample receipt form on arrival. The information requested in the sample receipt included the following: ID numbers of the test aliquots, arrival date, material condition on receipt (i.e., intact, damaged, notes), storage conditions, and the name of the person receiving the materials.

3.2. RM HOMOGENEITY STUDY

A homogeneity study was performed on a number of sub units representing the whole batch. Twenty units (including ten redundant units) were selected by using random stratified sampling software (TRaNS) and reserved for the study of homogeneity between units. Homogeneity tests were carried out for all candidate CRMs by measuring three sub samples under the same

repeatability conditions. The method used for these measurements was validated and the samples to be analysed were introduced to the instrument in random order to determine any trend arising from analytical and/or filling sequences. Certified reference materials and samples were analysed during the same run. All measurements were carried out using elemental analysis isotope ratio mass spectrometry (EA IRMS). Homogeneity tests were performed by the JSI. The results indicated that the isotopic composition of rice flour material was not significantly different between the vials (ten random vials analysed in triplicate gave a SD of 0.07 ‰). Statistical results (ANOVA) for homogeneity are given in Table 2.

TABLE 2. ANOVA RESULTS FOR THE HOMOGENEITY STUDY OF RICE FLOUR

	ANOVA Test for Homogeneity	
Analyte	F statistic	F critic
Rice flour	1.66	2.39

3.3. INSTRUCTIONS FOR PARTICIPANTS

There were no specific storage requirements for the vials of rice flour, although it was recommended that they were kept at room temperature. Vials could be opened multiple times during use. The amount of material used for isotopic measurements was defined by the usual protocols of the participant's laboratory. The instructions also asked that a delta value with uncertainty be reported as well as the results from at least 5 independent replicates. Participants were also instructed to provide full details of their methods including the amount of sample analysed, any corrections applied to the instrumental data (including but not limited to ^{17}O, drift, linearity, carryover and blank corrections) and the method applied for scale calibration.

The participants were free to choose any suitable method provided that they included a full description of the method used. It was recommended that at least two organic reference materials should be used for scale calibration and that the delta values assigned to these reference materials should be those recommended in the IUPAC technical report [1]. Each laboratory was encouraged to report a full uncertainty budget as part of their results. Contributions to the overall uncertainty would arise from the repeatability of the sample preparation, the repeatability of instrumental determination, scale calibration using suitable reference materials and any other parameter specific to the method of analysis chosen by the participant.

4. RESULTS AND DISCUSSION

4.1. MEASUREMENT TECHNIQUES

All participants used IRMS to determine the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of the rice flour, with an elemental analyser (EA) to convert the flour into gaseous CO_2 , N_2 and SO_2 , respectively (isotope ratio mass spectrometers are gas source instruments). IsoLab, FEM and JSI used an Elementar Vario PYRO Cube EA coupled to an Isoprime 100 mass spectrometer, while other participants used Thermo Scientific systems: Flash EA 1112 coupled via a Conflo interface to a Delta V Plus or Delta V Advantage mass spectrometer.

4.2. REFERENCE AND QUALITY CONTROL (QC) MATERIALS

None of the laboratories used the same suite of RMs for δ scale calibration. All participants used a one, two and multi point linear calibration approach. Table 3 lists the different RMs used by each participant. The values assigned to these RMs were those from the IUPAC Technical

Report [1]. The advantage of multiple point linear regression where *n* is greater than 2 is that random error associated with the analysis of any one reference material can be detected via the correlation coefficient [2]. To check the quality of the obtained delta values within a single sequence it is common to analyse one or more quality control material(s) within each sequence for which the delta value is well known. This can be another RM or a well characterised in house standard, or in an ideal situation, a material matrix matched to the sample. If the results for the QC material(s) following all corrections (including scale calibration) are within the expected range then the results for unknown samples can be assumed to be reliable. The following quality control material(s) were used: TRACE wheat flour (IsoLab, FEM), Sorghum flour and protein (IVA, Germany) (INCT) and SERCON protein (JSI). All the other participants did not report the use of any additional quality control materials.

TABLE 3. REFERENCE MATERIALS USED BY PARTICIPANTS

Lab. No.	**Reference materials (RMs)**					
	$\delta^{13}C$		**$\delta^{15}N$**		**$\delta^{34}S$**	
	Name	**Material**	**Name**	**Material**	**Name**	**Material**
1	BCR glucose NBS 22	Glucose Mineral oil	IAEA N 1 IAEA N 2 IAEA N 3	Ammonium sulfate Ammonium sulfate	IAEA S 1	Silver sulfide
2	USGS 42	Tibetan Human Hair	USGS 42	Tibetan Human Hair	USGS 42	Tibetan Human Hair
3	Caffeine		Caffeine			
4	Leucine		Leucine			
5	NBS 127				IAEA S 1	Silver sulfide
6	USGS 40 USGS 41	l Glutamic Acid l Glutamic Acid	USGS 40 USGS 41	l Glutamic Acid l Glutamic Acid		
7	IAEA CH 3 IAEA CH 6	Cellulose Sucrose	USGS 40 IAEA N 1 IAEA N 2	l Glutamic Acid Ammonium sulfate Ammonium sulfate	IAEA S 1	Silver sulfide
8	Caffeine					
9	USGS 40 USGS 41	l Glutamic Acid l Glutamic Acid	USGS 40 USGS 41	l Glutamic Acid l Glutamic Acid		

4.3. STATISTICAL EVALUATION OF RESULTS

4.3.1. Determination of the consensus value and standard deviation of the interlaboratory comparison

The statistical evaluation of the results was performed using an International Harmonised Protocol [3], where the following sequential steps were considered:

- Detection and removal of outlier data
- Calculation of the summary parameters
- Calculation of the performance indicators
- Graphical presentation of the obtained results.

4.3.2. Scores and evaluation criteria

Individual laboratory performance was expressed in terms of z scores (z) in accordance with the International Harmonised Protocol [3]:

$$z = \frac{(X_{lab} - X_{assigned})}{\sigma_p}$$

where X_{lab} is the measurement result reported by a participant, $X_{assigned}$ is the assigned value and σ_p is the target standard deviation for proficiency assessment. The z scores can be interpreted as follow:

$|z| \leq 2$ satisfactory result;

$2 < |z| \leq 3$ questionable result (95 %);

$|z| > 3$ unsatisfactory result (99 %).

4.4. REPORTED CARBON, NITROGEN AND SULFUR ISOTOPE RATIO DELTA VALUES

The δ values reported by the participants can be found in Table 4, while the evaluation of results are graphically presented in Figures 1 3.

TABLE 4. REPORTED $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ VALUES TOGETHER WITH THE AVERAGE, STANDARD AND TARGET DEVIATION (SD), MAXIMUM AND MINIMUM VALUES FOR RICE FLOUR

Lab. No.	$\delta^{13}C$ – ‰	$\delta^{15}N$ - ‰	$\delta^{34}S$ - ‰
1	-27.80	5.10	4.10
2	-27.70	4.68	3.26
3	-28.16	4.70	
4	-28.30	4.73	
5	-27.71	4.79	2.42
6	-28.12	4.98	
7	-28.25	5.21	3.14
8	-28.02		
9	-28.08	5.48	
Mean	**-27.99**	**4.95**	**3.19**
STD	**0.23**	**0.27**	**0.60**
Target SD	**0.15**	**0.30**	**0.50**
Max	**-27.70**	**5.48**	**4.10**
Min	**-28.30**	**4.68**	**2.42**

All participants reported $\delta^{13}C$ and $\delta^{15}N$ data, however $\delta^{15}N$ value from participant 8 was excluded from the evaluation, since the statistical evaluation detected their reported value as an outlier. Participant 8 was informed about this issue and suggestions for remedial actions made to check the measurements and normalization of results. $\delta^{34}S$ data were obtained from only of the participating laboratories due to the technical challenges of routinely measuring SO_2 gas.

Lab. No.	Sample	Z-score
4	-28.30	-2.09
7	-28.25	-1.74
3	-28.16	-1.16
6	-28.12	-0.89
9	-28.08	-0.62
8	-28.02	-0.22
1	-27.80	1.25
5	-27.71	1.85
2	-27.70	1.91

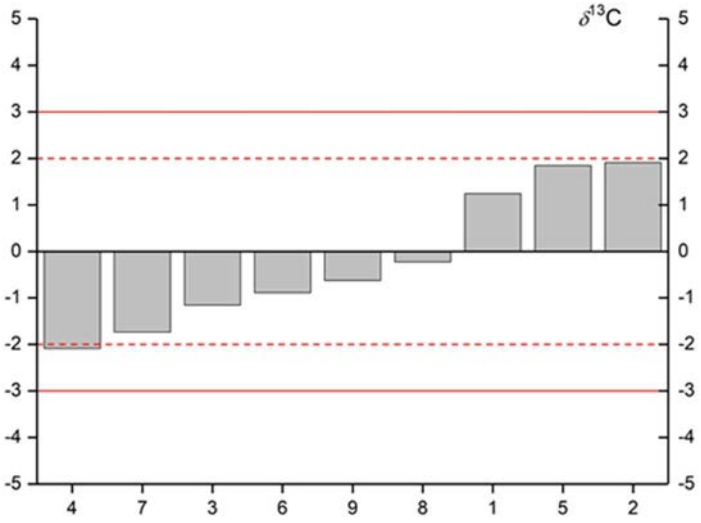

FIG. 1. Interlaboratory z score results for $\delta^{13}C$ values in rice flour.

Lab. No.	Sample	Z-score
2	4.70	-0.88
3	4.73	-0.82
4	4.79	-0.72
5	4.84	-0.52
6	4.98	0.12
1	5.10	0.52
7	5.21	0.88
9	5.48	1.78

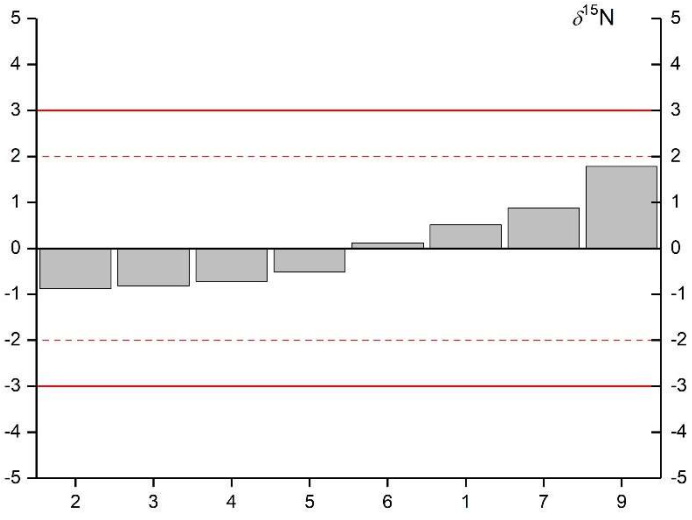

FIG. 2. Interlaboratory z score results for $\delta^{15}N$ values in rice flour.

Lab. No.	Sample	Z-score
5	2.42	-1.28
7	3.14	-0.08
2	3.26	0.12
1	4.10	1.52

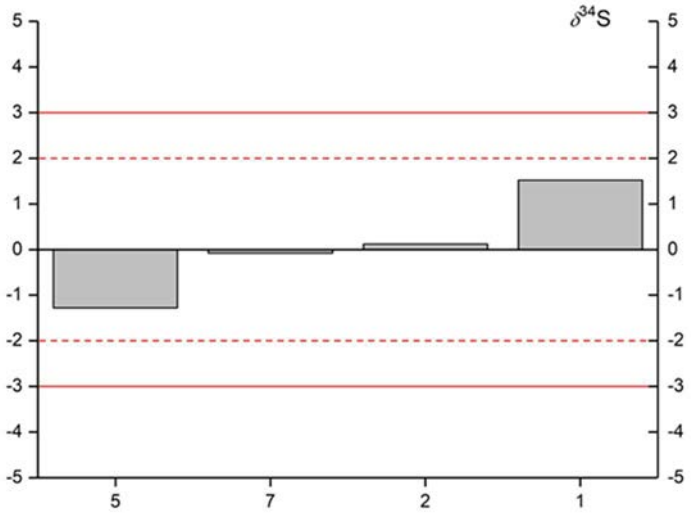

FIG. 3. Interlaboratory z score results for $\delta^{34}S$ values in rice flour.

5. CONCLUSION

The performance of all interlaboratory exercise participants was very good, illustrating their ability to obtain accurate results for carbon, nitrogen and sulfur isotope ratios, within the calibration range afforded by internationally agreed reference materials. This was despite the fact that no two participants used exactly the same approach in terms of instrumentation or data treatment.

ACKNOWLEDGEMENTS

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DIFFERENTIATION OF THE GEOGRAPHICAL ORIGIN OF RETAIL MILK IN SINGAPORE USING STABLE ISOTOPE SIGNATURES AND ELEMENT CONCENTRATIONS

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Abstract

Singapore's heavy reliance on imports to meet her peoples' food requirements means the country is especially susceptible to food safety and food fraud related incidents. With food fraud concerns increasing globally due to the involvement of organised crime syndicates, systems that can independently assure the traceability of food along the supply chain in addition to current paper based approaches is becoming increasingly important. The setting up of a scientific traceability programme in Singapore using stable isotope and trace element concentration (SITE) measurements was demonstrated using dairy milk as an example matrix. 296 dairy milk samples from ten countries of origin were analysed over a period of two years. Differences in their isotope signatures and elemental content were found to be suitable to be used to differentiate them based on their country of production.

1. INTRODUCTION

Singapore is a city state with limited agricultural capacity that relies on imports to meet its food supply requirements. Such reliance on imported food makes Singapore susceptible to food safety and food fraud related incidents. With food fraud concerns increasing globally, systems that can assure the traceability of food along the supply chain is becoming of rising importance. However, paper based traceability may be vulnerable to documentary errors or even fraud and manipulation. Consequently, there is a need to establish science based analytical methods to verify and compliment current paper based traceability approaches.

The Agri Food and Veterinary Authority of Singapore (AVA) is the national food safety authority under the Ministry of National Development (MND). Its primary role is to ensure a resilient supply of safe food, and to safeguard the health of animals and plants for the wellbeing of the nation. The Veterinary Public Health Centre (VPHC) under the Laboratories Group (LG) is the cornerstone of AVA's integrated food safety programmes through its provision of scientific expertise and comprehensive laboratory testing services. Recognising Singapore's vulnerability to food safety incidents given its heavy reliance on imports to meet its food supply requirements, LG initiated a traceability programme in 2013 to build up its capacity in developing the necessary skills and expertise in food origin determination.

Dairy milk was selected to be one of the matrices of interest because it is an important food commodity that is widely consumed locally. Almost all of the milk that the country consumes is imported since the local dairy industry is too small to cater for domestic demand. Singapore imports both pasteurised and UHT milk from a large number of countries, which is subsequently sold in the local markets. These include regional countries such as Australia, Malaysia and Thailand as well as international suppliers such as France and the United States. It is therefore important that AVA has in place a system that can verify the geographical origin of milk to support food safety systems and underpin food origin labelling so that consumers can make informed choices, with confidence, about the origin of the food they consume.

Many methods to discriminate foods for geographical origin have been developed, among which isotope ratio measurements and elemental profiling have been recognised as arguably the best hypothesis driven technique to link a food to its production origin. For instance, Crittenden et al. [1] found that multi element stable isotope ratios of carbon, oxygen and sulfur were useful in distinguishing between dairy products produced in different regions of Australasia. Similarly, Luo et al. [2] showed that isotope signatures of carbon, nitrogen, hydrogen and oxygen could be used to discriminate milk from Australia, China, France, Germany, New Zealand and the United States. With regard to elemental profiling, previous studies to discriminate the geographical origin of milk have mainly focused on regional distinction. For example, Brescia et al. [3] identified Ba, Mn, Zn, Al, Fe and Cu as the key elements that could be used for the discrimination of cow's milk samples from the Apulia region in southern Italy. Meanwhile, Huque et al. [4] found that Ca, Mg, Na, K, Mn, Zn and Cu were the key elements selected for the differentiation of raw milk samples from four different climatic zones of Bangladesh. In this study, we demonstrate the use of both isotope ratios and element concentrations in the geographical discrimination of dairy milk sold on the Singaporean retail market.

2. MATERIALS AND METHODS

2.1. SAMPLE COLLECTION

Singaporean milk samples were collected directly from the dairy farms while samples from Australia, France, Germany, Indonesia, Japan, Malaysia, New Zealand, Thailand and the United States were purchased from local supermarkets over a period of 2 years, whilst acknowledging that their provenance was not guaranteed. It was noted that all samples imported from Malaysia were reconstituted milk while others were either fresh pasteurised or ultra-heat treated (UHT) milk. To investigate the differences between reconstituted milk and fresh/UHT milk, 8 additional reconstituted milk samples were purchased from a supermarket in Indonesia while 3 more were purchased from a supermarket in Malaysia. A summary of the number of samples collected as well as the number of samples analysed for their isotope ratios and elemental concentrations is given in Table 1.

TABLE 1. TOTAL NUMBER OF MILK SAMPLES COLLECTED FROM EACH COUNTRY AND THE NUMBER OF SAMPLES ANALYSED FOR (A) ISOTOPE RATIOS ONLY (B) ELEMENT CONCENTRATIONS ONLY AND (C) BOTH ISOTOPE RATIOS AND ELEMENT CONCENTRATIONS

Country	**Total Number of Samples Collected**	**Total Number of Samples Analysed**		
		(a) Isotope Ratios ONLY	**(b) Element Concentrations ONLY**	**(c) Both**
Australia	59	30	9	20
France	10	6	1	3
Germany	8	6		2
Indonesia	18	6		4
Japan	8	4	2	2
Malaysia	23	12		8
New Zealand	25	12	6	7
Singapore	116	113	3	
Thailand	15	6	5	4
United States	25	12	6	7
Total	296	207	32	57

Numbers in brackets indicate the additional reconstituted samples purchased from supermarkets in Malaysia and Indonesia to investigate differences between reconstituted milk and fresh pasteurised/UHT

2.2. REAGENTS AND SOLUTIONS

2.2.1. Isotope Ratio Measurements

2 M hydrochloric acid (HCl) used for the extraction of casein from milk was prepared from a 37% HCl purchased from RCI Labscan (Bangkok, Thailand).

2.2.2. Elemental Profiling

All solutions were prepared using deionized water (18.2 MΩ/cm) produced from a PURELAB® Ultra ELGA (High Wycombe, United Kingdom) water purification system. 70 % nitric acid (ULTREX ® II Ultrapure Reagent, J.T. Baker, Phillipsburg, NJ) and 30 % hydrogen peroxide (ULTREX ® II Ultrapure Reagent, J.T. Baker, Phillipsburg, NJ) were used for sample digestion.

The multi element reference solutions were prepared from five 100 mg L^{1} stock solutions (Inorganic Ventures, Christiansburg, VA and Accustandards®, New Haven, CT). The multi element standards included 52 elements, such as Ag, Al, As, Ba, Be, Bi, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Ga, Gd, Ge, Hf, Ho, In, La, Li, Lu, Mn, Mo, Nb, Nd, Ni, Pb, Pd, Pr, Pt, Re, Ru, Sb, Se, Sm, Sn, Sr, Ta, Tb, Th, Ti, Tl, Tm, U, V, W, Y, Tb and Zr. Single element reference solutions (Ca, Fe, K, Mg, Na, P, S, Si, Zn) were prepared from 1000 mg L^{1} and 10 000 mg L^{1} stock solutions (Inorganic Ventures and Accustandards). Single element reference solutions (Ir, Rh) were prepared from 1000 mg L^{1} stock solutions (Inorganic Ventures) as internal standards.

Standard reference material, milk powder (NIST 1549b, National Institute of Standards and Technology (NIST), Gaithersburg, MD) was used to evaluate accuracy and normalize data for day to day analysis. A powdered milk sample was also used as an in house reference material for the normalization of data for day to day analysis.

2.3. SAMPLE PREPARATION

2.3.1. Isotope Ratio Measurements

Carbon and nitrogen isotope ratio measurements were made on the casein fraction in milk. Casein was extracted from the milk samples following procedures described in Camin et al. [5] with slight modifications. Briefly, 40 ml of milk sample was poured into a 50 ml centrifuge tube then centrifuged at 7000 x g (Sorvall® Evolution, Thermo Fisher Scientific, Waltham, MA) for 10 mins at 4 6 °C. Any fat present in the milk would form a layer on the surface of the milk where it could be skimmed off. The process was repeated twice to ensure that all fat present in the milk was removed prior to the precipitation of casein. Casein was precipitated from the defatted milk by adjusting the pH of the milk to 4.3 using 2 M HCl. The extracted casein was transferred to a new 50 ml centrifuge tube. Ultra-pure water from an ELGA water system (High Wycombe, United Kingdom) was added to the 50 mL mark and the mixture was centrifuged at 3400 x g for 10 mins under ambient conditions. The process was repeated another 4 times to wash the casein before it was dried using a freeze dryer (SP Scientific, Warminster, PA) for a minimum of 36 hours.

2.3.2. Elemental Profiling

15 g of liquid milk samples were weighed into a 50 mL polypropylene tube and dried using a freeze dryer (Martin Christ, Germany) for at least 30 hours until a consistent dry weight was obtained. 0.4 g of freeze dried milk was microwave digested (UltraWAVE®, Milestone, Shelton, CT) using 5 mL 70 % nitric acid (HNO_3). The microwave digestion program was as shown in Table 2.

TABLE 2. TEMPERATURE PROGRAM FOR MICROWAVE ASSISTED ACID DIGESTION

Stage	Duration /min	Max Voltage /W	T1 /ºC	T2 /ºC	Pressure /bar
1	10	800	110	70	90
2	10	1200	180	70	90
3	10	1500	240	70	120
4	10	1500	240	70	120

Digested solutions were cooled to room temperature and 1 ml of 30 % hydrogen peroxide was added to the solutions before being transferred to 60 mL LDPE bottles and diluted with deionized water up to 30 g by weight. Further dilution was performed for the analysis of elements of high concentrations beyond the calibration solution range.

2.4. INSTRUMENTAL ANALYSIS

2.4.1. Isotope Ratio Measurements by IR MS

Carbon and nitrogen isotope ratios were measured using a Flash 2000 elemental analyser linked online via a Conflo IV interface to a Delta V Advantage continuous flow isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The system was operated in the dual isotope mode, allowing carbon and nitrogen isotope ratios to be measured simultaneously on the same sample. A summary of the operating conditions for the elemental analyser is shown in Table 3. For analysis, approximately 0.75 mg of sample was weighed into a tin capsule (Santis Analytical, Teufen, Switzerland) using a precision micro balance (Mettler Toledo, Columbus, OH). Standard and sample weights were selected so that peak areas were as closely matched as possible (± 50 %) to minimize errors associated with source linearity effects. All samples and standards were analysed in triplicate.

TABLE 3. OPERATING CONDITIONS FOR THE FLASH 2000 ELEMENTAL ANALYSER

Item	Chemical / Condition
Oxidation catalyst	Chromium oxide
Reduction catalyst	Reduced copper
Water trap	Magnesium perchlorate
Flow rate for He carrier gas	100 ml / min
Temperature of GC oven	40 ºC

A two point normalization approach was used to calibrate the raw data from the instrument to the relevant international δ scale. Certified reference materials USGS 40 and 41 (United States Geological Survey (USGS), Reston, VA) were chosen to be the normalization standards as their

significantly different $\delta^{13}C$ and $\delta^{15}N$ values cover a range that should encompass a large proportion of naturally occurring materials (USGS 40: $\delta^{13}C$ = -26.369 ‰ and $\delta^{15}N$ = -4.5 ‰; USGS 41: $\delta^{13}C$ = +37.626 ‰ and $\delta^{15}N$ = +47.6 ‰). Sample isotope ratios were expressed as relative differences to a reference standard using the conventional delta notation (δ scale).

$$\delta = \left(\frac{R_{sample}}{R_{reference}}\right) - 1 \qquad (1)$$

where R is the isotope abundance ratio of the heavier to the lighter stable isotope in the sample (R_{sample}) and standard ($R_{standard}$), respectively. Differences are expressed in units of per mil (‰) by multiplying calculated δ values according to Eqn. (1) by 1000.

Milk casein samples were analysed alongside a set of reference materials comprising IAEA 600 (Caffeine, $\delta^{13}C$ = -27.771 ‰), IAEA N 2 (Ammonium sulfate, $\delta^{15}N$ = +20.3 ‰) (International Atomic Energy Agency (IAEA), Vienna Austria) as well as an in house casein reference material which has been previously calibrated for its $\delta^{13}C$ and $\delta^{15}N$ values. All results are presented relative to international standards carrying an assigned δ value of zero absolute, i.e., Vienna Pee Dee Belemnite (V PDB) for $\delta^{13}C$ and air N_2 for $\delta^{15}N$, respectively. Average measurement repeatability for independent analysis of the same sample was approximately 0.15 ‰ for both carbon and nitrogen isotope ratio measurements.

2.4.2. Elemental Profiling by ICP MS and ICP OES

Digested solutions were analysed by ICP MS (Agilent 7700, Agilent Technologies, Santa Clara, CA) and ICP OES (Agilent 5100, Agilent Technologies, Santa Clara, CA). Plasma operating conditions are listed in Table 4 and Table 5.

TABLE 4. OPERATING CONDITIONS USED IN ICP MS

Parameter	**Operating Condition**
RF applied power (W)	1600
Sample Depth (mm)	8.0
Carrier gas flow rate (L/min)	0.70
Dilution gas flow rate (L/min)	0.40
Helium gas flow rate (mL/min)	4.5
Hydrogen gas flow rate (mL/min)	5.0
Integration time (s)	0.15 (no gas mode)
	0.30 (H_2 gas mode)
	0.51 (He gas mode)

TABLE 5. OPERATING CONDITIONS USED IN ICP OES

Parameter	Operating Condition	
RF applied power (W)	1100	
Nebulizer gas flow rate (L/min)	0.70	
Plasma gas flow rate (L/min)	12.0	
Auxiliary gas flow rate (L/min)	1.00	
Read time (s)	5	
Viewing mode	Axial and radial	
Viewing height (mm)	4	
Emission lines (nm)	Ca II 317.933 (radial)	P I 213.618 (radial)
	Fe II 238.204 (axial)	S I 180.669 (axial)
	K I 766.491 (radial)	Si I 251.611 (axial)
	Mg I 285.213 (radial)	Zn I 213.857 (radial)
	Na I 589.592 (radial)	
Internal standard emission lines (nm)	Rh I 343.488	Ir II 212.681

Note: I – atomic lines / II – ionic lines

3. RESULTS AND DISCUSSION

3.1 ISOTOPE RATIO MEASUREMENTS

The carbon and nitrogen isotope ratios of milk casein from different countries are summarised in Table 6 and the box plots (Figures 1A and 1B) below. The $\delta^{13}C$ of casein samples showed significant differences among the different countries while differences in $\delta^{15}N$ were smaller.

TABLE 6: RANGE OF CARBON AND NITROGEN ISOTOPE RATIOS OF MILK CASEIN FROM DIFFERENT COUNTRIES

Country	Range of $\delta^{13}C$ values (‰)	Range of $\delta^{15}N$ values
Australia	-28.96 to -22.84	4.80 to 7.68
France	-25.35 to -19.72	4.62 to 5.96
Germany	-24.82 to -22.35	5.61 to 6.14
Indonesia	-22.10 to -17.85	3.88 to 4.79
Japan	-21.32 to -19.02	4.13 to 5.57
Malaysia	-29.35 to -24.13	5.38 to 6.74
New Zealand	-29.76 to -22.96	4.61 to 6.88
Singapore	-26.08 to -22.57	3.52 to 7.48
Thailand	-25.18 to -19.64	4.79 to 5.62
United States	-27.17 to -14.79	4.91 to 6.50

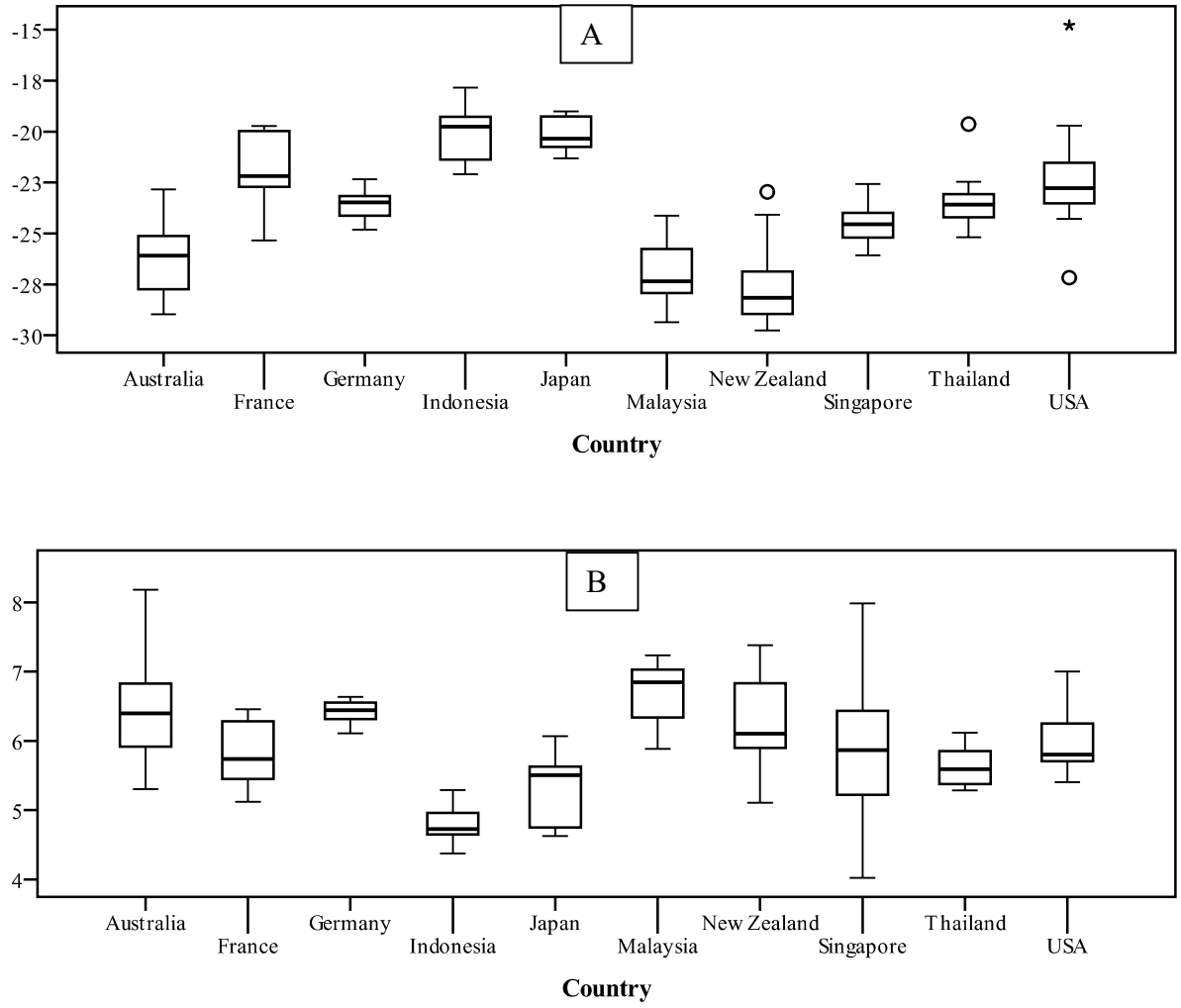

FIG. 1 Boxplots showing the distribution of the (A) carbon and (B) nitrogen isotope ratios of milk casein from different countries.

Differences in the carbon and nitrogen isotope signatures of milk from different countries arose due to different dairy cattle feeding regimes in different geographical regions. Depending on their photosynthetic pathways, terrestrial plants can have differences in their carbon isotope ratios. C_3 plants such as wheat and soy have bulk $\delta^{13}C$ values ranging from -24 to -34 ‰, while C_4 plants such as maize and sorghum have bulk $\delta^{13}C$ values from -10 to -23 ‰ (Bender [6]; Smith and Epstein [7]). Hence the carbon isotope ratios of milk may reflect both the grazing pasture and/or the type of feed given to the cows in extensive and intensive dairy systems. Milk casein from Australia, Malaysia, New Zealand, and Singapore tend to have $\delta^{13}C$ values lower than -24 ‰. Dairy cows from these regions are likely to be grazing on C_3 pasture or given (supplementary) feed containing mostly material from C_3 plants. On the other hand, the slightly elevated (i.e., greater than -24 ‰) $\delta^{13}C$ values of milk casein from France, Germany, Indonesia, Japan, Thailand, and the United States is indicative of some C_4 plant material being included in the dairy cattle's' feed ration.

Nitrogen isotope ratios on the other hand can be used to distinguish the fertilization method of feed crops and the proportion of nitrogen fixing crop species in the animal's diet. Nitrogen isotope values of synthetic fertilizers lie in the range between -4 ‰ and 4 ‰ while that for organic nitrogen fertilizers, which include manure and compost, can spread anywhere from

around 0 to more than 30 ‰ (Bateman and Kelly [8]). Meanwhile, minimal isotopic fractionation occurs during N_2 fixation by nitrogen fixing crop species such as leguminous plants and such plants are shown to exhibit $\delta^{15}N$ values close to 0 ‰ (Bedard Haughn et al. [9]). Most of the milk casein have similar $\delta^{15}N$ values. Milk casein from Indonesia appeared to have significantly lower $\delta^{15}N$ values probably as a result of a higher proportion of either artificially fertilised feed material or nitrogen fixing crop species in the animals' diet.

3.2. ELEMENTAL PROFILING

The large number of element concentrations which were measured constitute a data trove which could be used to discriminate dairy milk on the basis of country of origin. Depending on the feed and water given to the dairy cow as well as the geological environment in which animal was reared, the milk produced showed differences in their elemental content. Milk from the United States for instance showed a higher concentration of Li and B compared to milk from other geographical regions. The distribution of Li and B concentrations in milk samples from the different countries is shown in Figures 2(A) and (B). Data for other elements will be published in a separate peer reviewed journal.

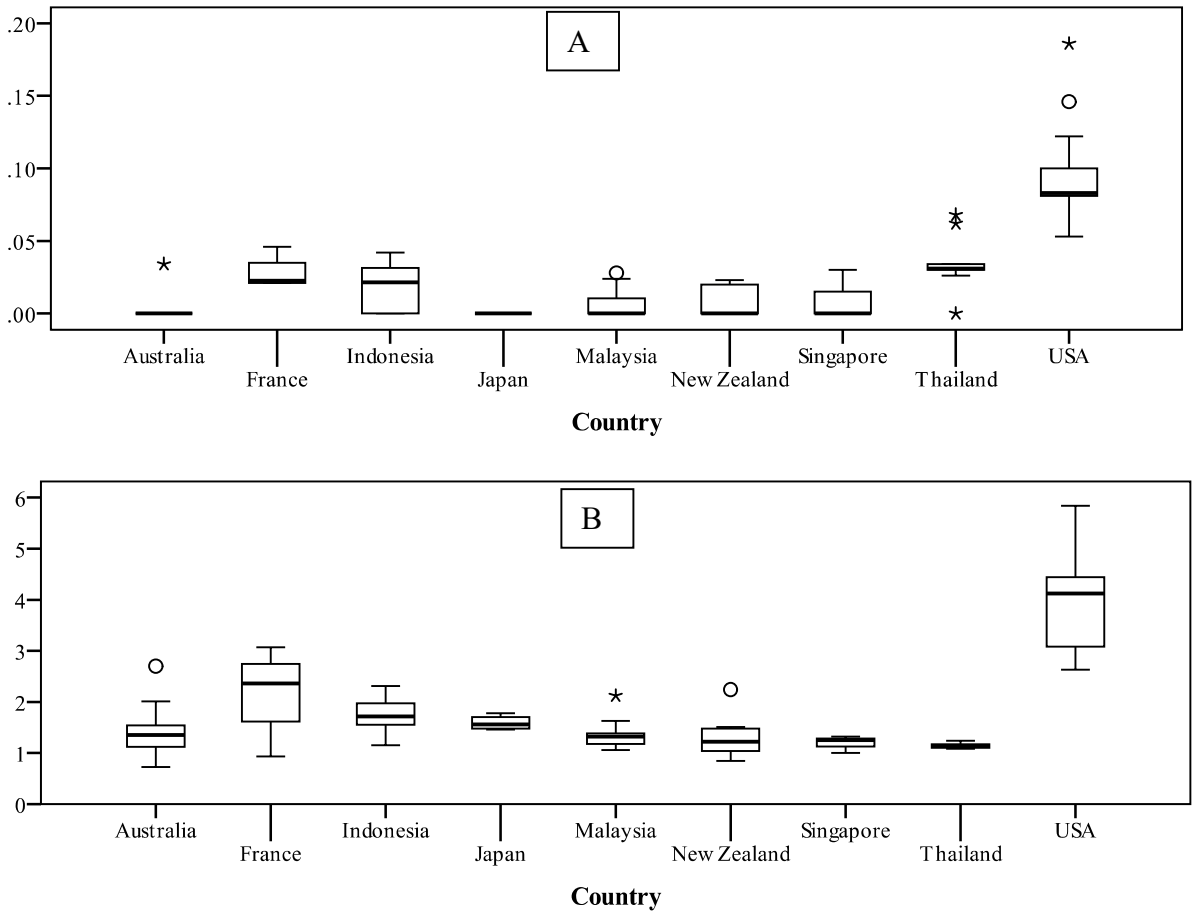

FIG. 2: Boxplots showing the distribution of (A) Lithium and (B) Boron concentrations in milk from different countries. (Data for Germany is not shown because there are only 2 data points).

4. CONCLUSION

Both isotope signatures and element concentrations were found to be useful parameters for discriminating milk from different countries of origins. Hence, this scientific approach can be used to supplement the current paper based approach to confirm the geographical origin of milk

sold on the retail market. The scientific approach to food traceability set up in this project will be used as a template to develop models for other food matrices which are of the most relevance to Singapore.

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STABLE CARBON AND NITROGEN ISOTOPE ANALYSES AS A POTENTIAL TOOL FOR VERIFYING ORIGIN OF CATTLE MILK PRODUCED IN DIFFERENT AGROCLIMATIC ZONES OF SRI LANKA

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Abstract

Producing safe and high quality food is a prerequisite to ensure consumer health as well as being a vital aspect of food security. Cows' milk is vulnerable to a range of food safety hazards that may arise at any stage from production to marketing. Consequently, having analytical methods to identify the source of any potentially unsafe milk would be of great benefit in undertaking investigations or targeted recalls. The dairy sector contributes to the economy of Sri Lanka by way of employment generation and reducing nutritional poverty. This study was aimed at establishing a method to verify the origin of cattle milk using stable isotope data of defatted milk coming from different agroclimatic zones of Sri Lanka. A total of forty five milk samples were collected from five different agroclimatic zones in Sri Lanka, namely dry zone, coconut triangle, mid country, upcountry, and wet zone. Although the $\delta^{13}C$ and $\delta^{15}N$ values of defatted milk samples were not significantly different among the agroclimatic zones except for the dry zone, a multivariate discriminant analysis based on variables $\delta^{13}C$, $\delta^{15}N$, nitrogen%, carbon%, and carbon to nitrogen molecular ratio, achieved good discrimination among the milk produced in different agroclimatic zones of Sri Lanka.

1. INTRODUCTION

The livestock sector has a vast potential in contributing to the economy of Sri Lanka by way of employment generation and reducing nutritional poverty. The Sri Lankan government has identified the dairy sector as a priority area for development as nearly 50% of the milk currently consumed is imported into the country at the cost of about LKR 50 billion annually. The main policy objective of the livestock sector is to achieve a higher level of self-reliance in milk. At present milk collection and marketing in Sri Lanka is carried out through a complex supply chain with many mid-level involvements at different stages from production to marketing. As a result, there is increased vulnerability of the dairy industry to a range of food hazards (microbiological, chemical, physical) that may arise at any stage of the milk supply chain. Economically motivated fraudulent practices such as the addition of low quality adulterants are widely used to increase the value of low quality milk. These fraudulent practices have become more complex and difficult to detect using conventional analytical methods. Isotopic fingerprinting provides a robust analytical tool to determine the origin of food as well as having the potential to detect certain types of adulteration. Stable isotope ratios of the foodstuff mostly depend on botanical, climatic or geographical conditions. Stable isotope ratios of carbon and nitrogen in animals and animal products reflect diet, local environment and agricultural practices. Carbon isotopes (expressed as $\delta^{13}C$) have recently been used to discriminate between milk and cheese produced in different areas, with different dietary regimes and production systems [1, 2, 3, 6]. Nitrogen isotopes (expressed as $\delta^{15}N$) of milk and cheese reflect agricultural conditions, e.g., different fertilisation practices, which vary between regions, closeness to the sea, drought in the area, and

the presence of leguminous species in cattle diets [3, 5, 6, 7, 8, 10]. Milk production in Sri Lanka differs among different agroclimatic zones based on the breeds of animals and husbandry practices, which in turn are closely related to the agroecology and climate. Five major agroclimatic zones with significant dairy production have been identified [4, 9] as up country, mid country, dry zone, coconut triangle and low country wet zone, described in more detail in Table 1.

TABLE 1. OVERVIEW OF AGRO CLIMATIC ZONES IN SRI LANKA

Zone	Elevation (m)	Rainfall (mm)	Temperature Range °C	Husbandry Practices
Up country	>1200	>2000	10 32	Feeding on cuttings from small plots of pasture above the tea lands or on foraged *Gliricidia, Erythrina* etc. from tea plantations. There is a seasonal supply of fodder and concentrate. European breeds of cattle such as Ayrshire, Friesian, Jersey and their crossbreeds are reared.
Mid country	450 1200	>2000	10 32	Breeds are mainly the European dairy breeds, their crossbreeds, and crossbreeds with Indian breeds.
Coconut triangle	< 450	1500 2500	21 38	Limited grazing of medium sized herds. Crossbreeds of exotic breeds e.g. Zebu types and indigenous animals and crosses.
Wet lowlands	< 450	1875 2500	24 35	Limited grazing of medium sized herds. Crossbreeds of exotic breeds e.g. Zebu types and indigenous animals and crosses
Dry lowlands	< 450	1000 1750	21 38	Free grazing large, nomadic herds. Sedentary small herds in irrigated schemes. Indigenous cattle, Zebu cattle and their crossbreeds

The isotopic composition of dairy products will reflect the dietary regime and agroclimatic conditions of an animal as well as its metabolism. Having nuclear based analytical methods will facilitate the regulatory authority to distinguish between authentic milk and adulterated milk and support the withdrawal of contaminated products from the market. Public awareness of such analytical tools can also act as a deterrent to the trading of adulterated or contaminated products. Ensuring the safety of cows' milk through effective control of adulteration and continuous monitoring, in turn, will have a major impact on the economy of the country by increasing the demand for good quality locally produced liquid milk and also by reducing the budget allocated for imported milk powder. The present study aimed to apply stable isotope measurements to discriminate dairy milk collected from different agroclimatic zones. Sri Lanka is a small tropical island with large variation in environment and cattle husbandry practices, the effects of which are expected to be reflected in the stable isotope composition of the milk. The data will be used to develop a tool to identify fraudulent practices in cattle milk production.

2. MATERIALS AND METHODS

2.1. SAMPLES

Forty five samples were collected from five different agroclimatic zones in Sri Lanka, including dry zone, coconut triangle, mid country, upcountry, and wet lowlands. Three large to medium scale dairy farms were selected from each of the above agroclimatic zones to carry out the sampling. Three milk samples were collected on three consecutive days from the bulk collection tank immediately after milking from each selected farm.

2.2. SAMPLE PREPARATION

An aliquot of 1 mL from the liquid milk samples was placed in a 1.5 mL microcentrifuge tube and centrifuged at 5000 rpm for 5 minutes (at the temperature at 4 °C). The suspension in the bottom of the tube was pipetted out and transferred to another microcentrifuge tube without disturbing the fat layer. This step was repeated two times. The defatted samples were freeze dried to a powder using freeze dryer (Freezone, Labconco Corporation, USA) for 60 hours and homogenized with a pestle and mortar.

2.3. CARBON (δ^{13}C) AND NITROGEN (δ^{15}N) STABLE ISOTOPES ANALYSIS

The δ notation was used to describe the isotopic difference between the sample and an international standard, which was defined in the following formula,

$$\delta(‰) = \frac{(R_{Sample} - R_{Standard})}{R_{Standard}} X1000$$

where R_{sample} is the isotope ratio of the sample, and $R_{standard}$ is the isotope ratio of the international standard Vienna Pee Dee Belemnite (V PDB) for carbon (δ^{13}C) and air for nitrogen (δ^{15}N). The powdered de fatted milk was weighed 1 mg into a small tin capsule. The capsule was folded and compressed using tools. The prepared samples were introduced into the isotope ratio mass spectrometer (Delta Advantage, Thermo Finnigan, Bremen, Germany) operating in continuous flow mode. The isotopic composition of the sample gases was normalized and measured relative to the V PDB and air, respectively. Normalization was made by three point calibration with laboratory standards; standards for carbon (USGS 40 = -26.24‰, USGS 41 = 37.76‰, EDTA = −38.52‰) and standards for nitrogen (USGS 40 = −4.52‰, USGS 41 = 47.57‰, EDTA = −0.73‰). Time based drift correction was calculated from the laboratory standard analysed at regular intervals with the samples. IAEA MP153 (Milk Powder) and EDTA OAS were used as quality control standards to monitor analytical performance.

2.4. STATISTICAL ANALYSIS

Statistical analysis was carried out using R version 3.3.0 (2016 05 03). Descriptive statistics (mean, standard deviation, sample size) were taken for isotopic values of each agroclimatic zone. One way ANOVA was used to assess whether the δ^{13}C and δ^{15}N values of milk samples differed between the agroclimatic zones. The means were separated using Duncan's multiple range test at a confidence level of 95%. The combined effect of isotopic parameters was analysed using principal component analysis (PCA) and least squares discriminant analysis (LDA).

3. RESULTS

The data set of stable isotopes of de fatted milk samples revealed that $\delta^{13}C$ values ranged from -23.76 ‰ to -15.53 ‰ (n=45) and $\delta^{15}N$ ranged from 8.79 ‰ to 5.58 ‰ (n=45). The mean and standard deviation values of $\delta^{13}C$ and $\delta^{15}N$ for the defatted milk powder samples collected from each agroclimatic zone are presented in Table 2.

TABLE 2. MEAN $\delta^{13}C$ AND $\delta^{15}N$ VALUES OF DEFATTED MILK SAMPLES COLLECTED FROM DIFFERENT AGROCLIMATIC ZONES

Agroclimatic Zone	$\delta^{13}C_{VPDB}$	$\delta^{15}N_{AIR}$	Sample size
Coconut Triangle	-20.59 ± 2.45	6.55 ± 0.58	9
Dry Zone	-16.97 ± 0.91	8.03 ± 0.53	9
Low Wet Zone	-21.46 ± 0.7	7.03 ± 0.99	9
Mid Country	-21.77 ± 0.72	6.71 ± 0.57	9
Up Country	-19.29 ± 0.91	6.65 ± 0.54	9

Box plots of de fatted milk samples elaborating regional variation of $\delta^{13}C$ and $\delta^{15}N$ values are shown in Figure 1. Samples collected from the dry zone showed significantly higher $\delta^{13}C$ and $\delta^{15}N$ values than samples collected from the other regions. The $\delta^{13}C$ values of samples collected from the coconut triangle scattered with a wider range compared to the other samples. Duncan's multiple range test and GLM revealed that the mean $\delta^{13}C$ values of de fatted milk from the dry zone and up country were significantly different from that of the low wet zone, coconut triangle and mid country. A significant difference in the mean $\delta^{13}C$ values was not observed in the samples collected from the low wet zone, coconut triangle and mid country. The mean $\delta^{15}N$ value of the dry zone was significantly different from that of other agroclimatic zones and no significant difference was observed among the other regions.

A)

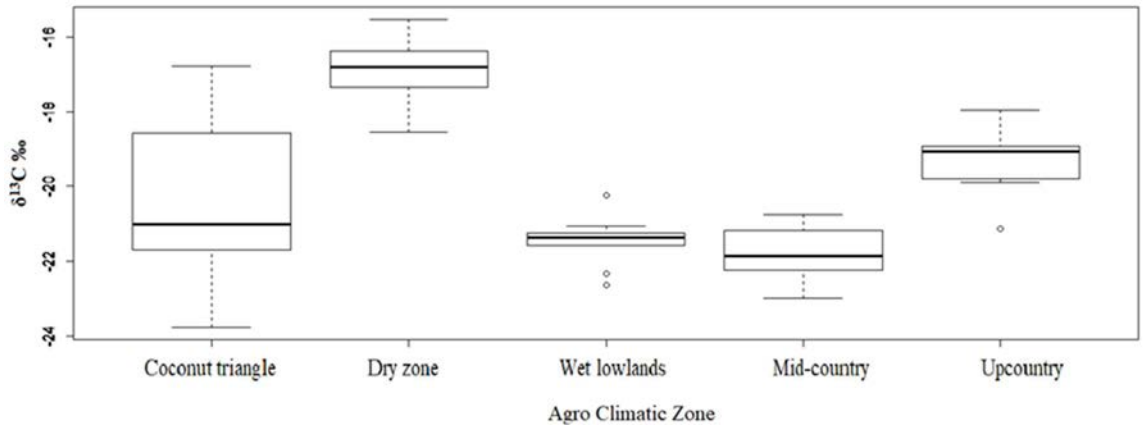

FIG. 1. Box and whisker plots of isotopic values for defatted milk samples collected from agroclimatic zones a) $\delta^{13}C$ values of defatted milk samples b) $\delta^{15}N$ values of defatted milk samples.

B)

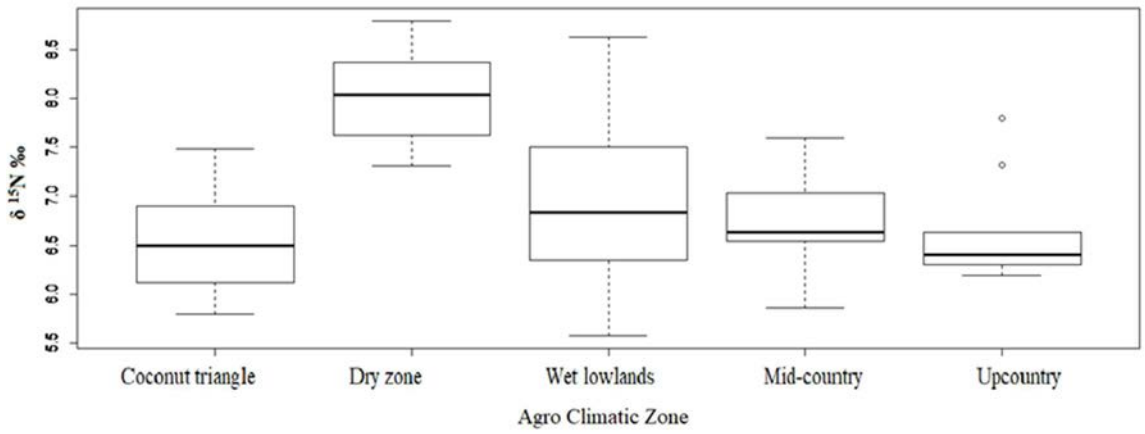

FIG. 1 contd. Box and whisker plots of isotopic values for defatted milk samples collected from agroclimatic zones a) $\delta^{13}C$ values of defatted milk samples b) $\delta^{15}N$ values of defatted milk samples.

A scatter plot of carbon and nitrogen isotopic composition of the defatted milk samples from different agroclimatic regions is presented in Figure 2. As shown in Figure 2, isotopic values among the production zones can be grouped together by observation except for the coconut triangle where the values are widely dispersed especially along the carbon isotope axis.

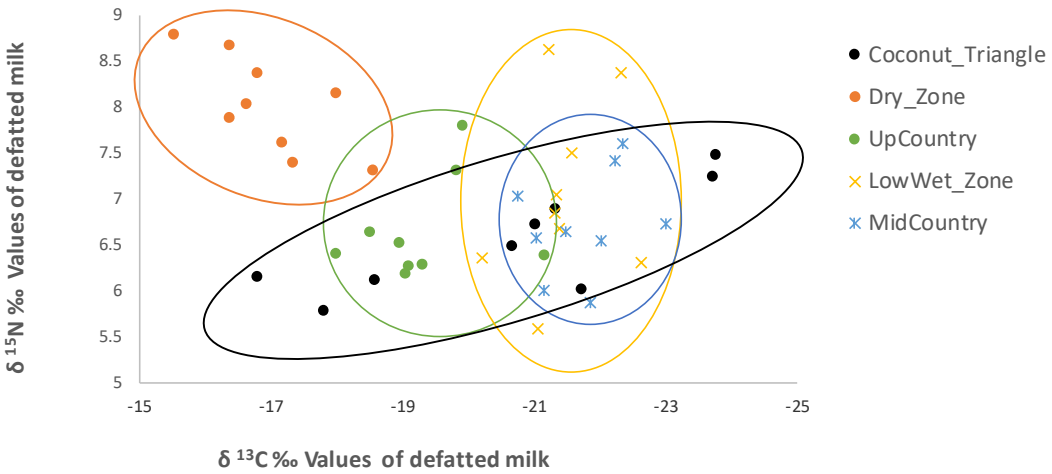

FIG. 2. Scatter plot of carbon and nitrogen isotopic composition of the defatted milk samples.

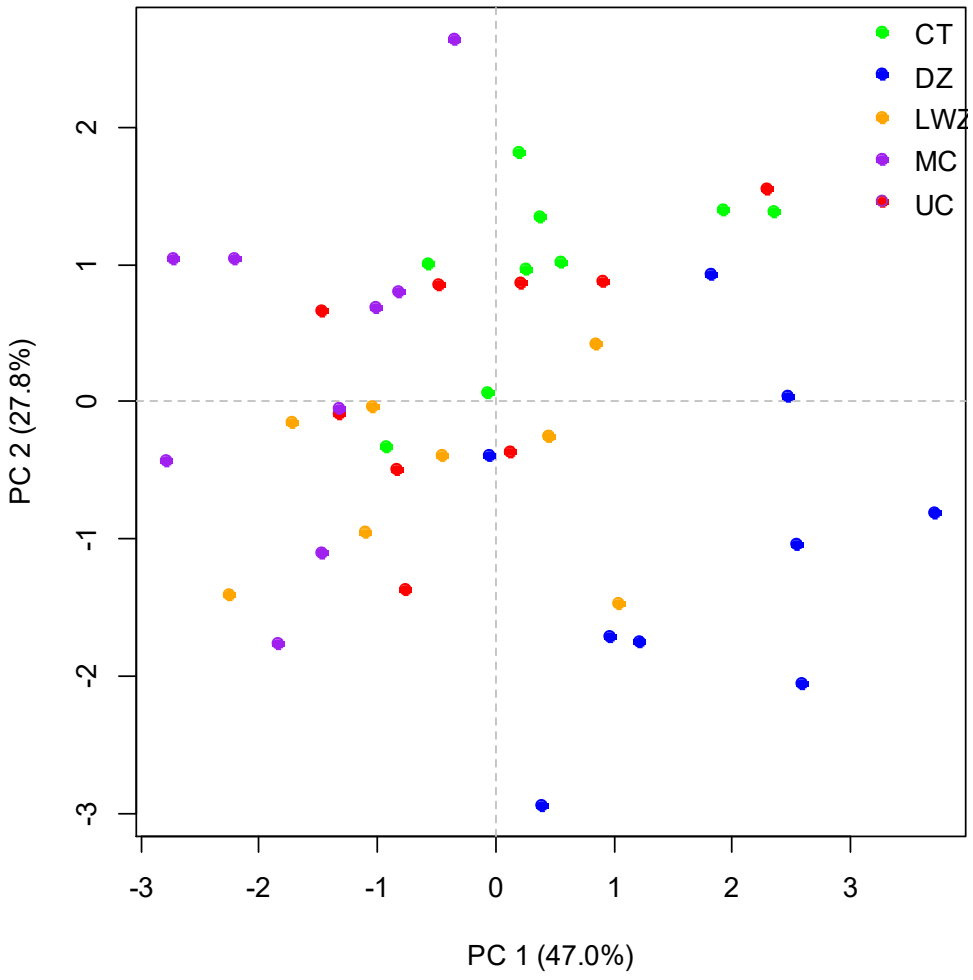

FIG. 3. Score plot of the first and second principal components.

Principal components analysis on variables of $\delta^{13}C$, $\delta^{15}N$, nitrogen%, carbon% and carbon to nitrogen molecular ratio is presented in Figure 3. The first two principal components were able to explain 74.8% variability in defatted milk powder. The influence on the PCA data structure was examined by variable loadings and are presented in Table 2.

TABLE 2. LOADING OF VARIABLES ON FIRST TWO PRINCIPAL COMPONENTS

Variable	**PC 1**	**PC2**
$\delta^{15}N$	0.213	0.609
Nitrogen %	0.617	0.100
$\delta^{13}C$	0.475	0.150
Carbon %	0.083	0.694
Carbon to Nitrogen Molecular Ratio	0.583	0.337

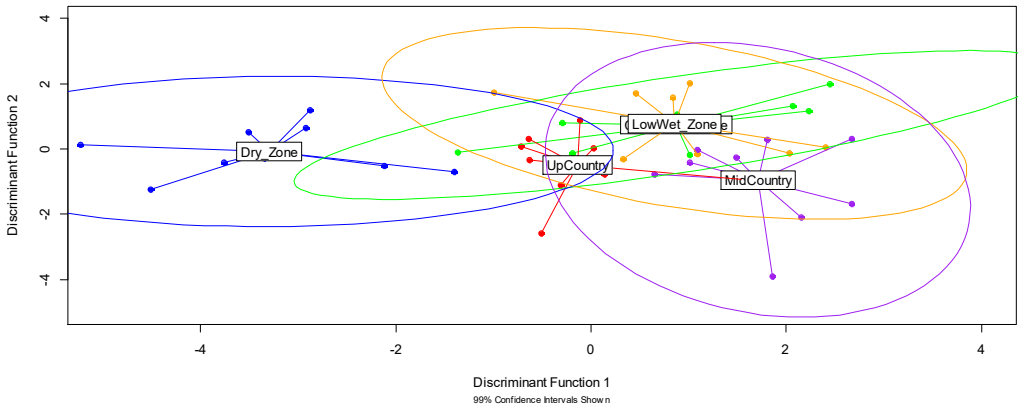

FIG. 4. Discrimination of milk from five agroclimatic zones based on variables $\delta^{13}C$, $\delta^{15}N$, nitrogen%, carbon% and carbon to nitrogen molecular ratio.

The LDA model prepared using values of $\delta^{13}C$, $\delta^{15}N$, nitrogen %, carbon % and carbon to nitrogen molecular ratio provided a clear separation of the milk samples among the agroclimatic zone except for the low wet zone and coconut triangle (Figure 4).

4. DISCUSSION

The study represents the first nationwide characterization of carbon and nitrogen stable isotope signatures of defatted milk samples processed from authentic cows' milk samples collected from five different agroclimatic zones of Sri Lanka. The stable isotope (C and N) composition of defatted milk powder sourced from Sri Lanka shows geographic variation, and there is considerable overlap in values between regions. An LDA model developed using five variables (i.e. $\delta^{13}C$, $\delta^{15}N$, nitrogen %, carbon % and carbon to nitrogen molecular ratio) was effective in distinguishing the geographic origin of samples except for samples from the low wet zone and coconut triangle. Nitrogen and carbon isotopic compositions of dairy products are primarily related to production systems. Based on the data obtained in the study, the composition of stable isotope ratios is applicable as a potential tool to discriminate the origin of cattle milk. Further work with other bio element isotope ratios such as hydrogen and sulfur could improve the discrimination of origin.

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Cannavan, A.	International Atomic Energy Agency

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Vienna, Austria: 26–29 November 2013
Rabat, Morocco: 3–7 October 2016
Vienna, Austria: 9–13 October 2017
Ljubljana, Slovenia: 3–6 September 2018

STAKEHOLDER MEETING

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