

Conference paper

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Transfer of chemical elements from milk to dairy products

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Abstract: A pilot study was carried out to evaluate the transfer of As, Cd, Pb, Ni, Cr and Hg from milk to dairy products and to correlate their behavior with that of some components and macro-elements of milk. Due to the low level of these chemical elements in most common commercial products, hard cheese was produced starting from cow's milk spiked with the analytes of interest. Several intermediate and final products coming from cheese making were sampled and analysed for content of fat, dry matter, proteins, macro and oligo-elements. The relationship between spiked elements and milk components was evaluated through both the study of concentration factors and the statistical analysis (Principal Component Analysis and correlation matrix). Except for As and Hg, a clear correlation between spiked elements and milk components was found so a likely bond with proteins, fat and dry matter was demonstrated. As for Pb, for which a legal limit (Maximum Level, ML) is set in the pertinent European regulations, it was found that the link with proteins could lead to an increase of this element concentration different from the mere concentration factor from milk to cheese. Furthermore, it was proven that the stage of ripening affected the variation of Pb concentration so this aspect should be deeply considered in case of setting a ML in cheese or extrapolating a ML from milk to cheese.

Keywords: cheese; chemical elements; dairy products; Eurasia 2018; heavy metals; maximum level; oligo-elements.

Introduction

In the last 10 years, the worldwide increase in production and consumption of milk and dairy products has led to growing interest in studies on dairy products.

In particular, milk consumption in 2010 reached a global average *per capita* of 104.7 kg representing +1% compared to 2009 and +10% compared to 2000 [1]. This trend is confirmed in the Outlook drawn up by the Food and Agricultural Policy Research Institute (FAPRI) whose task is to elaborate baseline projections for the US agricultural sector and international commodity market [2]. A similar picture is reported in the

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OECD-FAO Report [3], where the dairy sector is considered among the fastest growing sectors covered by this project with a long-term forecast of growth in milk production.

In particular, the world production will rise steadily in the next decade of around 1.6–1.9% per year according to both outlooks. This will be the consequence of an increasing consumption of dairy products that is expected to be around 2% for the decade 2014–2023. A big contribution to this growth should come from developing countries due to both the rise of incomes and the westernization of diets.

The development of the dairy sector [4–7] has stimulated a lot of researchers to investigate the nutritional and/or toxicological properties of the relevant minerals and trace elements [8–11]. On the contrary, the presence of some chemical elements (e.g. lead), mainly due to the contamination from environmental conditions and/or manufacturing process, was only partially considered by researchers [7, 12]. Although certain contaminants such as cadmium, mercury and arsenic can be recognized as potentially toxic within specific limiting values, no maximum levels (MLs) for these elements in milk and dairy products are established in the European regulations. In fact, Commission Regulation (EC) 1881/2006 and following amendments [13–17] only defines a ML of 0.020 mg kg^{-1} for Pb in milk, which justifies the great interest in the study of its distribution in cheese and dairy products.

The chemical affinity of Pb for certain milk components can produce some dairy products with Pb concentration higher than the one obtained by simply applying the concentration factor from milk to cheese.

Conversely, a milk with a content of Pb higher than the ML could yield a product with Pb lower than 0.020 mg kg^{-1} due to its chemical behavior in specific dairy products (e.g. whey and its derivatives). This could happen for other chemical elements as well. Therefore, the objective of this work was to evaluate the partition of As, Cd, Pb, Hg, Cr and Ni among the different dairy products. The study was focused on these six toxic or potentially toxic elements for specific reasons. In particular, Pb was selected because of its ML in milk while the other analytes were considered because they are of toxicological interest.

As for Ni, dietary exposure provides most of its intake. Ni is absorbed from the gastrointestinal tract and can cross the placenta. The oral exposure to soluble Ni compounds is associated with toxic effects during pregnancy (interference with the development of the embryo and fetus) and high consumption of food with high content of Ni may arouse eczematous flare-up reactions in sensitized individuals. According to the 2015 European Food Safety Authority (EFSA) opinion on the risks to public health related to the presence of nickel in food and drinking water, the current dietary exposure raises concern for chronic exposure levels. Due to the absence of MLs for Ni in EU Regulation and the poor availability of occurrence data, EU Commission recommended Member States to monitor nickel content in foodstuffs (CR (EU) 2016/1111), milk and dairy products included.

For arsenic, EU Commission asked EFSA to draw up an opinion on the risks to human health related to the presence of arsenic in the food chain. According to this scientific opinion many adverse effects are associated with long term ingestion of inorganic arsenic, so EU Commission published a specific recommendation on the monitoring of arsenic in food (CR(EU) 2015/1381) including milk and dairy products.

Mercury was considered of interest for this study because many EU Member States plan to search for this contaminant when draw up their annual monitoring plan for certain substances in the chain of production of foods derived from animals.

As for Cd, food is the main route of exposure in non-smoking population and, once ingested, it is adsorbed in the gastro-intestinal tract and accumulates in liver and kidneys. It can cause kidney failure and can be associated with an increased risk of cancer. In 2012, an EFSA overview concluded that the exposure of children and adults at 95th percentile exceeded the tolerable weekly intake established by the EFSA Panel on Contaminants in the Food Chain. Therefore, it was deemed of particular relevance the continuous monitoring of Cd content in foodstuffs as well as the reduction of limit of detection/quantification of the methods used for this monitoring so as to get a more precise and accurate calculation of the human exposure.

As far as Cr is concerned, it can be present in food and drinking water due to both natural and anthropogenic sources. Milk and dairy products are considered among the main contributors to the exposure of the general population and the infants and toddlers. Cr (VI) is the form that can cause the main adverse effects. Fortunately, the oral exposure to potentially toxic Cr (VI) is lowered by its extracellular reduction to Cr (III) but a portion may however enter the stomach cells and potentially initiate tumour formation.

The aim of the present work was to evaluate the transfer of As, Cd, Pb, Ni, Cr and Hg from milk to dairy products and to correlate their behavior with that of some components and macroelements of milk. As the most common dairy products available on the market have a negligible level of these chemical elements, a procedure was studied to produce hard cheese and intermediate products from cow's milk enriched with the analytes of interest. Therefore, the raw cow's milk was spiked with As, Cd, Pb, Hg, Cr and Ni at concentration values such as to overcome analytical problems. In fact, the initial low content could have made less evident and/or not significant possible differences.

In this study the following intermediate and final products were sampled and analysed: spiked milk, whey, ricotta and cheeses at two different ripening stages (after 2 weeks and 3 months).

All these products were characterized by determining the percentage of fat, proteins and dry matter.

Analytical determination of Mg, Zn, Ca, Mn, Cu and K was carried out in milk and in dairy products (intermediate and final products) so as to highlight possible correlation between the spiked analytes and the oligo/macroelements.

In particular, the behavior of Pb was investigated by spiking milk with a concentration of Pb roughly twice the ML. The possibility that the spiked analytes might bind themselves to milk components was investigated and their distribution from milk up to intermediate/final dairy products was discussed with the aim of highlighting the likely correlation with the behavior of some endogenous factors.

Experimental

Reagents

Reagents for this work were at least of Suprapur grade of purity. In particular, the following chemicals were used: Suprapur concentrated nitric acid (HNO_3) 67–69 % (v/v) (Romil, Cambridge, UK); Suprapur hydrogen peroxide (H_2O_2) 30 % (v/v) (Romil, Cambridge, UK); single element 1000 mg L^{-1} stock standard solutions of As, Cd, Pb, Cr, Ni, Mg, Zn, Ca, Mn, Cu in 2 % (v/v) HNO_3 (High-Purity Standards, Charleston and Perkin Elmer, Shelton); Rh in 10 % (v/v) HCl (High-Purity Standards, Charleston and Perkin Elmer, Shelton); Setup/Stab/Masscal Solution (Perkin Elmer, Shelton); ICP multi-element standard solution VI (Merck, Darmstadt).

High purity deionised water with a specific resistance $\geq 18 \text{ M}\Omega \text{ cm}$ (Zeener UP 900 Water Purification System, Human Corporation, Texax, USA) was used for cheese making and for preparation of all samples and standard solutions. Suprapur and ultrapure grade guaranteed that the concentration of the analytes or interfering substances in reagents was negligible compared to the level of concentration to be determined.

Cheese making

The plan of cheese making provided the addition of a proper volume of spiked milk to the whole mass of milk that would be used to prepare dairy products. Therefore, a preliminary experiment was conducted to verify whether to spike milk with a moderately acidic standard solution could produce any significant modification (e.g. curdling). A milk standard solution containing the elements of interest was prepared to simulate the same acidity as the spiking milk that would be added for cheese making. The tested milk was maintained under continuous stirring. It was noticed that formation of curdling occurred when the solution was added to the milk at coagulation temperature (about 37°C). On the contrary, no modification was evident when the standard solution was poured out into the milk at room temperature under stirring and then slowly heated to coagulation temperature. It was concluded that the spiked solution had to be added at room temperature before heating milk in order to avoid coagulation of the sample.

All containers and tools used to make cheese were disposable and brand-new or properly decontaminated so as to minimize contaminations from chemical elements.

The spiking milk contained the elements of interest at the following concentration levels: 40 mg/kg for Pb; 20 mg kg⁻¹ for Cd and Hg; 50 mg kg⁻¹ for As, Cr and Ni.

Milk and rennet were supplied by the farm, ferments were BIOCHEM MSO-1 series 1000. One hundred liters of milk obtained from the evening/morning milking in the winter season (late January) were filtered. Five liters of this filtered milk were sampled and spiked with stock standard solutions under stirring (30 min).

This “spiked” milk was then added to the remaining volume and heated at coagulation temperature (37–38 °C) under manual stirring and, finally, milk let coagulate very quickly by adding enzymes to acidify the mass up to pH 5–6. Once temperature and optimal pH were reached, the milk was left to coagulate with lamb rennet paste for about 30 min. The breaking-up of the curd took place in two different phases: the first one was slow and soft, whilst the second one was as strong as to reduce it to the dimension of rice grains.

Keeping the temperature around 40 °C, the mass was gently agitated so as to promote complete separation from the whey. After separating the two phases, the stirring was stopped and the curd was left to stand for about 10 min. The curd, placed into plastic baskets, was pressed to facilitate the discharge of the whey and finally left standing again.

As for cheese, once the first “quadrant” was formed, it was turned upside down, pressed again and transferred to a heated (35 °C) and damp (90–100 % humidity) room for about 24 h. Subsequently, it was left at room temperature and repeatedly turned over inside the cheese molds so it could take the proper shape with slightly convex faces. Twenty-four hours after production dry salting was carried out on the entire surface of the product with sea salt. This operation was performed by turning the cheese and adding salt every 2 h. Subsequently cheese was washed and, once dry, was left to mature. Ripening was carried out in a natural environment at about 8–12 °C, keeping cheese in typical plastic containers (fucelle) for the first 2 days and afterwards turning it over once a day. Maturing occurred up to the formation of a compact dry rind and cheese was sampled in two different times: halfway and at the end of the ripening period.

Ricotta preparation

Ricotta is a fresh typical Italian “whey cheese” without external rind and with a white compact smooth paste, obtained through the heating of whey.

Whey, kept under continuous stirring, was heated up to the boiling temperature: whey proteins coagulated into particles at about 85 °C and started to separate from the liquid phase coming afloat like small flakes that condensing on the surface. The clotting was gently skimmed with a proper ladle and placed in special baskets so as to form ricotta.

Sampling

Samples of spiked milk and whey were stored in proper plastic decontaminated containers and kept at –20 °C. Ricotta was sampled at time of preparation. Cheese was sampled after two different ripening stages: after 2 weeks (Primo sale cheese) and after 3 months (ripe cheese). Samples of cheese were prepared in vacuum-sealed packs and stored at –80 °C till the analysis.

Analysis of fat, proteins and dry matter

Analyses were performed by the “Laboratorio Standard Latte (LSL), Maccaresse, Italy” accredited according to ISO/IEC 17025:2005. All products were characterized by determining the percentage of fat, proteins and dry matter following adequate international standards (ISO 1211/IDF 1, ISO 8968-2/IDF 20-2 and ISO 6731/IDF21).

Analysis of chemical elements

The chemical element quantification (i.e. spiked analytes, macro and oligo-elements) was performed using analytical methods in-house validated or accredited according to ISO/IEC 17025 [18].

All samples were defrosted and processed as follows: liquid products were suitably shacked; ricotta and curd were carefully homogenized; seasoned cheese, without rind, was finely grated. All samples underwent microwave assisted acid digestion (MILESTONE-1200 or MILESTONE-900 equipped with a probe for temperature control, FKV, Italy). 0.5 g of solid samples were weighed into Teflon digestion vessels. Afterwards, 3 mL H₂O, 5 mL HNO₃ and 1 mL H₂O₂ were added and the sealed vessels underwent a pre-digestion treatment (130 °C for 5 min, 140 °C for 2 min and 140 °C for 10 min). This step was deemed necessary so as to avoid possible explosions during the dissolution of samples with a high fat content.

As for liquid samples (whey and milk), 1.5 mL was put into vessels with 3 mL H₂O, 3 mL HNO₃ and 0.5 mL H₂O₂.

The vessels with both liquid samples and pre-digested solid samples were sealed and the digestion program was started (ramp from 100 °C to 200 °C and held at 200 °C for 25 min). The vessels were then degassed in an ultrasonic bath (Starsonic 35, PBI International, Milan) and then the resulting solutions were diluted up to 20 mL with deionized water.

The analytical determination of As, Cd, Pb, Cr, Ni, Mg, Zn, Ca, Mn and Cu was carried out by Inductively Coupled Plasma Mass Spectrometry (ELAN DRC II, Perkin Elmer, Norwalk, CT). In particular, quantification was based on the following isotopes: ⁷⁵As, ¹¹⁴Cd, ²⁰⁸Pb (as sum of isotope 206 and 207), ⁵²Cr, ⁵⁸Ni, ²⁴Mg, ⁶⁶Zn, ⁵⁵Mn, ⁴³Ca and ⁶⁵Cu. As for Cr and Ni, DRC mode with ammonia as reaction gas (flow = 0.6 mL min⁻¹ and RPq = 0.7) was used to minimize possible interferences. A preliminary semi-quantitative analysis was performed in order to roughly estimate both concentration of analytes and type/level of interferences. Before starting the semi-quantitative analysis, calibration was refreshed with the ICP multi-element standard solution Merck VI. Calibration curves for quantification were prepared on the basis of the preliminary data from semi-quantitative analysis. Samples were differently diluted depending on the elements to be quantified. External standard calibration or addition calibration approach were used according to the level of dilution. The scheme of dilution factor and the type of calibration for the ICP-MS determination is reported in Table 1. Internal standardization was applied for all elements and Rh was used as internal standard.

In case of high level of dilution matrix effect was negligible so external calibration was successfully applied. The addition calibration approach was used for low diluted samples as the matrix effect could be remarkable.

K was analyzed by Flame Atomic Absorption Spectrometry (AAAnalyst 800, Perkin Elmer) with external calibration approach. Standard solution and samples were prepared with the same percentage of Lanthanum contained in the ionization buffer. The dilution was carried out at the following levels: milk and whey 1:75 (v/v), cheese 1:20 (v/v) and ricotta 1:50 (v/v). This technique was also used to confirm the level of Ca found with ICP-MS. Hg was analyzed by means of Flow Injection Mercury System (Perkin Elmer) with a 500 µL loop and after having degassed both sample and blank of digestion in which the calibration curve was prepared.

Table 1: Scheme of ICP-MS dilution factor (v/v) and calibration approach for each element/sample.

Element	Sample	Instrumental dilution factor	Calibration ^a
As, Cd, Pb	Milk/whey	1:2	AC
As, Cd, Pb	Curd/ricotta/cheese	1:3	AC
Mg	Milk/whey/curd/cheese	1:1000	EC
Mg	Ricotta	1:100	EC
Ca, Zn	Ricotta/whey	1:100	EC
Ca, Zn	Milk/curd/cheese	1:1000	EC
Mn, Cu	All	1:3	AC
Cr, Ni	All	1:2	AC

^aAC, Addition calibration; EC, external calibration.

Accuracy was tested both analyzing Certified Reference Materials (BCR063R, BCR150 and SRM1549) and in-house spiked materials. Recovery rates were nearly satisfactory (>95 %) for all analytes.

Statistical analysis

Principal components analysis (PCA) was performed by means of Minitab statistics package developed at the Pennsylvania State University. The aim of PCA was to uncover relationships between all elements of milk, cheese and dairy products as to highlight the presence of common behavior. The number of principal components was established considering only the eigenvalues greater than 1.

Linear relationship between variables was considered using Pearson product-moment correlation coefficient. The more this coefficient value is close to 1, the more the linear correlation is strong. The sign of the coefficient indicates the direction of the relationship: a positive value means that the two variables vary in the same direction together (increase or decrease), whereas a negative coefficient represents two variables that go on different direction (i.e. the first increases and the second on decreases). The correlation was considered strong and moderate in case of a Pearson coefficient greater than or equal to 0.8 and 0.5, respectively.

Results and discussion

Cheese composition in macro/essential elements and heavy metals, including toxic or potentially toxic elements, can vary depending on: the starting milk; the environmental condition; the stage of lactation; the cheese making procedure and the level of ripening. Furthermore, the concentration factor can depend on the way the cheese was produced and seasoned [4]. Since the results of this work were closely connected with the specific procedure used for cheese making, each dairy product was characterized through the assessment of the main characteristics (i.e. content of fat, proteins and dry matter). This approach allows to better evaluate the results obtained and to compare them with data reported in literature. The results are summarized in Table 2.

Based on the content of fat and moisture, primo sale cheese and ripe cheese were classified as hard medium fat and extra hard medium fat, respectively. The classification was made according to the Codex Alimentarius [19]. As for endogenous elements (Table 3), the concentration of Mn, Cu and Zn in “primo sale”

Table 2: Composition (%) in fat, protein and dry matter of the dairy products analysed in the study.

Type of product	Fat ($x \pm s$)	Protein ($x \pm s$)	Dry matter ($x \pm s$)
Milk	3.5 ± 0.2	3.5 ± 0.02	12.6 ± 0.1
Whey	0.9 ± 0.1	1.0 ± 0.01	7.6 ± 0.01
Ricotta	13.1 ± 0.9	7.8 ± 0.1	26.1 ± 0.5
Curd	20.5 ± 0.8	16.8 ± 1.3	42.2 ± 0.8
Primo sale	29.4 ± 0.1	23.3 ± 0.1	62.0 ± 0.4
Ripe cheese	38.8 ± 0.2	30.9 ± 0.2	81.7 ± 0.1

Table 3: Concentration ($\mu\text{g g}^{-1}$) of macro and oligo-elements in the dairy samples (n = 3).

Sample	Mg	Zn	Ca	Mn	Cu	K
Milk	106 ± 1	6.0 ± 0.5	1113 ± 30	0.024 ± 0.001	0.0520 ± 0.006	1590 ± 16
Whey	77 ± 1	0.12 ± 0.01	320 ± 10	0.018 ± 0.001	0.0191 ± 0.002	1563 ± 15
Ricotta	81 ± 2	0.82 ± 0.07	545 ± 11	0.018 ± 0.001	0.210 ± 0.014	1591 ± 12
Curd	210 ± 4	25.3 ± 1.3	4379 ± 67	0.110 ± 0.001	0.244 ± 0.008	1230 ± 30
Primo sale	344 ± 3	37.0 ± 3.0	7641 ± 102	0.180 ± 0.002	0.353 ± 0.012	1164 ± 33
Ripe cheese	376 ± 4	48.1 ± 2.0	9221 ± 89	0.171 ± 0.001	0.440 ± 0.011	1501 ± 36

and ripe cheese was in accordance with the range reported by Favretto [20] for products with comparable characteristics. Similarly, the level of Ca, Mg, Cu, Zn and Mn in milk, whey and curd was in line with the ones reported by Martin-Hernandez and Juarez [21] if the ratios whey/milk and curd/milk were considered. This outcome proves that the procedure followed for cheese making allowed us to prepare products with characteristics similar to those of standardized processes (industrial and/or traditional productions).

The concentration values of spiked elements in starting, intermediate and final products are summarized in Table 4. As for Cd and Pb the limits of quantification (LoQs) of the method were $0.7 \mu\text{g kg}^{-1}$ and $4.2 \mu\text{g kg}^{-1}$, respectively.

Some conclusions can be drawn about the fate of endogenous and spiked elements from milk to cheese. In particular, the behavior of K and As was different from that of the other analytes. In fact, except for these two elements the other concentration values decreased from milk to whey and increased from milk to curd. The concentration of all the elements in cheeses (i.e. primo sale and ripe cheese), except for K, increased significantly.

As for ricotta, the behavior of the analytes was more complex and diversified probably due to the characteristics of this product, which has a high water content (about 74 %), as well as to the highest fat/proteins ratio (~ 1.7) compared to the other products.

The increase of concentration value from whey to ricotta was noteworthy for Hg, Cu and Zn, whilst K, As and Mn held nearly steady their concentration.

Furthermore, comparable concentrations of some spiked elements in starting milk did not result in similar concentrations in dairy products (e.g. As and Ni in cheese and curd), whilst others (e.g. Cd and Pb) showed a very similar fate (Table 4).

PCA was used to study the behavior of the spiked elements highlighting possible similarities with the endogenous components. This statistical analysis provides an overview of the phenomenon as it reduces the number of variables. The variance of the first principal component was equal to the largest eigenvalue (12.463) and accounted for 83.1 % of the total variation in the data. The second one (1.667) accounted for 11.1 % of the total variability. Since the sum of variances associated to the first two components was equal to 94.2 %, two components were considered sufficient to explain most of the variance.

According to the eigenvalues, Mg, Zn, Ca, Mn, Cu, Cd, Pb, Cr, Ni, fat, dry matter and proteins weighed on the first component, whereas K, As and Hg weighed on the second one (Table 5). This could mean that K, As and Hg took part in the phenomena differently from the other variables that seemed to be correlated to each other. The outcome is clearly evidenced in the loading plot of the variables (Fig. 1).

Table 4: Concentration ($\mu\text{g kg}^{-1}$) of spiked elements in the dairy samples ($n=6$).

Sample	As	Cd	Pb	Hg	Cr	Ni
Milk	50.0 ± 0.4	19.1 ± 0.2	40.2 ± 1.2	18.3 ± 0.4	49.5 ± 0.7	53.2 ± 1.8
Whey	53.2 ± 1.3	<LOQ	<LOQ	11.1 ± 0.5	34.9 ± 0.5	14.1 ± 0.7
Ricotta	55.1 ± 1.3	8.3 ± 0.4	<LOQ	141 ± 6	60.5 ± 3.2	47.7 ± 2.4
Curd	36.0 ± 0.8	108 ± 4	235 ± 8	69.2 ± 2.4	157 ± 4	202 ± 9
Primo sale	62.3 ± 2.8	162 ± 2	349 ± 11	94.3 ± 3.1	222 ± 8	400 ± 13
Ripe cheese	81.0 ± 6.4	205 ± 7	464 ± 4	118 ± 1	219 ± 6	395 ± 6

Table 5: Principal components.

Variable	Mg	Zn	Ca	Mn	Cu	K	As	Cd	Pb	Hg	Cr	Ni	Fat	Protein	Dry matter
PC1	0.279	0.279	0.280	0.277	0.271	-0.172	0.107	0.281	0.279	0.160	0.280	0.279	0.279	0.282	0.280
PC2	-0.036	-0.026	-0.013	-0.146	0.127	0.677	0.582	-0.027	-0.015	0.320	-0.122	-0.071	0.095	0.055	0.103

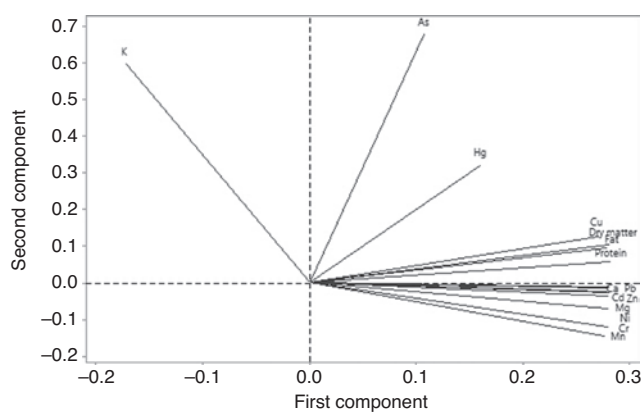


Fig. 1: Loading plot for first two components.

The graph showed that there was a relationship between Mg, Zn, Ca, Mn, Cu, Cd, Pb, Cr, Ni, fat, dry matter and proteins as they were projected in the same direction (positive PC1). In particular, several variables (Mg, Zn, Ca, Mn, Cd, Pb, Cr and Ni) were strictly correlated in a direct way since they were in the same direction and with the same versus (positive PC1 and negative PC2). On the contrary, K represented an independent information (negative PC1 and positive PC2). As for arsenic and Hg, the coefficients resulted positive on both components weighing more on PC2 (0.582 for As and 0.320 for Hg) than on PC1 (0.107 and 0.160 for As and Hg, respectively).

Correlation matrix approach was used to assess the strength and direction of relationship between endogenous milk components and spiked elements. The results are reported in Table 6 where the first line and the second line represent the Pearson's correlation coefficient (r) and the p -value, respectively.

Pearson's coefficient assesses whether two continuous variables are linearly correlated. It falls between -1 and $+1$: the closer the absolute correlation is to 1, the more tightly the data points fall on a line. On the contrary, a correlation close to 0 indicates no linear relationship. The sign of the coefficient depends on the direction of the relationship: the sign is negative if one variable tends to increase as the other decreases. Conversely, if two variables increase together the coefficient is positive.

In order to determine if the correlation is significant, the p -value is compared to a level of significance (α) of 0.05.

The associations, highlighted through the loading plot, were confirmed by means of the correlation matrix. As expected, correlations among fat, proteins and dry matter resulted to be very strong ($r > 0.7$) and statistically significant as the p -value was lower than $\alpha = 0.05$. According to their affinity for the same or very similar ligands, also Ca, Mg, Zn strictly correlated among them ($r > 0.7$, $p = 0.000$), thus points perfectly fitting along a straight line. These elements were also strongly correlated with fat, proteins and dry matter but the power of the correlation was slightly lowered (p range: 0.001–0.004) since their enrichment in ricotta did not result proportional to that of fat and proteins. As for K, no correlation was found neither with the endogenous components of milk nor with added elements, probably because K was mainly present as free ion in milk [22]. As for spiked elements, a very strong correlation was found between Cd and Pb ($r = 0.999$; $p = 0.000$). Correlations with the same significance occurred between these spiked elements and Zn, whilst slightly less powerful correlations, but still strong, were evident with Ca (Ca/Cd: $r = 0.997$, $p = 0.000$; Ca/Pb: $r = 0.996$, $p = 0.000$) and Mg (Mg/Cd: $r = 0.992$, $p = 0.000$; Mg/Pb: $r = 0.990$, $p = 0.000$). Cr and Ni resulted to have a significant relationship ($r = 0.988$, $p = 0.000$). They were both strongly correlated to proteins ($r = 0.970$ and $r = 0.963$, respectively; $p \leq 0.05$), dry matter ($r = 0.957$ and $r = 0.959$, respectively; $p \leq 0.05$) and others main component in milk (i.e. Mg, Zn, Ca and Mn).

Arsenic did not correlate with any variables, therefore a link with milk components was highly unlikely confirming a possible transfer of As into water. Also Hg did not show any correlation with milk components and spiked elements, thus weighing on the PCA similarly to As. Nevertheless, the partition of As and Hg

Table 6: Correlation matrix between spiked elements and components of dairy products.

	Mg	Zn	Ca	Mn	Cu	K	As	Cd	Pb	Cr	Ni	Hg	Fat	Protein
Zn	0.990													
	0.000													
Ca	0.998	0.996												
	0.000	0.000												
Mn	0.986	0.975	0.981											
	0.000	0.001	0.001											
Cu	0.904	0.903	0.913	0.888										
	0.014	0.014	0.011	0.018										
K	-0.608	-0.589	-0.587	-0.729	-0.496									
	0.200	0.218	0.221	0.101	0.317									
As	0.376	0.383	0.634	0.401	0.437	0.488								
	0.462	0.453	0.431	0.660	0.386	0.326								
Cd	0.992	0.999	0.997	0.980	0.916	-0.603	0.376							
	0.000	0.000	0.000	0.001	0.010	0.205	0.462							
Pb	0.990	0.999	0.996	0.973	0.906	-0.582	0.398	0.999						
	0.000	0.000	0.000	0.001	0.013	0.225	0.435	0.000						
Cr	0.979	0.974	0.978	0.994	0.924	-0.723	0.232	0.981	0.973					
	0.001	0.001	0.001	0.000	0.008	0.105	0.658	0.001	0.001					
Ni	0.995	0.977	0.989	0.992	0.914	-0.658	0.316	0.982	0.975	0.988				
	0.000	0.001	0.000	0.000	0.011	0.155	0.541	0.001	0.001	0.000				
Hg	0.441	0.430	0.455	0.426	0.776	-0.154	0.371	0.458	0.437	0.505	0.480			
	0.381	0.395	0.364	0.400	0.070	0.771	0.469	0.361	0.386	0.307	0.335			
Fat	0.945	0.950	0.955	0.927	0.991	-0.523	0.444	0.959	0.953	0.954	0.946	0.689		
	0.004	0.004	0.003	0.008	0.000	0.287	0.378	0.002	0.003	0.003	0.004	0.130		
Protein	0.967	0.976	0.977	0.951	0.973	-0.551	0.425	0.982	0.978	0.970	0.963	0.613	0.995	
	0.002	0.001	0.001	0.004	0.001	0.257	0.400	0.000	0.001	0.001	0.002	0.196	0.000	
Dry matter	0.963	0.966	0.972	0.938	0.980	-0.505	0.479	0.973	0.969	0.957	0.959	0.642	0.997	0.997
	0.002	0.002	0.001	0.006	0.001	0.307	0.337	0.001	0.001	0.003	0.002	0.169	0.000	0.000

among the dairy products was not similar. In fact, the As concentration was steady in milk, whey and ricotta but it decreased in curd. On the contrary, Hg showed affinity for curd (increased concentration value from milk to curd) but its level decreased in whey and increased in ricotta. Arsenic seemed to move entirely into water and not be bound to any component of milk. This assumption was confirmed by comparing the content of As, normalized for the water content of samples, in the different dairy products.

Milk, whey and curd had 87.4 %, 92.4 % and 57.8 % of water, respectively: the level of As seemed to vary proportionally with the water content of these samples. On the other hand, the concentration of As in ricotta was the same as in whey (55 vs. 53 $\mu\text{g kg}^{-1}$), thus highlighting the absence of binding to whey proteins. In fact, ricotta was obtained through the precipitation of whey proteins, mainly albumin, so if As had been bound to albumin its concentration in ricotta would have increased.

The reduction of As concentration in curd could be attributable to the preferential transfer of As into water. The increase in concentration of As from curd to primo sale and ripe cheese was consistent with the decrease in water content, thus indicating that a link with casein was highly unlikely.

The relationship between As concentration and water content in all samples is represented as an histogram (Fig. 2) in which values of As and water are normalized for the corresponding level in milk. The desiccation entailed the halving of water content from primo sale to ripe cheese but the expected doubling of the As concentration did not occur. This could be due to the significant loss of water by dripping during the maturing process (from primo sale to ripe cheese), resulting in the reduction of the residual concentration of As.

Mercury did not show any statistically significant correlation with the other variables but it seemed to be linked to whey components. In fact, the percentage of its reduction from milk to whey was comparable to the variation of dry matter in these samples (39 % vs. 40 %). In particular, a relationship between Hg and fat was

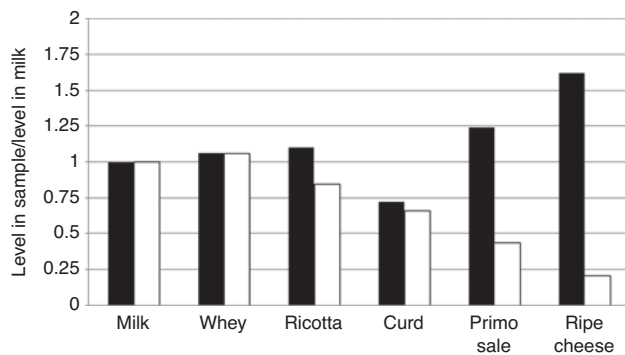


Fig. 2: Histogram of As (black bar) distribution in dairy products in respect to water (white bar).

conceivable given the increase of Hg similar to that of Cu and fat in ricotta. As for curd, the Hg level increased by about the same factor as dry matter. Furthermore, the content of Hg, Cu and fat increased similarly from curd to primo sale and ripe cheese (Fig. 3).

Cadmium and Pb seemed to have a similar behavior (Fig. 4). In particular, their concentration was extremely reduced from milk to whey becoming lower than LoQs. Consequently, the level of these analytes in ricotta was significantly lowered (about 50 % of Cd level in milk and below the LoQ for Pb). Therefore, these elements did not bind to whey components but were linked to curd components. In fact, their level increased about six-fold from milk to curd and this was the same concentration factor as fat. The change in concentration of Cd and Pb in primo sale and ripe cheese was similar to the change in dry matter, thus confirming that these chemical elements were strongly correlated to the curd components. In addition, the concentration factors were compared with those of Zn that has similar characteristics to Cd belonging to the same group of

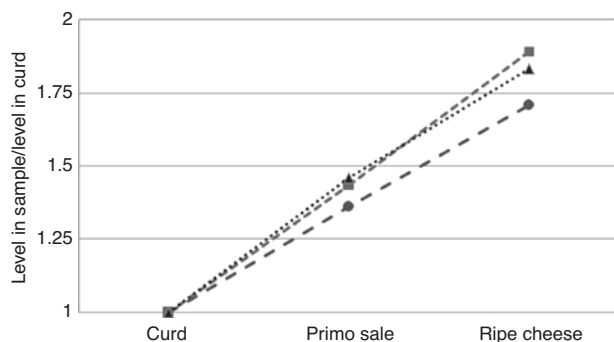


Fig. 3: Graphical representation of variation of Hg (solid circle), Cu (solid triangle) and fat (solid square) from curd to cheeses.

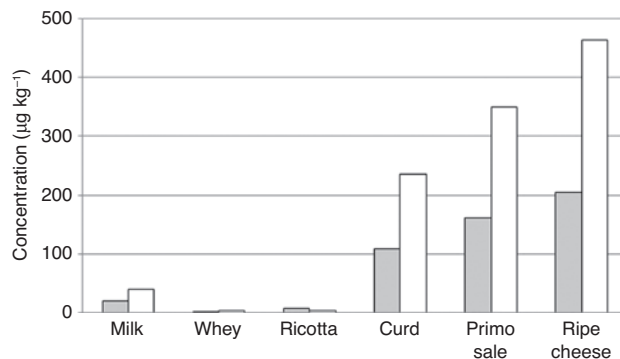


Fig. 4: Histogram of Cd (grey bar) and Pb (white bar) concentration in the dairy products.

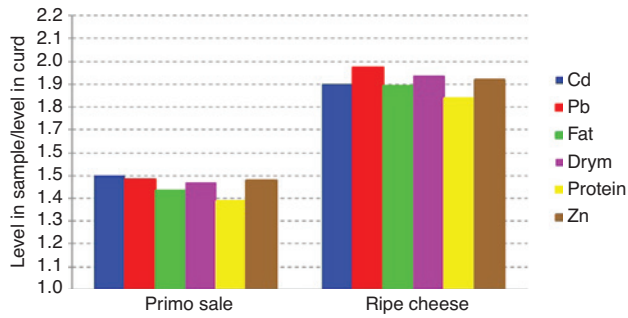


Fig. 5: Trend for Cd, Pb, Zn, dry matter, fat and protein in cheeses compared to curd.

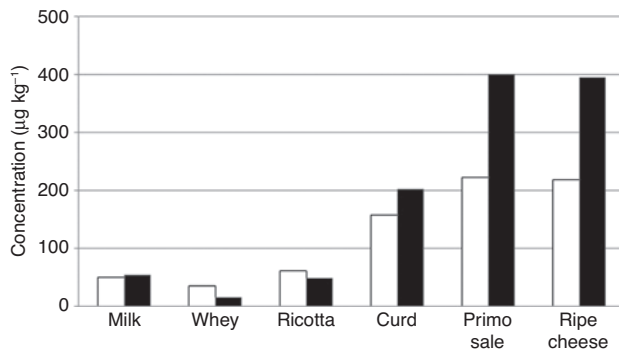


Fig. 6: Histogram of Ni (black bar) and Cr (white bar) concentration in the dairy products.

the periodic table. The concentration of Pb, Cd and Zn in primo sale and ripe cheese varied in the same way so it could be assumed that Pb and Cd were bound to curd components as Zn was. The behavior of Cd, Pb, Zn, dry matter, fat and protein is represented in Fig. 5.

The concentration of Ni and Cr had an analogous trend as shown in Fig. 6. The lowering of their content from milk to whey indicated that these elements did not simply dissolve in water since the water content in whey was higher than that in milk (92% vs. 87%). Furthermore, they moved from milk to whey with the same concentration factor as fat/proteins and dry matter, confirming a possible relationship with whey components.

As for the concentration factor ricotta/whey, Ni had the same factor as dry matter (3.4) and Cr the same factor as Ca (1.7). As far as cheese was concerned, both Cr and Ni seemed not to be affected by different level of salting and ripening since the concentration factor primo sale/curd and ripe cheese/curd was exactly the same (1.4 and 2.0 for Cr and Ni, respectively). This behavior could be compared to that of Mn, which had the same concentration factor (1.6) primo sale/ripe cheese.

The increase in concentration of Cu, Mn, Zn, Cd, Pb and Cr in curd confirmed results obtained in other studies [9]. The shift into curd could be explained as a result of a preferential bond of these elements to casein and fat. Ni and Mg acted differently from how observed by Coni and seemed to follow the same fate as Cu, Mn, Zn, Cd, Pb, Cr and Hg. That could be attributable to the curdling and salting used for the specific cheese making performed in this study as these phases affect changes in the element concentrations.

Conclusions

The likely link between spiked chemical elements and milk components was demonstrated through the analysis of concentration factors, the evaluation of the PCA and the correlation matrix. In case of unbound

elements, in fact, the cationic forms should be prone to pass preferentially into whey that has water content higher than curd. Except for As, all the other spiked elements seemed to shift preferentially into curd indicating a possible bond to certain proteins and fat components. Bivalent positive ions (Cd, Pb, Ni and Cr) could be complexed to casein through its phosphate groups that are negatively charged. It should be also conceivable a similar association with low molecular weight ligands (e.g. citrate), but the first hypothesis can be the most explicative due to the high casein concentration and the preferential shift into curd. This assumption could be supported by specific studies on Zn binding in milk [23, 24]. This essential element behaves similarly to some of the spiked analytes as confirmed by the correlation with Cd, Pb, Ni and Cr. As for Hg, the link to fat could be only assumed taking into account their similar concentration factor in the intermediate and final products. Furthermore, this hypothesis could be corroborated by the concentration factor of Cu that was comparable to that of Hg. Copper, in fact, is also strongly associated with the lipids of fat-globule in milk [25, 26]. However, the behavior of Hg should be further investigated as its correlations were not statistically confirmed unless transformed variables were used. As for Pb, the binding with milk proteins could lead to an increase in its concentration different from the mere concentration factor from milk to cheese.

The level of Pb seemed to also depend on the stage of ripening so this aspect should be deeply considered in case of setting or extrapolating a legal limit (Maximum Level) in/to cheese.

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References

- [1] International Dairy Federation. *The World Dairy Situation 2011. Bulletin of the International Dairy Federation*, **451**, 23 (2011).
- [2] Food and Agricultural Policy Research Institute. *FAPRI-ISU 2011 World Agricultural Outlook* (2011).
- [3] Organization for Economic Cooperation and Development–Food and Agriculture Organization. *OECD-FAO Agricultural Outlook 2011–2020* (2011).
- [4] M. Merdivanb, E. Yilmaza, C. Hamamcia, R. S. Aygunc. *Food Chem.* **87**, 163 (2004).
- [5] A. Anastasio, R. Caggiano, M. Macchiato, P. Catellani, M. Ragosta, S. Paino, M. L. Cortesi. *Acta Vet. Scand.* **47**, 69 (2006).
- [6] D. Mendil. *Food Chem.* **96**, 532 (2006).
- [7] R. Tahvonen, J. Kumpulainen. *Food Addit. Contam. Part A*, **12**, 789 (1995).
- [8] L. Gambelli, P. Belloni, G. Ingraio, L. Pizzoferrato, G. P. Santaroni. *J. Food Compos. Anal.* **12**, 27 (1999).
- [9] E. Coni, A. Bocca, P. Coppolelli, S. Caroli, C. Cavallucci, M. Trabalza Marinucci. *Food Chem.* **57**, 253 (1996).
- [10] E. Coni, B. Bocca, S. Caroli. *J. Dairy Res.* **66**, 589 (1999).
- [11] E. C. Pappa, A. C. Pappas, P. F. Surai. *Sci. Total Environ.* **372**, 100 (2006).
- [12] D. Bakircioglu, Y. B. Kurtulus, G. Ucar. *Food Chem. Toxicol.* **49**, 202 (2011).
- [13] Commission Regulation (EC) No. 1881/2006 of 9 December 2006 setting maximum levels for certain contaminants in foodstuffs. *OJL* **364**, 5 (2006).
- [14] Commission Regulation (EC) No. 629/2008 of 2 July 2008 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. *OJL* **173**, 6 (2008).
- [15] Commission Regulation (EU) No. 420/2011 of 29 April 2011 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. *OJL* **111**, 3 (2011).
- [16] Commission Regulation (EU) No. 488/2014 of 12 May 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs. *OJL* **138**, 75 (2014).
- [17] Commission Regulation (EU) No. 1005/2015 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs. *OJL* **161**, 9 (2015).
- [18] A. Sorbo, A. C. Turco, M. Di Gregorio, L. Ciaralli. *Food Control* **44**, 159 (2014).
- [19] Codex Alimentarius Commission. Codex general standard for cheese. CODEX STAN 283 (1978).
- [20] L. G. Favretto. *Food Addit. Contam. Part A* **7**, 425 (1990).

- [21] M. C. Martin-Hernandez, M. Juarez. *J. Dairy Sci.* **72**, 1092 (1989).
- [22] F. Gaucheron. *Reprod. Nutr. Dev.* **45**, 473 (2005).
- [23] G. Harzer, H. Kauer. *Am. J. Clin. Nutr.* **35**, 981 (1982).
- [24] P. Blakeborough, D. N. Salter, M. I. Gurr. *Biochem. J.* **209**, 505 (1983).
- [25] J. S. Aulakh, C. M. Stine. *J. Dairy Res.* **54**, 1605 (1970).
- [26] G. B. Fransson, B. Lönnerda. *Am. J. Clin. Nutr.* **39**, 185 (1984).