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Separation of Selenium, Zinc, and Copper Compounds in Bovine Whey Using Size Exclusion Chromatography Linked to Inductively Coupled Plasma Mass Spectrometry

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To study the role of trace elements for the quality and nutritional value of bovine milk, the distribution of selenium, zinc, and copper in whey was investigated using a method linking size exclusion chromatography to inductively coupled plasma mass spectrometry (SEC-ICP-MS). Three major peaks were detected for selenium, two peaks for zinc, and five peaks for copper. More than 65% of the selenium was found in protein fractions, mainly in fractions coinciding with the major whey proteins β-lactoglobulin and α-lactalbumin. All zinc was associated with low molecular weight compounds (<5 kDa) and one of these compounds was probably citrate. More than 60% of the copper eluted in protein fractions and two of the five major peaks probably contained metallothionein and citrate. This method was used to compare milk and whey produced by organic and conventional feeding procedures. The selenium content in whey and desalted milk produced using organic regimens was significantly lower than that in conventional samples. Moreover, the proportion of selenium in protein fractions of organic whey was significantly smaller than that in conventional whey, but the distributions of zinc and copper did not differ. This study showed that with the SEC-ICP-MS technique the distribution profiles of several trace elements in whey could be studied in the same run and that the selenium profile differed in whey produced by organic and conventional procedures.

KEYWORDS: Trace element distribution; ICP-MS; milk; whey; selenium; copper; zinc

INTRODUCTION

Milk and other dairy products are an important source of several trace elements in the human diet (*1*). For selenium and zinc it has been estimated that these foods account for 17 and 22%, respectively, of their intake in Sweden (*2*) and for both elements other animal foods make major contributions. Many factors regulate the concentration of trace elements in milk (*3*) and recently new knowledge has accumulated regarding the transporters mediating the transfer of zinc and copper to milk (*4*). Milk contains many proteins and peptides binding to minerals and trace elements and also other factors influencing the absorption of these nutrients (*5*, *6*). Some of them occur in whey and since whey is increasingly used as a food ingredient, there is a need to gain further knowledge on the distribution of trace elements in whey.

Many studies on selenium in foods have concerned its total amount (*7*) but only few have assessed the occurrence of different selenium compounds in milk and other foods (*8*−*14*). Also for zinc and copper, only a few speciation studies in milk have been performed (*14*, *15*), although several proteins and other ligands binding zinc and copper have been identified (*16*−*18*). The chemical form of trace elements can affect their bioavailability and for selenium this has been studied for several foods (*19*−*22*). Much interest has also been devoted to the bioavailability of trace elements in different animal species (*23*).

Both atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) have been used for selenium speciation studies, and for both techniques such factors as the limit of detection and the composition of the matrix affect their applicability. Milk is a complex matrix and for this reason studies of trace elements in whey are more feasible. The aim of this investigation was to study the distribution of selenium, zinc, and copper of bovine whey using size exclusion chromatography (SEC) and on-line detection with ICP-MS, and

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to apply this technique to a comparison of the distribution patterns in organically and conventionally produced bovine whey.

MATERIALS AND METHODS

Chemicals. Superdex 200 10/300 GL (1 cm × 30 cm) and PD-10 columns (containing Sephadex G-25) were supplied by Amersham Biosciences AB, Uppsala, Sweden. Superdex columns were calibrated using ferritin (type I from horse spleen, trimer) and a marker kit including β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) obtained from Sigma Chemical Co. (St. Louis, MO). As reference substances for the identification of the separated fractions, selenomethionine, selenocystine, bovine erythrocyte superoxide dismutase (Zn, Cu), rabbit metallothionein-II, bovine ceruloplasmin, bovine β-lactoglobulin, bovine α-lactalbumin, orotic acid, and urate were used (Sigma Chemical Co, St. Louis, MO). Other chemicals were of reagent grade.

Sampling of Milk and Preparation of Whey. In experiment 1, fresh bovine milk was obtained from the Experimental Station of the Swedish University of Agricultural Sciences, Alnarp, and it was defatted by centrifugation at 4 °C for 30 min at 4000*g* (J2-21, Beckman, Palo Alto, CA). To precipitate caseins, the pH of the milk was then adjusted to 4.6 using 10% (v/v) lactic acid followed by centrifugation at 5000*g* and 4 °C for 30 min to obtain the whey supernatant. The pH of whey was re-adjusted to 6.7 using 10 M sodium hydroxide and the sample was stored at −80 °C until analysis.

In the second experiment ten milk samples from cows reared on organic regimens and seven conventional milk samples were collected from different farms by the Danish Institute of Agricultural Sciences. Milk samples were defatted as described above before further preparation. Desalted milk was prepared using PD-10 columns. Whey samples from individual cows were prepared from defatted milk using lactic acid addition as described above. For separation of trace element compounds whey samples were pooled into four organic and three conventional samples according to their selenium content.

Size Exclusion Chromatography of Whey. Whey samples were diluted 1:2 with Tris acetate buffer (20 mM Tris acetate containing 0.15 M ammonium acetate and 3% methanol (v/v), pH 6.7) and filtered through a 0.22 μm filter before injection into a 250 μL sample loop (Valco Instruments Co. Inc., Houston, TX). The whey sample and the eluent (Tris acetate buffer) were then pumped by a Shimadzu LC-10AD pump (Kyoto, Japan) through a Superdex 200 column (fractionation range for globular proteins 10−600 kDa) with a flow rate of 0.7 mL min^{-1}. The separated fractions were detected by a Lambda Max detector (Waters, Milford, MA) at 280 nm and the outlet was on-line connected to a quadropole-based ICP-MS instrument (Thermo X7, Thermo Elemental, Winsford, UK) for element determination. Between runs the column was regularly washed according to the recommendations of the manufacturer to remove remaining material.

Calibration of Size Exclusion Chromatography Columns. A molecular weight marker kit was used for calibration of the Superdex 200 columns. Ferritin and water were used to determine the void and the total volume, respectively. The linearities of the selectivity curves of two columns in the range of 0.3−0.9 for the partition coefficient (K_{av}) were log MW= $-4.21K_{av} + 6.98$ ($r^2 = 0.97$) and log MW = $-3.72K_{av} + 6.44$ ($r^2 = 0.98$), respectively.

Inductively Coupled Plasma Mass Spectrometry. Selenium, zinc, and copper in whey and milk were quantified by the quadropole-based ICP-MS instrument equipped with a conical glass nebulizer (Glass Expansion, Melbourne, Australia) with 1 mL min^{-1} uptake and a Peltier chilled conical impact bead spray chamber (Thermo Elemental, Winsford, UK). The gas flows were 13 L min^{-1} for the cooling gas, 1.1 L min^{-1} for the auxiliary gas, and 0.93 mL min^{-1} for nebulizer gas. The samples were analyzed in peak-jumping mode for ^{45}Sc, ^{65}Cu, ^{66}Zn, ^{79}Br, ^{82}Se, and ^{89}Y (1 point per peak, 70 sweeps, and 10 ms dwell time for Sc, Y, Cu, Zn, and 30 ms for Br and Se). Interference corrections were made for ^{82}Se for the spectral overlap of brominehydride (BrH). Sample preparation and sample introduction were performed as described previously (*24*). The analytical accuracy was checked against reference material (Seronorm trace elements serum; batch JL4409, Sero AS, Billingstad, Norway). The results obtained were for Cu 1.1 (0.04) mg L^{-1} (mean (SD)), vs recommended 1.1 (0.06) mg L^{-1}, for Zn 1.1 (0.06) mg L^{-1} vs 0.85 (0.08) mg L^{-1}, and for Se 67 (5.9) μg L^{-1} vs 72 (6.1) μg L^{-1}.

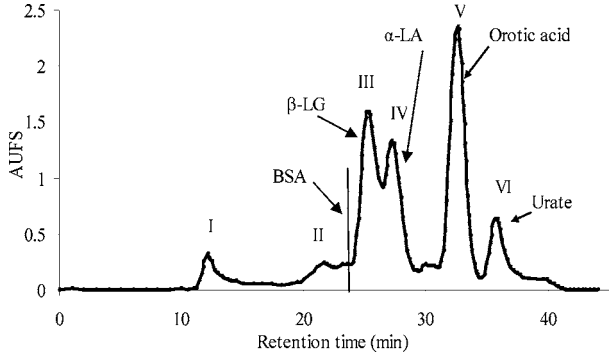

Figure 1. Fractionation of bovine whey using a Superdex 200 column and detection at 280 nm. The chromatogram represents a typical profile from three runs. Peaks III, IV, V, and VI coeluted with β-lactoglobulin (β-LG), α-lactalbumin (α-LA), orotic acid, and urate, respectively. The retention time for the standard substance bovine serum albumin (BSA; 66 kDa) is also indicated in the chromatogram.

For the speciation of selenium, zinc, and copper, the ICP-MS instrument was connected on-line to the SEC equipment. The eluate was analyzed in peak-jumping mode for ^{77}Se, ^{82}Se, ^{65}Cu, and ^{66}Zn (3 points per peak, 30 ms dwell time) in time-resolved analysis mode. The isotope ^{77}Se was used to confirm the spectral overlap of BrH on ^{82}Se. Thus, peaks detected at only ^{82}Se and not at ^{77}Se were not considered to contain selenium.

Statistical Analysis. To compare data obtained on organic and conventional whey and milk samples, they were subjected to a Mann−Whitney U test using SPSS 12.0.1. P values less than 0.05 were considered significant.

RESULTS

Separation of Whey on Superdex 200 Columns. In experiment 1 fractionation of whey resulted in six main peaks by detection at 280 nm (**Figure 1**). Peak I at the void volume represented high molecular weight proteins (>600 kDa), while peaks II, III, IV, and V eluted at apparent molecular weights of 195, 36, 14, and <2 kDa, respectively, and peak VI eluted approximately at the total column volume. Peaks III, IV, V, and VI coeluted with β-lactoglobulin, α-lactalbumin, orotate, and urate, respectively, in agreement with a previous study (*25*).

Selenium, Zinc, and Copper Compounds in Whey. With use of SEC-ICP-MS, four major peaks were found in whey samples by measurement of ^{82}Se (**Figure 2**) but the last peak eluting at about 35 min was excluded because it was not observed in the ^{77}Se chromatogram. Of the three remaining selenium peaks no. I and no. II eluted at mean K_{av} of 0.58 and 0.67, respectively, which corresponded to molecular weights of 36 and 14 kDa, and these two peaks comigrated to a large extent with the protein peaks of β-lactoglobulin and α-lactalbumin. Selenium peak III was found at a $K_{av} = 0.86$ with an apparent molecular weight of 2 kDa and this peak coeluted with selenomethionine and selenocystine, although these compounds have lower molecular weights. Most of the selenium (66%) was associated with the protein fractions (**Table 1**).

Two zinc peaks were found in the low molecular weight fractions eluting with apparent molecular weights of 4 and <2 kDa, respectively (**Figure 3**), and the major part of it (81%) was found in the 4 kDa fraction (**Table 1**). The pattern of copper-containing peaks differed markedly from that of zinc since five copper peaks were found, occurring in both protein

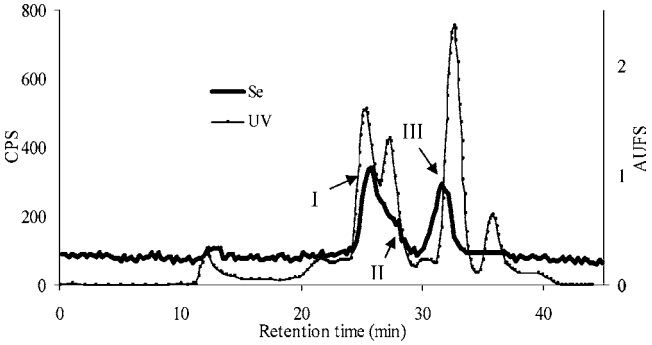

Figure 2. Distribution of selenium in whey using a Superdex 200 column connected to an ICP-MS. The chromatogram represents a typical profile from three runs. A *m*/*z* 82 peak at ~35 min representing interference from BrH was omitted and is indicated by a horizontal line.

Table 1. Partition Coefficients (K_{av}), Estimated Molecular Weights (MW), and Distribution of Selenium, Zinc, Copper, and A280 Peaks in Bovine Whey Using SEC-ICP-MS ($n = 3$)[a]

peak	K_{av}	MW (kDa)	%
		A280	
I	–0.05	>600 (n.d.[b])	5 (4–5)
II	0.40	195 (183–209)	11 (9–13)
III	0.58	36 (32–38)	25 (23–26)
IV	0.67	14 (13–16)	19 (17–20)
V	0.93	<2 (n.d.[b])	32 (31–33)
VI	1.08	<2 (n.d.[b])	8 (6–10)
		Se	
I	0.58	36 (32–38)	66[c] (63–68)
II	0.67	14 (14–19)	
III	0.86	2 (2–2)	34 (32–37)
		Zn	
I	0.8	4 (4–4)	81 (77–90)
II	0.97	<2 (n.d.[b])	19 (10–23)
		Cu	
I	–0.05	>600 (n.d.[b])	17 (14–20)
II	0.48	91 (88–92)	31 (30–32)
III	0.71	10 (10–11)	15 (12–16)
IV	0.79	5 (5–5)	33 (29–37)
V	0.93	<2 (n.d.[b])	4 (2–5)

[a] Data Are Expressed as Means (Range). [b] n.d., not determined. [c] The percentage of both peaks I and II because these peaks overlapped.

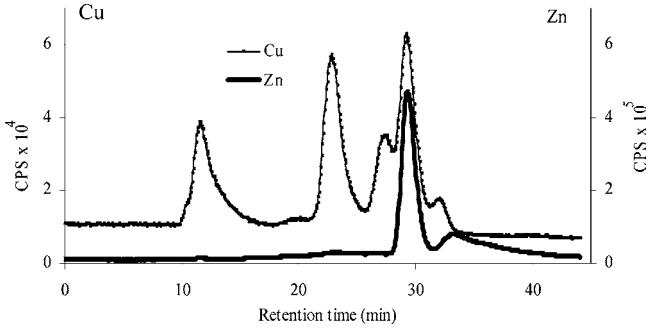

Figure 3. Distribution of zinc and copper in bovine whey using a Superdex 200 column connected to an ICP-MS. The order of chromatograms is the same as that in the legend. The chromatogram represents a typical profile from three runs.

and low molecular weight fractions (**Figure 3**). The first peak eluted at the void volume and the other peaks at K_{av} of 0.48, 0.71, 0.79, and 0.93 with calculated molecular weights of 91, 10, 5, and <2 kDa, respectively. More than 60% of the copper

Table 2. Mean Content of Selenium, Zinc, and Copper of Bovine Whey and Desalted Milk Produced by Organic and Conventional Regimes[a]

	desalted milk	whey		
	Se (μg L^{-1}) mean (range)	Se (μg L^{-1}) mean (range)	Zn (mg L^{-1}) mean (range)	Cu (μg L^{-1}) mean (range)
organic	4.6[b] (2.4–6.1)	2.2[c] (1.5–2.7)	2.0 (0.7–2.7)	15 (11–18)
conventional	7.3 (4.2–9.7)	3.0 (2.7–3.4)	2.2 (1.0–3.1)	18 (12–21)

[a] n = 7–10 for selenium and n = 3–4 for zinc and copper. [b] Milk selenium from organic regimens was significantly lower than that of conventional milk, $p = 0.02$. [c] Whey selenium from organic regimens was significantly lower than that of conventional whey, $p = 0.001$.

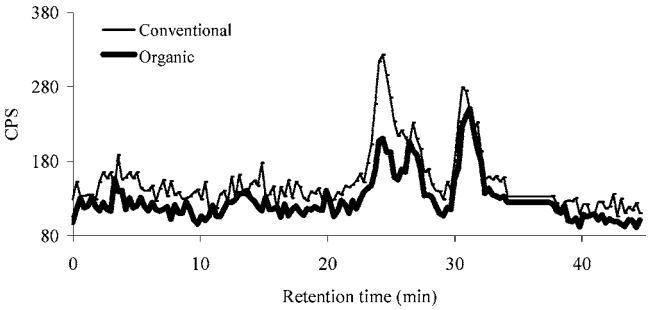

Figure 4. Distribution of selenium in organic and conventional bovine whey using a Superdex 200 column connected to an ICP-MS. The non-selenium peaks at ~35 min were excluded from the chromatogram as indicated by horizontal lines. The order of chromatograms is the same as that in the legend. The chromatograms represent typical profiles from three to four runs.

was found in the protein fractions (peaks I−III, **Table 1**). The migration rate of peak II (91 kDa) was close to but probably not identical to albumin whereas that of peak III migrated as the reference compound metallothionein-II, although the latter has a molecular weight of 6 kDa. The retention time of peak III was also close to that of α-lactalbumin. Since both zinc and copper occurred in 4−5 kDa peaks, this might represent another trace-element binding ligand(s) but it was not identified. The peaks of zinc and copper at <2 kDa could at least partly represent citrate.

Trace Element Patterns in Whey Obtained from Organic and Conventional Regimens. The selenium content as measured in both whey and desalted milk samples from organic regimens was significantly lower than that in the conventional samples (**Table 2**). Upon chromatography of organic whey samples, the selenium peaks I and II were more clearly separated than those in conventional whey (**Figure 4**), which is consistent with the result shown in **Figure 2**. The proportion of selenium in the protein fractions of organic whey was significantly lower than that in conventional whey ($p = 0.03$; **Table 3**) but there were no significant differences between the elution volumes of selenium peaks in organic and conventional whey. No significant differences in the content of zinc and copper were found between organic and conventional whey samples (**Table 2**). As in experiment 1, two peaks of zinc were found in whey samples from both farming systems and almost 90% of the zinc was located in peak I (**Table 3**). Also in this experiment five copper-containing fractions were found in both organic and conventional whey samples (**Figure 5**). The distributions and elution volumes

Table 3. Distribution of Selenium, Zinc, Copper, and A280 Peaks Using SEC-ICP-MS in Bovine Whey Produced by Organic and Conventional Regimens ($n = 3–4$)[a]

peak	organic whey %	conventional whey %
	A280	
I	5 (3–6)	5 (4–6)
II	12 (11–13)	12 (10–14)
III	29 (27–32)	27 (25–29)
IV	16 (14–17)	15 (14–16)
V	31 (29–33)	33 (32–33)
VI	7 (6–7)	8 (7–10)
	Se	
I	33[b] (29–37)	64[c] (63–65)
II	22[b] (18–24)	
III	45 (41–49)	36 (35–37)
	Zn	
I	89 (83–95)	88 (79–94)
II	11 (5–17)	12 (6–21)
	Cu	
I	32 (27–35)	30 (29–32)
II	28 (25–33)	32 (28–37)
III	18 (17–20)	16 (14–18)
IV	21 (19–23)	20 (15–24)
V	1 (1–1)	2 (2–4)

[a] Data are expressed as mean (range). Peak migration data are shown in **Table 1**. [b] The percentage of selenium in peaks I and II of whey produced by organic regimens was significant lower than that of conventional whey, $p = 0.03$. [c] The percentages of peaks I and II were combined because these peaks overlapped.

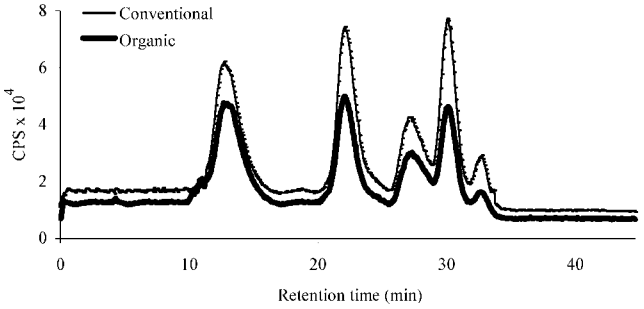

Figure 5. Distribution of copper in organic and conventional bovine whey using a Superdex 200 column connected to an ICP-MS. The order of chromatograms is the same as that in the legend. The chromatograms represent typical profiles from 3 to 4 runs. The baseline of the curve for the conventional sample was adjusted upward to increase clarity.

of zinc and copper of organic whey were not significantly different from those of conventional samples.

DISCUSSION

The speciation of trace elements in milk is a complicated task for several reasons (*12*, *13*). The milk matrix with its content of protein and lipids can interfere with the analytical methods. Our preliminary experiments showed that chromatography of whole or defatted milk was more complicated to link to trace element analysis and therefore this study focused on the analysis of whey. To yield a pure whey fraction, acid whey was used because sweet whey prepared by centrifugation or rennet treatment contains small amounts of non-micelle caseins or κ-casein (*26*). This report shows the feasibility of a method for the study of the distribution of several trace elements in whey, which is suitable for application to other types of whey samples and maybe also milk samples. There was a good reproducibility when the same sample was chromatographed several times as indicated by the low variation in retention times and percent distributions (**Tables 1** and **3**). The major advantages of using ICP-MS as a selenium detection technique compared to AAS used previously (*9*) are that selenium measurement can be performed on-line, which is time- and labor-saving. In addition the ICP-MS technique has a lower limit of detection. The trace element peaks were tentatively identified from their calculated molecular weights but this method can in some instances be misleading since such factors as the carbohydrate content and structure of proteins may influence the chromatographic mobilities. To obtain more detailed information on trace element compounds in the protein and low molecular weight fractions in milk and whey, it would be desirable to develop procedures using several columns with different separation mechanisms (*27*).

Distribution of Selenium in Whey. In the present study most of the whey selenium was found in the 14−36 kDa fractions using Superdex 200 columns. Since a large part of the selenium comigrated with β-lactoglobulin and α-lactalbumin, this suggests a nonspecific incorporation of selenium as selenomethionine into these two major whey proteins. β-Lactoglobulin contains four methionine residues per molecule while α-lactalbumin contains only one (*28*), which may explain the lower incorporation into the latter protein. Nonspecific selenium incorporation might also have occurred in less abundant whey proteins like immunoglobulins and serum albumin, but no signicant selenium peaks could be detected at the corresponding migration rates. Previously it was found that 12, 6, 42, and 19% of the whey selenium was associated with immunoglobulins, serum albumin, β-lactoglobulin, and α-lactalbumin fractions, respectively, which is close to our findings (*26*).

In addition to the nonspecific incorporation of selenium it is incorporated into specific selenoproteins as selenocysteine. The essentiality of selenium is attributed to the 25 specific selenoproteins demonstrated in the human genome (*29*) such as the glutathione peroxidase (GSHPx) family, the thioredoxin reductase family, the iodothyronine deiodinase family and the selenophosphate synthetase family (*30*). So far only extracellular GSHPx has been demonstrated in milk and whey (*31*, *32*). However, no selenium peak could be found migrating at the expected molecular weight of 96 kDa for extracellular GSHPx (**Figure 2**), confirming that it represents a low proportion (3%) of whey selenium (*32*). In a preliminary experiment using another chromatographic system we found that GSHPx in whey eluted in one major peak corresponding to 115 kDa and in milk one peak with several shoulders was observed at an apparent molecular weight of 155 kDa, using detection by immunoassay. It can be speculated that in milk and whey GSHPx might exist in a complex attached to other compounds as proposed previously based on studies of bovine and human milk (*33*, *34*). The other blood plasma selenoprotein, selenoprotein P, could not be demonstrated in bovine whey using SEC-ICP-MS. Previously, it was found to migrate as a 174 kDa peak in human serum, although its peptide weight is 43 kDa (*35*).

In the present study approximately one-third of the whey selenium was found in the low molecular fraction, which is similar to the somewhat lower percentage (20%) found previously (*26*). This may represent selenomethionine, selenocystine, or other selenium compounds with the same chromatographic mobility. This agrees with the demonstration of selenomethionine in ultrafiltered human milk and formula based on cow milk (*36*). With use of an advanced analytical scheme, several other low-molecular selenium compounds were found in human milk (*37*).

Turning to the selenium distribution in bovine milk, several studies have shown that only a small proportion of selenium (<6%) was associated with the lipid fraction and most of the selenium was bound in milk proteins (*26*, *33*, *38*, *39*). Some authors found that selenium was mainly associated with the casein fraction, while others found more selenium in whey than in caseins (*26*, *33*, *38*). The explanation for these divergent findings is not apparent.

Distribution of Zinc and Copper in Whey. The ICP-MS technique also permitted the measurement of other trace elements in the same run, making feasible the study of zinc and copper. All zinc in whey occurred in compounds <5 kDa, which agrees with previous findings that the binding of zinc to milk proteins is decreased at low pH (*17*, *18*, *40*, *41*). Much of the zinc is associated with citrate as studied in human milk (*42*), which may explain the <2 kDa zinc peak. Regarding the zinc in the 4 kDa peak, its identity is unclear. The high proportion of low-molecular weight zinc agrees with previous findings showing that >50% of the zinc occurred in fractions with <5 kDa in whey prepared from human milk or formula based on cow milk, although the variation between samples was considerable (*43*).

In the present study copper in whey was found to be associated to compounds with a large range of molecular sizes with five major peaks. Also in a previous study copper was distributed in many fractions of whey from human milk and formula based on cow milk (*44*), although our use of ICP-MS on-line better demonstrated the shape of the peaks. Analogous studies on human milk also revealed a distribution of copper in many fractions (*14*−*16*), and nearly 40 and 25% of copper was found in fractions containing serum albumin and low-molecular-weight compounds, respectively (*16*). The copper-binding ligand in the low-molecular-weight fraction in both human and bovine milk was identified as citrate (*45*) and this may explain the <2 kDa copper peak found by us. Peak III (10 kDa) migrated as metallothionein and can probably be accounted for by a metallothionein, although its different forms have molecular weights in the range 6−7 kDa. The migration of peak III was also close to that of α-lactalbumin and in a previous study of human milk the major copper peak was tentatively identified as α-lactalbumin (*46*). The copper-containing ceruloplasmin (*47*) and superoxide dismutase (*48*) have been demonstrated in bovine milk, but in our study no copper was detected at elution times matching these proteins (132 and 32 kDa), probably due to their low concentrations in whey. In human milk ceruloplasmin was estimated to account for 5−25% of the copper (*47*, *49*). The absence of a ceruloplasmin peak may also be due to the formation of a ceruloplasmin−lactoferrin complex as demonstrated in human milk (*50*). The identities of the copper peaks I, II, and IV in the present study are difficult to interpret. The 91 kDa peak moved close to but is not identical to albumin and another hypothetical possibility is that it represented a degradation product of ceruloplasmin (*51*). Regarding the copper 5 kDa peak, it is suggestive that a large part of the zinc also had this migration rate and it may be hypothesized that it represented a compound binding both metals.

Trace Element Profiles in Milk and Whey Produced by Organic and Conventional Regimens. In the recent decades the agriculture in Europe is converting toward more sustainable production systems due to the increased awareness of environmental pollution. The nutrient composition of organic foods may differ from that of conventional ones due to restrictions in feed composition for organically reared animals. It is argued that organic animal foods may be safer compared to conventional foods because of the reduced use of synthetic chemicals. A study of trace elements in milk in relation to the breeding system showed that the content of zinc and copper tended to be lower in milk from cows reared on organic than conventional regimens but no such difference was seen for selenium (*52*). In contrast the present study showed the selenium content in organic whey and desalted milk to be significantly lower than that in the conventional samples, which is in agreement with previous findings in bovine milk (*53*). Furthermore, the selenium in organic whey seemed to be shifted to low molecular compounds, but the mechanism for this is not clear. The lower content of selenium in organic milk may be due to the absence of selenium supplementation of the feed, which is often used since the selenium content in the soil in Scandinavian countries is low (*54*). This is supported by results obtained in Norwegian farm animals in which the blood selenium content was lower on farms not using selenium supplementation of feed (*55*).

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