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The Influence of the Addition of Polyacrylic Hydrogel on the Content of Proteins, Minerals and Trace Elements in Milk Protein Solutions

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Summary

Solutions of milk protein concentrate, whey protein concentrate and bovine serum albumin (BSA) were treated with polyacrylic hydrogel to establish whether the hydrogel could be used for decontamination of heavy metal ions from milk protein-based products. The obtained results indicated that swelling of hydrogel in these solutions had different effects on their mineral, trace element and total protein content. Total protein and phosphorus content increased in milk protein concentrate and whey protein concentrate solutions after swelling of hydrogel without changes in their protein compositions. On the other hand, the protein content in BSA solution decreased after swelling. The content of Na did not change in milk protein concentrate solution, whereas it significantly increased in whey protein concentrate solution after hydrogel swelling. The content of Ca and Mg was reduced after the swelling in milk protein concentrate and whey protein concentrate solutions for 20.3–63.4 %, depending on the analysed sample and the mineral. The content of Zn did not change during swelling, whereas the content of Fe, Cu, Mn, Ni and Pb significantly decreased after hydrogel swelling in all analysed samples. According to the obtained results, the addition of polyacrylic hydrogel to milk and whey protein concentrate solutions can significantly decrease the content of heavy metal ions without affecting their protein composition. Therefore, this work could be useful in developing a new technological process for heavy metal purification of milk protein-based products.

Key words: milk protein solutions, polyacrylic hydrogel, mineral content, trace element content

Introduction

Hydrogels are polymeric networks with defined degree of cross linking which have enormous possibility of swelling in aqueous solutions (*1*). During swelling, these materials do not dissolve in water. Great power of hydrogel swelling in water is based on the ability of water molecules to penetrate into the porous structure of the hydrogels, which causes an increase in their total volume. When hydrogel is swollen, the other components present in the aqueous solution can also penetrate, depending on their affinity to bind to the hydrogel struc-

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ture. Previous studies have shown that hydrogels can bind different heavy metal ions: Pb^{2+} (*2*,*3*), Cu^{2+} and Cd^{2+} (*3*) and Ni^{2+} (*4*,*5*). Also, these materials can interact with different biomacromolecules, such as bovine serum albumin (BSA) (*6*,*7*), lysozyme (*8*), α-lactalbumin (*9*), cytochrome C and protamines (*10*). Thanks to these and other properties such as biocompatibility, nontoxicity and hydrophility, polyacrylic hydrogels have found widespread application in the field of medicine (*11*,*12*), pharmacy (*13*), environmental protection and agriculture (*14–16*). Recently, there have been reports on the use of hydrogels based on natural materials for the adsorption of heavy metal ions, which could be used in the field of environmental chemistry and chemical technology. Oxidized cellulose hydrogel (*17*) and cation-induced hydrogels of cellulose (*18*) have shown a high capacity for binding metal ions such as Cu^{2+} (*17*,*18*), Ca^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+} (*18*) and can be used as reusable heavy metal ion adsorbents (*17*). Also, semi-interpenetrating polymer network (SIPN) hydrogel based on chitosan and gelatin (*19*) was used for fast and efficient adsorption of Cu^{2+} ions, while granular alginate-based hydrogel (*20*) has been used in same way for the removal of Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} ions. At the same time, these types of materials can also interact with some protein molecules. For example, superabsorbent hydrogel based on starch has been used as carrier for albumin (*21*), methacrylated dextran (*22*,*23*) has been used for controlled release of immunoglobulin G (IgG), whereas some other derivatives of dextran have been used for adsorption of BSA (*24*).

Industrialization during last centuries resulted in heavy metal contamination of soils and water worldwide. There is evidence that vegetables and crops are able to accumulate Cd, Zn, Ni, Cu, Pb and Cr in their edible and inedible parts at various concentration levels (*25*). Furthermore, recent studies have shown that milk (*26*,*27*), baby food (*28*) and dairy products (*29*,*30*) could also be contaminated with heavy metals. Although some heavy metals such as Cu, Zn, Mn and Fe are considered as essential micronutrients and have a variety of biochemical functions in living organisms, they can be toxic when taken in excess. For example, manganese plays an important role in some neurological disorders (*31*). Toxicity and negative impact of nonessential heavy metals on human health are well documented.

Milk is one of the most abundant foods in human nutrition and is considered as an important food product for all age groups. Apart from fresh milk, cheese, yogurt and a wide range of milk protein-based products can be obtained during technological processing of milk. Milk protein concentrate, whey protein concentrate and individual milk proteins are widely used in food industries. Despite the benefits of milk and milk products as one of the major food products for infants and children, the presence of contaminants, such as heavy metals, may represent health risks. Moreover, infants and children tend to be exposed to relatively higher levels of contaminants, since they consume more food relative to their body mass compared to adults.

Taking into account that milk can be contaminated with heavy metals and their concentration and retention in milk and milk products are closely related to animal feeding, the time of the year of sample collection, environmental conditions and manufacturing processes (*29*, *32–34*), their determination and possible decontamination are of great importance for human health, especially for children.

To the best of our knowledge, studies about the application of hydrogel for food decontamination of heavy metals are not available. For these reasons, the objective of our research is to investigate whether polyacrylic hydrogel could be used for heavy metal decontamination of milk protein products. Milk protein-based products, milk protein concentrate, whey protein concentrate and bovine serum albumin dissolved in Ultrapure water were used as model systems. The obtained results could be useful in developing new technological processes for purification of milk protein-based products.

Materials and Methods

Materials

Polyacrylic hydrogel used in this investigation was synthesized by simultaneous radical polymerization reaction, following procedure described by Kostic *et al*. (*1*). *N'*,*N*-methylenebisacrylamide was used as a cross-linking agent, while sodium persulphate and sodium thiosulphate were used as redox initiators coupled with 30 % hydrogen peroxide. The obtained gel was converted to Na^+ salt (60 %) by neutralization of polyacrylic acid with a 3 % solution of sodium carbonate. Milk protein concentrate (MPC) with max. 85.5 % of protein was obtained from Ingredia (Arras Cedex, France), and whey protein concentrate (WPC) with max. 78 % of protein was supplied from DMV International (Veghel, The Netherlands). Crystallized bovine serum albumin (BSA) with 98 % of protein was obtained from Gerbu (Gerbu Biotechnik GmbH, Heidelberg, Germany). Heavy metal standards were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All chemicals and reagents used in this study were dissolved in Ultrapure water (Ultrapure water system, v. 1.11, SG Water USA LLC, Londonderry, NH, USA).

Sample preparation

Volumes of 100 mL of 2.8 % MPC, 2.2 % WPC and 0.26 % BSA solutions were prepared by dissolving in Ultrapure water. The pH of the prepared solutions was 6.72, 6.60 and 6.92 for MPC, WPC and BSA, respectively. Sodium azide (0.02 g) was added to all solutions to prevent bacterial growth. After that, 0.1 g of dried hydrogel (xerogel) was added to the investigated solutions and left to swell for a day to reach equilibrium degree of swelling (when hydrogel does not change its mass anymore). For all experiments 5 mL of each sample were taken before and after hydrogel swelling for further analysis.

Determination of total nitrogen, protein and phosphorus contents

Total nitrogen content was determined using official AOAC method 991.20 (*35*). Total protein content was calculated by multiplying total nitrogen with 6.38. Total phosphorus was established by the molybdovanadate method (*36*) from samples digested in concentrated sulphuric acid.

SDS-PAGE

SDS-PAGE was performed under reducing conditions according to the method of Fling and Gregerson (*37*). The mass per volume ratio of separating gel was 12.5 %, pH=8.85, and of stacking gel 5 %, pH=6.8. The running buffer was Tris-glycine (0.05 M Tris, 0.19 M glycine, and 0.1 % (by mass per volume) of SDS). All samples were heated in boiling water for 5 min before analysis. Aliquots of 11 μL of investigated samples were loaded per well. The gel was run for 3 h to completion. After electrophoresis, the gel was fixed and stained for 1 h in Coomassie® Blue dye solution (0.23 %, by mass per volume, of Coomassie® Brilliant Blue R250, 3.9 %, by mass per volume, of trichloroacetic acid, 6 %, by volume, of acetic acid and 17 %, by volume, of methanol). This was followed by destaining step using destaining solution (18 %, by volume, of ethanol and 8 %, by volume, of acetic acid), and then the gel was scanned using PS scanner (HP scanner 2400, Hewlett-Packard Company, Houston, TX, USA).

Determination of Ca, Mg, Fe, Zn, Cu, Mn, Ni and Pb

The content of Ca, Mg, Fe, Zn, Cu Mn, Ni and Pb was determined as described by Jones and Case (*38*) using atomic absorption spectrometer (Varian Spectra AA220 FS, Varian Inc., Palo Alto, CA, USA) in an acetylene/air flame. Briefly, a volume of 5 mL of all samples was digested with 20 mL of concentrated HNO_3 (Merck, Darmstadt, Germany) at 150 °C for 2 h. Samples were closed in beakers and left overnight. The next day, 3 mL of 33 % hydrogen peroxide were added to all samples and heated at 120 °C for 15 min to ensure complete dissolution and oxidation of samples. After cooling, the procedure with H_2O_2 was repeated once again. After that, 2 mL of $HClO_4$ were added to the samples and they were placed on a hot plate until white fumes of $HClO_4$ appeared. The solution was then quantitatively transferred into a volumetric flask containing Ultrapure water and diluted to 25 mL. The samples were stored in the polypropylene flasks under refrigeration until analyzed.

Standard calibration curves were used for quantitative determination. The mean recoveries of Ca, Mg, Fe, Zn, Cu, Mn, Ni and Pb were 103.8, 101.3, 98.6, 101.5, 102, 100.5, 96.1 and 95.4 %, respectively. The detection limits (in mg/L) for each metal were calculated as the standard deviation of the mean of the blank values multiplied by three (*39*) and were: Ca 0.003, Mg 0.009, Zn 0.042, Fe 0.021, Cu 0.001, Mn 0.003, Ni 0.013 and Pb 0.003. The quantification limits were obtained by multiplying the standard deviation by ten (*33*) (in mg/L): Ca 0.01, Mg 0.03, Zn 0.14, Fe 0.07, Cu 0.003, Mn 0.012, Ni 0.045 and Pb 0.01.

Determination of Na and K

Na and K were determined in the samples digested in a concentrated sulphuric acid for 30 min, after which a mixture of concentrated H_2SO_4 and $HClO_4$ (in a ratio of 1:1) was added to the samples and left until they became transparent. Thereafter, the available contents of alkali metals were determined by a flame photometric method (*40*).

Statistical analysis

All experiments were made in triplicate. The obtained data were analysed using STATISTICA software v. 6.0 (*41*). The results are presented as mean values±standard deviations (S.D.). The mean values obtained for each analysed parameter before and after hydrogel swelling were compared using *t*-test at $p<0.05$.

Results and Discussion

Protein content and composition

Total protein content in the investigated samples significantly ($p<0.05$) increased, by 11.06 % in MPC solution and by 14.53 % in WPC solution, but significantly decreased, by 15.38 %, in BSA solution after the treatment with polyacrylic hydrogel (Table 1). The reduction of total protein content in BSA solution after the treatment with polyacrylic hydrogel can be expected, since other authors noticed that the polyacrylic hydrogels can bind to the BSA molecules (*6,7*). SDS-PAGE analysis was performed to establish whether the protein composition of these solutions changes during swelling of hydrogels.

Table 1. Protein content in the investigated samples of MPC, WPC and BSA before and after hydrogel swelling

Samples		*w*(protein)/%
MPC	before swelling	$(2.26\pm0.10)^a$
	after swelling	$(2.51\pm0.10)^b$
WPC	before swelling	$(1.72\pm0.08)^a$
	after swelling	$(1.97\pm0.08)^b$
BSA	before swelling	$(0.26\pm0.01)^a$
	after swelling	$(0.22\pm0.01)^b$

^{a,b}mean values with the same superscript letters in the same column for the specific protein solutions were not significantly different at $p<0.05$; MPC=milk protein concentrate solution, WPC=whey protein concentrate solution, BSA=bovine serum albumin solution

The electrophoretic patterns of the investigated samples revealed that the intensity of all protein bands in MPC and WPC solutions slightly increased, but decreased in BSA solution after swelling of hydrogel (Fig. 1). This observation was also confirmed by densitograms (Fig. 2), which indicated that milk protein composition did not change after the treatment. The increase of protein content in the analysed milk and whey protein solutions after the treatment was probably caused by the absorption of water molecules by hydrogel during swelling.

The interactions between proteins and polyacrylic hydrogels could be a result of the reaction between free carboxylate groups of polyacrylic hydrogels and the oppositely charged groups of protein molecules. All major milk proteins, caseins and whey proteins, have isoelectric points below natural pH of milk (caseins at pH= 4.6 and whey proteins at pH=4.8–5.2). Since the swelling of hydrogels occurred in protein solutions at pH under isoelectric points of major milk proteins, the net charge of these proteins was negative. This could be the main

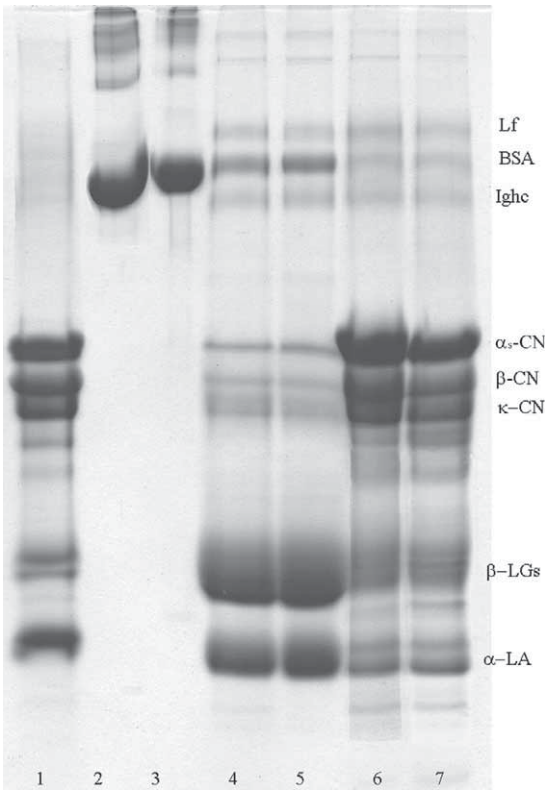

Fig. 1. SDS-PAGE analysis of milk protein concentrate (MPC), whey protein concentrate (WPC) and bovine serum albumin (BSA) solutions before and after swelling of polyacrylic hydrogel. Lane 1=milk protein standards, lane 2=BSA solution before swelling, lane 3=BSA solution after swelling, lane 4=WPC before swelling, lane 5=WPC after swelling, lane 6=MPC after swelling, lane 7=MPC before swelling. Lf=lactoferrin, BSA=bovine serum albumin, Ighc=immunoglobulin heavy chain, α_s-CN=α_s-casein, β-CN=β-casein, κ-CN=κ-casein, β-LGs=β-lactoglobulins, α-LA=α-lactalbumin

reason for the results obtained in this study. The interactions of BSA molecules with hydrogels in the BSA solution could be explained by low ionic strength of this solution. It has been found that even proteins with a net negative charge may adsorb to the negatively charged hydrogels when the ionic strength of the protein solution is low (*7*). High ionic strength of MPC and WPC solutions could explain why the basic minor protein, lactoferrin, did not adsorb to hydrogel during swelling. This is very important to note because this protein is a biologically active protein (*42*).

Na and K content

Na and K content of the samples is given in Table 2. In all samples potassium was under the detection limit. Sodium content significantly increased in WPC solution after swelling of hydrogel, but no significant changes of its content were detected in the MPC solution. In BSA solution its concentration was under the detection limit. The difference in Na content in WPC solution before and after hydrogel swelling could be a consequence of two simultaneous processes: (*i*) absorption of water by the hydrogel during swelling, which concentrates Na^+ ions in the solution, and (*ii*) squeezing out of Na^+ ions from

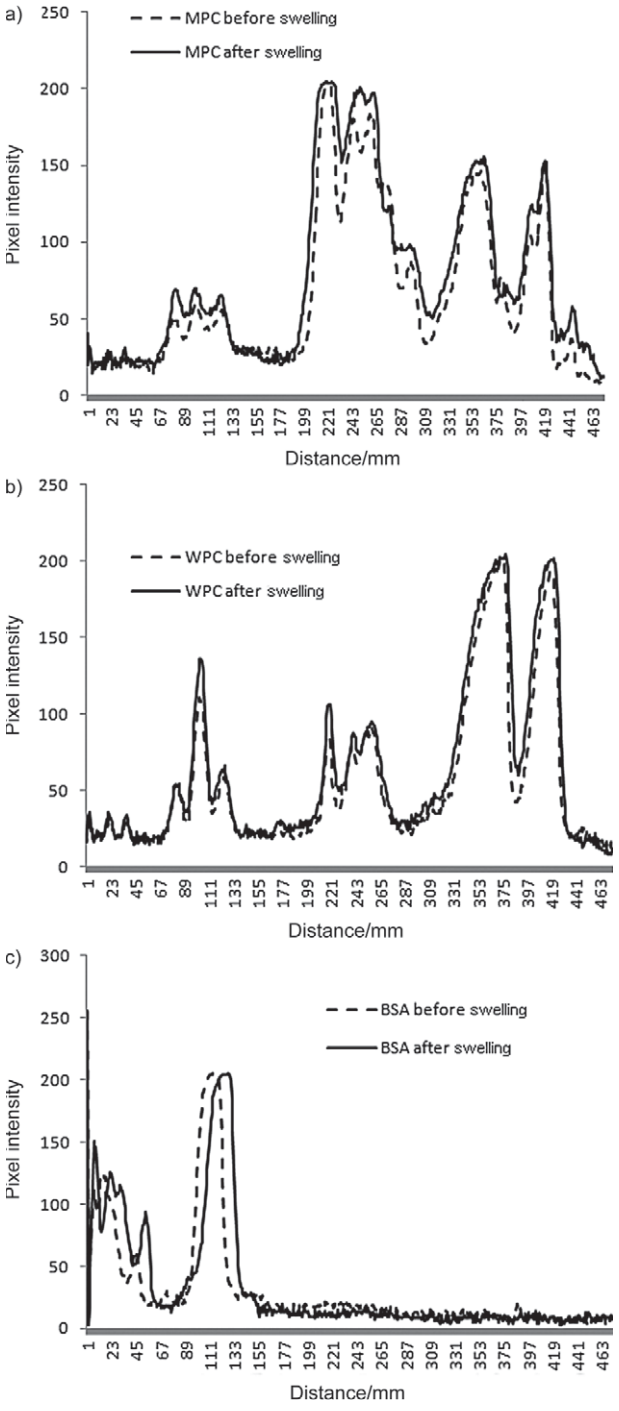

Fig. 2. Densitometric patterns of: a) milk protein concentrate (MPC), b) whey protein concentrate (WPC), and c) bovine serum albumin (BSA) solutions before and after swelling

the hydrogel into the solution by some other ions that have a higher affinity for binding to the hydrogel. Namely, about 60 % of hydrogel was in the form of sodium salts because of its synthesis procedure (*1*).

Phosphorus content

Phosphorus content increased in all analysed solutions after hydrogel swelling. The increase was by about 57, 21 and 150 % in MPC, WPC and BSA solutions, re-

Table 2. Content of minerals in protein solutions before and after swelling of hydrogel

Mineral	Milk protein concentrate		Uptake	Whey protein concentrate		Uptake	Bovine serum albumin		Uptake
	before swelling	after swelling	%	before swelling	after swelling	%	before swelling	after swelling	%
γ(K)/(mg/L)	n.d.	n.d.	–	n.d.	n.d.	–	n.d.	n.d.	–
γ(Na)/(mg/L)	(28.2±0.4)^a	(27.5±1.2)^a	–	(50.0±1.6)^a	(75.8±3.0)^b	–	n.d	n.d.	–
γ(P)/(mg/L)	(0.84±0.04)^a	(1.32±0.05)^b	–	(0.43±0.02)^a	(0.52±0.02)^b	–	(0.28±0.01)^a	(0.70±0.02)^b	–
γ(Ca)/(mg/L)	(765±31)^a	(476±14)^b	37.8	(66.1±1.7)^a	(24.2±0.6)^b	63.4	n.d.	n.d.	–
γ(Mg)/(mg/L)	(28.9±0.7)^a	(20.2±0.6)^b	30.1	(9.18±0.25)^a	(7.32±0.18)^b	20.3	n.d	n.d.	–
essential trace elements									
γ(Zn)/(mg/L)	(2.27±0.10)^a	(2.29±0.11)^a	–	(0.48±0.02)^a	(0.45±0.02)^a	–	n.d.	n.d.	–
γ(Fe)/(mg/L)	(0.51±0.02)	n.d.	100	(1.93±0.08)^a	(1.65±0.07)^b	14.5	(0.44±0.02)	(0.34±0.02)	22.7
γ(Cu)/(μg/L)	(14±0.5)^a	(12±1)^b	14.3	(380±20)^a	(170±10)^b	55.3	(7.24±0.35)	(3.60±0.10)	50.3
γ(Mn)/(μg/L)	(44±2)^a	(29±1)^b	34.1	(82±3)^a	(47±2)^b	42.7	n.d.	n.d.	–
nonessential trace elements									
γ(Ni)/(μg/L)	n.d.	n.d.	–	(110±5)^a	(50±3)^b	54.5	(95±4)^a	(80±4)^b	15.8
γ(Pb)/(μg/L)	n.d.	n.d.	–	(79±3)^a	(11±0.1)^b	86.1	(42±1)	n.d.	100

^{a,b}mean values with the same superscript letters in the same row for the specific protein solutions were not significantly different at $p<0.05$; n.d.=not detected

spectively (Table 2). In all three cases hydrogel did not absorb phosphorus, probably because it is mainly present in the solutions as a negatively charged phosphate ion, and polyacrylic hydrogel is negatively charged. The increase in the content of phosphorus in all three solutions could be the result of water adsorption by the hydrogel.

Ca and Mg content

Ca content in MPC and WPC samples decreased after hydrogel swelling, whereas in BSA solution its concentration was under detection limits (Table 2). Percentage of Ca^{2+} ion uptake by the hydrogel was 37.8 and 63.4 % in MPC and WPC solutions, respectively. On the other hand, average percentage of Mg^{2+} ion uptake by the hydrogel was in the range from 20.3 to 30.1 % in all analysed solutions. It can be concluded that hydrogel did not show great selectivity for binding these ions, which was probably affected by the ionic force of the solutions. Ionic force in MPC solution was higher than in WPC solution, and in WPC solution it was higher than in BSA solution, as the concentrations of these solutions were different and decreased in the order: MPC>WPC>BSA. There was a greater electrostatic potential difference inside and outside the hydrogel in the solution with the lower ionic force than in the solution with a higher ionic force (*43*). So, in accordance with that, the ability of ions to diffuse inside and outside of hydrogel is higher in the solution with a lower than in those with a higher ionic force.

Zn content

In MPC and WPC solutions, concentration of Zn^{2+} ions did not change after hydrogel swelling (Table 2). In BSA solution, the content of zinc was under the detection limit. These results indicated that hydrogel did not bind Zn^{2+} ions during swelling. This characteristic of polyacrylic hydrogel may be valuable, because zinc is an essential trace element (*44*).

Fe content

The uptake of Fe^{3+} ions in MPC solution was 100 %. On the other hand, binding of Fe^{3+} ions to polyacrylic hydrogel was significantly weaker in WPC and BSA solutions (Table 2). The reason for this could be the difference in the ionic force of the analysed solutions, but more likely it was due to the competition among ions present in the solutions for binding of free carboxylate groups, which are located in the polyacrylic hydrogel network. This competition occurred among all divalent and polyvalent ions. It is known that ions with smaller diameter (which easily penetrate through polymer network) or with larger electric charge (which show more electrostatic affinity to bind to hydrogel) have a greater ability to bind to hydrogels (*45,46*). Nevertheless, ions with higher ability to form complexes with free carboxylate ions (such as chelate complex) have also greater ability to bind to hydrogels (*47,48*).

Cu content

Percentage of Cu^{2+} ion uptake in MPC was lower than in WPC solution (Table 2), which was consistent with the reduction of ionic force in the WPC solution compared to the MPC solution. However, it should be noted that the initial concentration of copper ions in MPC samples was significantly lower. Also, it is possible that they compete with other ions, especially with Fe^{3+} ions, which are completely attached to the hydrogel after swelling in MPC solution. Initial concentration of Cu^{2+} ions in BSA solution was also low, but uptake after swelling of hy-

drogel was 50.3 %, which shows that there is a significant preference of polyacrylic hydrogel for binding Cu^{2+} ions from the solutions with lower ionic force.

Mn content

A reduction of manganese content in MPC and WPC solutions before and after the addition of hydrogel was noticed (Table 2). Percentage of uptake of Mn^{2+} ions was slightly higher in WPC samples than in MPC samples, which is consistent with the reduction of ionic force in WPC compared to the MPC solution. In BSA solution, Mn content was under the detection limit. The reduction of Mn content by the addition of hydrogel could be valuable, taking into account that Mn has a negative role in the development of some neurological disorders (*31*).

Ni and Pb content

Ni^{2+} and Pb^{2+} ions were not detected in MPC samples, but their content was determined in WPC solution. They could originate from whey, because WPC used in this study was prepared by ultrafiltration process. According to literature data, ultrafiltration membrane permeability of some milk contaminants such as Pb, Cu, Zn, Fe and Mn was very low, resulting in low reduction or even increase of the level of these elements in the retentate (*29*,*49*,*50*). After swelling of hydrogel in WPC solution, the hydrogel bound a significant amount of nickel (54.5 %), as well as lead (86.1 %). In BSA solution, hydrogel had weak affinity for binding Ni^{2+} ions (15.8 %), but the removal of Pb^{2+} ions was complete.

Conclusion

In conclusion, treatment of MPC, WPC and BSA solutions with polyacrylic hydrogel had different effects on their content of total proteins and the investigated minerals and trace elements. Total protein and phosphorus content increased in MPC and WPC solutions after the addition of hydrogel, without changing their protein compositions, whereas mineral content increased, decreased or remained unchanged, depending on the analysed solution and the mineral. The content of trace elements significantly decreased after swelling of hydrogel in all analysed protein solutions. It seems that the affinity of hydrogel for different elements probably depends on their concentration in the solutions and competition for binding sites on hydrogel. The reduction of Mg and Ca contents could represent a negative effect of the addition of hydrogel to MPC and WPC solutions, but significant reduction of the content of heavy metal ions, such as Mn, Cu, Ni and Pb, and a slight increase in the protein mass fraction in MPC and WPC solutions could be considered as a positive effect. Milk protein and whey protein concentrates are prepared mainly by ultrafiltration/diafiltration processes, which cannot effectively remove all milk contaminants. Taking into account that polyacrylic hydrogels showed high affinity to bind trace elements without any interactions with milk proteins, and with low production cost, it can be concluded that they could be used for efficient purification of milk protein-based products. However, further research is needed to test possible applications of hydrogel for removal of heavy metal ions from milk protein-based solutions.

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