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요 약. This paper has described about preparation of Zr^{4+} affinity column based on the poly(styrene-*co*- glycidyl methacrylate) prepared by emulsion polymerization of styrene and glycidyl methacrylate in order to isolate phosphopeptide. The Zr^{4+} ions were introduced after the phophonation of an epoxy group on polymeric microspheres. The successful preparation of Zr^{4+}-immobilized polymeric microsphere stationary phase was confirmed through Fourier transform infrared spectra, optical microscopy, scanning electron microscopy, X-ray photoelectron spectra and inductively coupled plasma-atomic emission spectrometer. The separation efficiency for Zr^{4+} affinity column prepared by slurry packing was tested to phosphonated casein and dephosphonated casein. The resolution time (min) of the phosphonated casein was higher than that of dephosphated casein for Zr^{4+} affinity polymeric microsphere by liquid chromatography. This Zr^{4+} affinity column can be used for isolation of phosphonated casein from casein using liquid chromatography.

주제어: Zr^{4+} affinity column; poly(styrene-*co*-glycidyl methacrylate); polymer microsphere; phosphonated casein; resolution time

ABSTRACT. This paper has described about preparation of Zr^{4+} affinity column based on the poly(styrene-*co*-glycidyl methacrylate) prepared by emulsion polymerization of styrene and glycidyl methacrylate in order to isolate phosphopeptide. The Zr^{4+} ions were introduced after the phophonation of an epoxy group on polymeric microspheres. The successful preparation of Zr^{4+}-immobilized polymeric microsphere stationary phase was confirmed through Fourier transform infrared spectra, optical microscopy, scanning electron microscopy, X-ray photoelectron spectra and inductively coupled plasma-atomic emission spectrometer. The separation efficiency for Zr^{4+} affinity column prepared by slurry packing was tested to phosphonated casein and dephosphonated casein. The resolution time (min) of the phosphonated casein was higher than that of dephosphated casein for Zr^{4+} affinity polymeric microsphere by liquid chromatography. This Zr^{4+} affinity column can be used for isolation of phosphonated casein from casein using liquid chromatography.

Keywords: Zr^{4+} affinity column; poly(styrene-*co*-glycidyl methacrylate); polymer microsphere; phosphonated casein; resolution time

INTRODUCTION

The preparation methods for polymeric microspheres have been investigated for many years.[1-5] Dispersion and emulsion polymerizations are commonly used for the preparation of polymeric microspheres. Polymer microspheres are applied a wide of fields, for example, immunoassay, cell labeling, latex reagents, drug carriers, and column packing materials for liquid chromatography.[6-9] The application fields can be expanded by adding functional groups on polymeric microspheres. Copolymerization is one of the efficient methods for introducing functional groups on polymer microspheres.

Glycidyl methacrylate (GMA) is one of the monomers which are easily modified into various functional groups. After GMA is polymerized, the epoxy groups of poly-GMA can be changed to alcohols,[10] amines,[11] phosphonic acid,[12] sulfonic acid,[13] or etc.[14]

In a previous paper,[15] the poly(methacrylate) (PM) was prepared by emulsion polymerization in order to apply the cosmetic supporters after coating of vitamin C. The spherical PM with various sizes can be obtained by changing reaction conditions. The coating of vitamin C on the surface of PM microspheres by using cyclodextrin as a binder can be achieved to 30 wt-% water/methanol mixture. We have also prepared the polymeric microsphere with an epoxy group by the radiation-induced polymerization of GMA and diethylene glycol dimethacrylate in reaction conditions with variations in solvents, irradiation dose, and monomer composition.[16] Lipase was immobilized to the epoxy group of the polymeric microsphere in experimental conditions with variations in the pH and the epoxy group content. The activity of the lipase-immobilized PM was in the range of 148-342 unit/mg min. However, these polymeric microspheres can not be used the packing materials for high performance liquid chromatography (HPLC) column because the polymeric microsphere have its soft properties and small diameter.

On the other hand, phosphonated casein can be obtained from casein by enzymatic hydrolysis during manufacture of milk products as well as during intestinal digestion.[17,18] It can be used for the food and drug materials as carrying supporter of calcium ions. However, there are no reports for isolation of phosphonated casein from casein by HPLC, to our knowledge.

In this study, we prepared the metallic affinity column based on poly(styrene-*co*-GMA) microsphere with Zr^{4+} ions. The Zr^{4+} ions were introduced on polymeric microsphere after phophonation. The Zr^{4+}-immobilized polymer microsphere stationary phase was characterized by FT-IR, OM, SEM, XPS and ICP-AES. The separation efficiency for Zr^{4+} affinity column prepared by slurry packing was tested for phosphonated casein and dephosphonated casein.

EXPERIMENTAL

Chemicals. Poly(vinylpyrrolidone) (PVP) and α,α-azobis(isobutyronitrile) (AIBN) as an initiator was obtained from Junsei Co. (Japan). Styrene, glycidyl methacrylate (GMA), pyridine, phosphorus oxychloride (99%), zirconyl chloride hydrate ($ZrOCl_2$, 99.9%), phosphonated casein and dephosphated casein separated from bovine milk was purchased from Sigma-Aldrich Co. Other chemicals were also purchased with reagent grades.

Instrumentation. The surface morphology of the samples was determined by using scanning electron microscopy (SEM, S-3000N, Hitachi Science System Ltd., Japan). Optical microscope image of the samples were obtained by Olympus, IMC-BOF, Japan. Fourier transform infrared (FT-IR) spectra were recorded in the range 400-4000 cm^{-1} with a 4 cm^{-1} resolution from KBr pellets on a Perkin-Elmer Spectrum 1000 system (Perkin-Elmer life and analytical sciences, USA). The X-ray photoelectron spectra of the samples were obtained using Thermo Fisher Scientific, MultiLab. ESCA2000, USA. The Zr contents were analyzed by inductively coupled plasma-atomic emission spectrometer (ICP-AES) (Jobin-Yvon, Ultima-C, USA). Also, the separation efficiency of the prepared column was determined by high performance liquid chroma-

tography (HPLC) (Aglient, 1100 series, USA) with UV detector.

Synthesis of Zr^{4+} affinity stationary phase based on poly(St-*co*-GMA) microsphere. *Scheme* 1 shows the preparation procedure of Zr^{4+} affinity stationary phase based on poly(styrene-*co*-GMA) with epoxy groups. Poly(styrene-*co*-GMA) microspheres were prepared by emulsion polymerization of comonomers (60 g), PVP (6.0 g) as stabilizer, AIBN (0.6 g) as initiator in ethanol (153 mL) with stirring 70 rpm at 65 °C under nitrogen. The ratios of comonomer to styrene:GMA were described in *Table* 1

Table 1. Effect of amount of GMA on poly(St-*co*-GMA) microsphere[a]

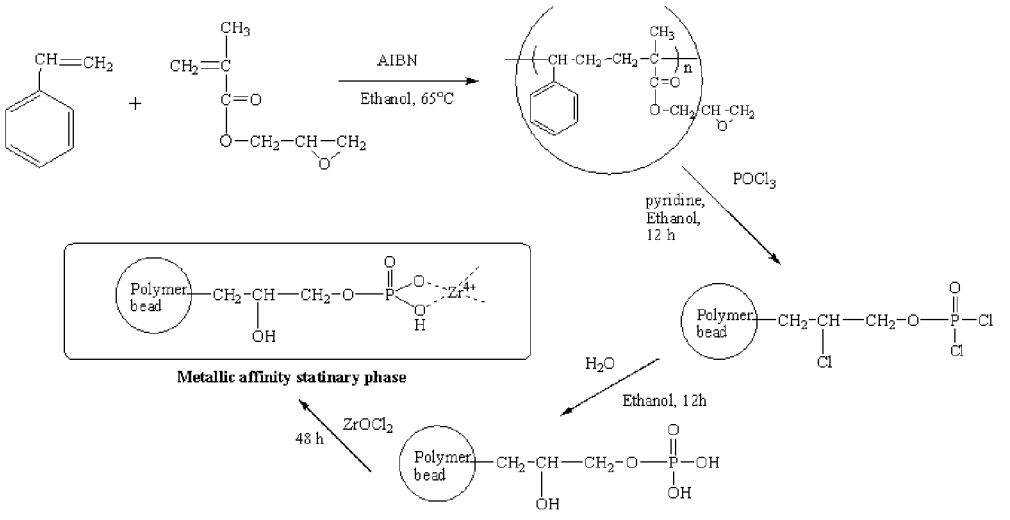
No.	GMA (mol)	Styrene (mol)	Diameter (μm)[b]
1	100	0.00	1.20 ± 0.002
2	90.0	10.0	4.54 ± 0.002
3	70.0	30.0	6.77 ± 0.002
4	50.0	50.0	5.24 ± 0.002
5	30.0	70.0	4.41 ± 0.002
6	10.0	90.0	8.79 ± 0.002
7	0.00	100	4.69 ± 0.002

[a]The copolymer was prepared by emulsion polymerization of comonomers in EtOH (153 mL) with PVP (6.00g, MW=40,000) and AIBN (0.60 g) at 65 °C with stirring of 70 rpm. [b]determined by OM.

and 2. Zr^{4+} ion was introduced in poly(styrene-*co*-GMA) with ratio of styrene:GMA=9:1 (run 6 in *Table* 1) as follows: The epoxy group-introduced polymeric microsphere (1.0 g) was reacted with $POCl_3$ (0.6 g, 1.0 mol) in acetonitrile (5.0 ml) in the presence of pyridine (0.93 g, 3.0 mol) under nitrogen for 12 hrs. The white powder was obtained by filtration, and then dried in vacuum oven at 50 °C for 8hrs. The powder was again dispersed in acetonitrile (5.0 ml). Subsequently, the deionized water (0.2 ~ 0.3 ml) was added under nitrogen at RT for 12hrs. In order to immobilize Zr^{4+} to phosphoric acid group, $ZrOCl_2$ (0.5 g) was added under nitrogen at RT, and was reacted for 48hrs. Finally, the obtained powder was washed with acetonitrile and dried in vacuum oven at 50 °C for 8hrs. The obtained Zr^{4+} affinity stationary phase was suspended in a hexane/2-propanol (9/1) mixture and packed in a stainless-steel column (100 × 2.1 mm I.D.) by a slurry packing method for use to HPLC.

RESULTS AND DISCUSSION

Firstly, we prepared Zr^{4+} affinity columns based on SiO_2 microsphere for the separation of phosphonated casein from casein using a HPLC system. Silica-based chromatography supports have numerous



Scheme 1. Preparation procedure of the Zr^{4+} affinity stationary phase based on polymer particle with epoxy group.

advantages, such as high mechanical stability, resistance to swelling, and excellent efficiency.[19] However, the silica-based affinity column cannot be used for the separation of phosphonated casein from casein because the mobile phase (buffer solution) is not passed on the affinity column. So, we selected styrene and GMA because of improving rigid properties to styrene and introducing functional group on GMA in order to prepare packing material for liquid chromatography. *Scheme* 1 explains the preparation procedure of the Zr^{4+} affinity stationary phase based on polymer particle with epoxy group.

Table 1 shows the results of copolymerization of styrene and GMA in methanol at 65 °C for 24 hrs as function of a molar ratio. The diameter of poly (styrene-*co*-GMA) with spherical form was in the range of 4.41-8.79 μm determined by an

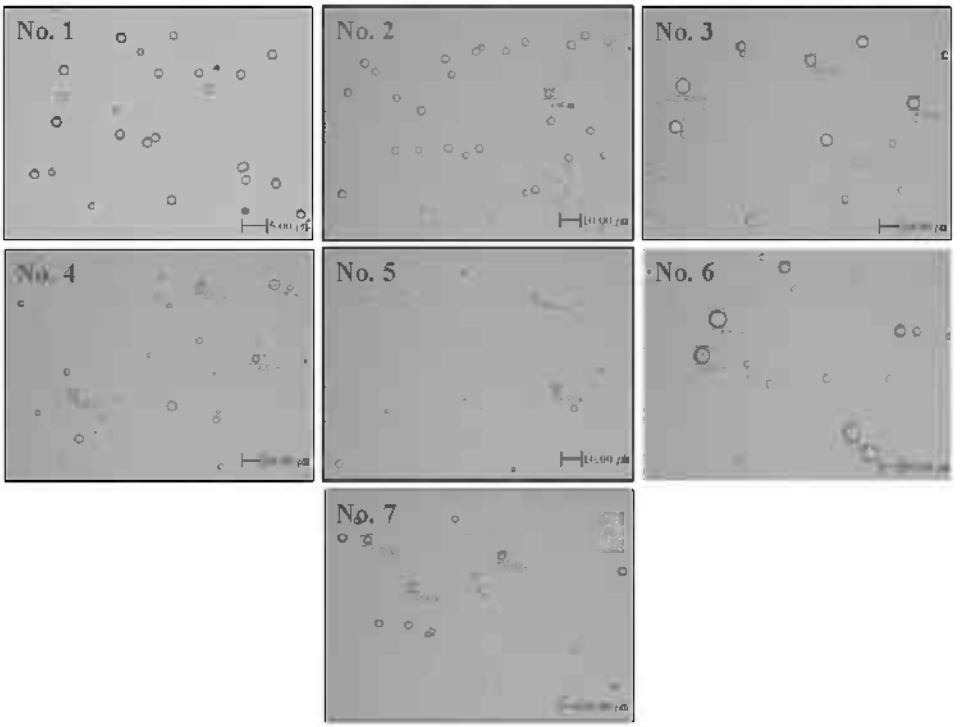


Fig. 1. OM images of No. 1, 2, 3, 4, 5, 6, and 7 in Table 1.

Table 2. Polymerization results of poly(St-*co*-GMA) as Function of GMA Content and PVP with various molecular weight[a]

Reactin Number	Styrene (wt-%)	GMA (wt-%)	Diameter (μm)	Remark
1	100	-	4.00 ± 0.002	Fig. 2(a,b)
2	99.0	1.00	4.83 ± 0.002	Fig. 2(c,d)
3	98.0	2.00	5.30 ± 0.002	Fig. 2(e,f)
4	99.1	0.90	6.28 ± 0.002	Fig. 3(a,b)
5	99.1	0.90	4.51 ± 0.002	Fig. 3(c,d)
6	99.1	0.90	2.25 ± 0.002	Fig. 3(e,f)

No. 4, 5, and 6 was used PVP with MW = 10,000, MW = 40,000, 360,000, respectively. [a]Polymerization condition: PVP 6g (MW = 40,000); AIBN 0.6 g; Ethanol 153 mL; at 65 °C, 70 rpm. [b]Determined by SEM.

optical microscopy (*Figure* 1). During phosphonation for poly(styrene-*co*-GMA), the poly(styrene-*co*-GMA) was become gelated. In order to keep the microsphere morphology of the copolymer, the molar ratio of the styrene monomer was highly increased above 99% as shown in *Table* 2. *Table* 2 shows the results of the copolymerization of styrene and GMA in methanol at 65 °C for 24 hrs as function of a molar ratio and stabilizer (PVP) with different molecular weights. The diameter of polystyrene with monodispersed microsphere form, No. 1 in *Table* 2 was about 4.0 μm (1 in *Table* 2). The diameter of poly(styrene-*co*-GMA), No. 2 and 3 were increased than polystyrene by adding GMA. The poly(styrene-*co*-GMA) was formed with microsphere and the diameter of the copolymer was increased as GMA contents were increased. However, the small size of microsphere was produced by

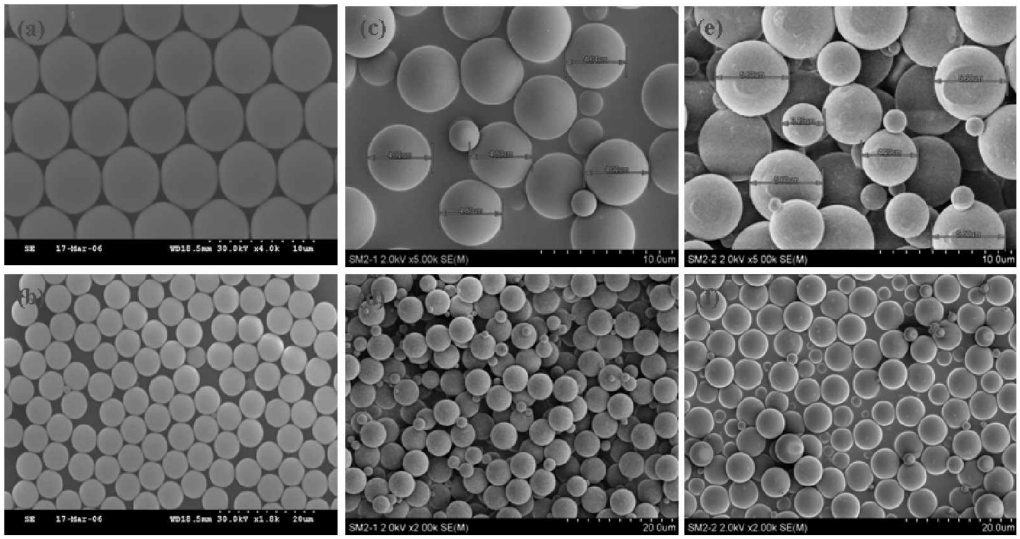


Fig. 2. SEM images of No. 1(a, b), 2(c, d) and 3(e, f) in Table 2.

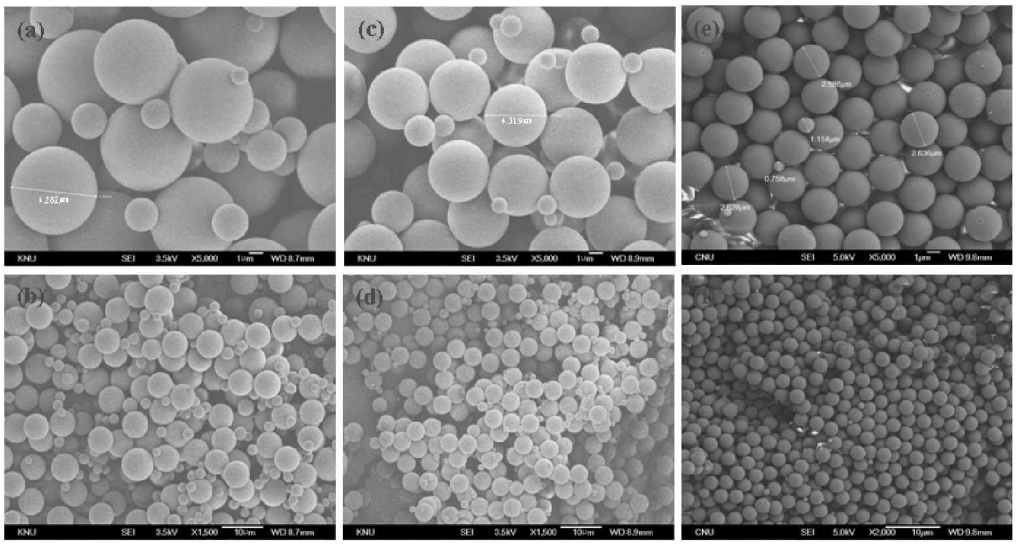


Fig. 3. SEM images of No. 4(a, b), 5(c, d) and 6(e, f) in Table 2.

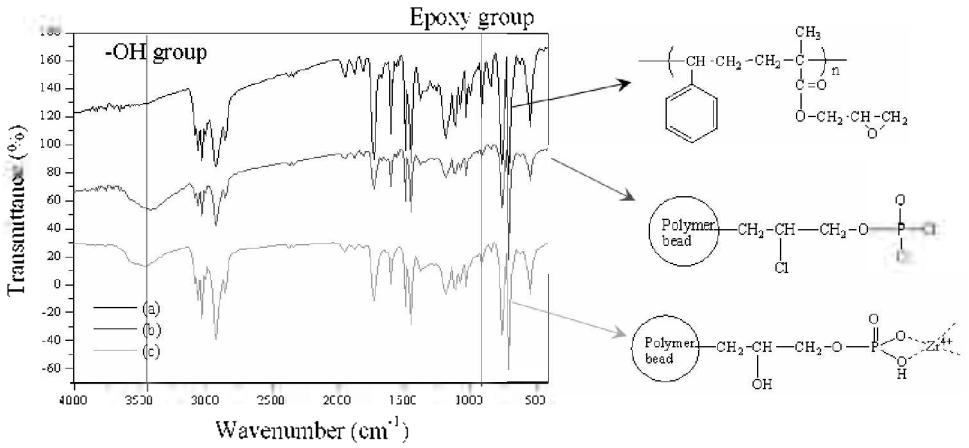


Fig. 4. IR spectra of Zr^{4+} affinity stationary phase.

adding GMA (*Figure* 2-c,d,e.f). In No. 4, 5, and 6 in *Table* 2, the diameter of copolymer was decreased with increasing molecular weights of PVP as shown in *Figure* 3. The poly(styrene-*co*-GMA) can be stabilized for microsphere morphology during phosphonation when the content of styrene was 99.0%. The microsphere morphology is very important factor in order to use HPLC packing materials. We used the copolymer of the diameter with above 5 μm as the packing materials for liquid chromatography (No. 4 in *Table* 2).

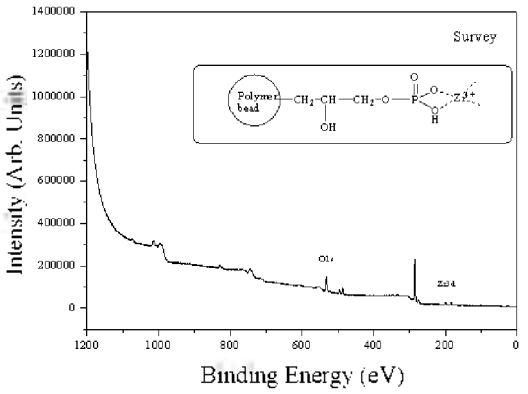


Fig. 5. XPS survey scan spectra of Zr^{4+} affinity stationary phase.

Table 3. Contents of Zr^{4+} affinity stationary phase[a]

Samples	Zr content (wt-%)
Zr^{4+} affinity stationary phase	2.07

[a]Metal content was determined by ICP-AES.

Figure 4 shows FT-IR spectra of the poly(styrene-*co*-GMA) (a), phosphoro chloride modified poly (styrene-*co*-GMA) (b), and Zr^{4+} affinity stationary phase (c). In *Figure* 3-a, the characteristic absorption peaks at 910 and 1730 cm^{-1} were appeared due to C-O-C (epoxy group) and O-C=O (carbonyl group), respectively. In *Figure* 4-b, c, the epoxy absorption peaks at 910 cm^{-1} was disappeared. On the other hand, around 3500 cm^{-1} due to -OH group was observed. These results may be considered that the phosphate groups was successfully introduced to epoxy group of the poly(styrene-*co*-GMA).

Figure 5 reveals the XPS survey scan spectra of the Zr^{4+} affinity stationary phase based on poly (styrene-*co*-GMA) microsphere. The O 1s and Zr 3d of the Zr^{4+} affinity stationary phase were appeared at 532 eV and 182 eV, respectively. As shown in *Table* 3, the content (wt-%) of Zr atom was to be 2.07% by ICP-AES. As results, the Zr^{4+} ion was successfully introduced to epoxy group of the poly(styrene-*co*-GMA). In order to know the separation efficiency of the prepared affinity column, we performed the separation for phosphonated casein and dephosphonated casein as model protein using HPLC.

Figure 6 exhibited the resolution results of phosphonated casein (a) and dephosphonated casein (b) using by Zr^{4+} affinity column. The resolution condition is as follows: Column, 100 × 2.1 mm I.

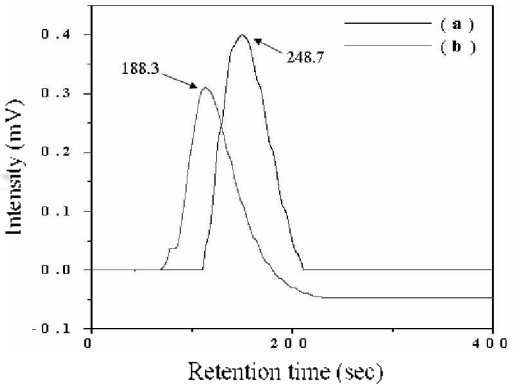


Fig. 6. Resolution of phosphorylated casein (a) and dephosphorylated casein (b) using by Zr^{4+} affinity column. (Column, 100 × 2.1 mm I.D.; triple distilled water; Flow rate, 0.1 ml/min; room temperature; Detect, UV 254 nm).

D.: triple distilled water; Flow rate. 0.1 mL/min; Room temperature; Detector. UV 254nm. The retention time (min) of the phosphonated casein and dephosphonated casein was at 2.5 min and 1.9 min, respectively. This means that the phosphonated casein was strongly interacted with Zr^{4+} ion of the affinity column. Therefore, we expect to use the affinity column for the isolation of phosphonated casein from casein using liquid chromatography.

CONCLUSION

The Zr^{4+} affinity column based on the poly (styrene-*co*-GMA) was prepared by emulsion polymerization of styrene and GMA. The retention time (min) of the phosphonated casein and dephosphonated casein was at 2.5 min and 1.9 min, respectively. The affinity column can be used for isolation of phosphonated casein from casein using liquid chromatography.

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