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Study of imidazole performance as pseudo-affinity ligand in the purification of IgG from bovine milk

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ABSTRACT

The spherical sepharose CL-6B beads were activated by epichlorohydrin in different epoxy contents (80, 120 and 160 $\mu mol_{epoxide}/mL_{gel}$) and, L-histidine and imidazole as pseudo-affinity ligands were covalently immobilized to them. Some linkers with different length, (1,2-ethanediol diglycidyl ether and 1,4-butanediol diglycidyl ether) were synthesized for activation of sepharose and the activated sepharose beads modified with imidazole and the performance of these adsorbents in the purification of immunoglobulin G from bovine milk were evaluated. Among the L-histidine bearing adsorbents, higher adsorption of IgG (0.28 mg/mL) was obtained by adsorbent with the lower concentration of L-histidine. The highest amount of IgG adsorption (0.53 mg/mL) was obtained by imidazole bearing adsorbent with the highest amount of imidazole and Among the adsorbents with synthesized linkers, the adsorbent with 1,2-ethanediol diglycidyl ether showed better performance and was able to purify 0.25 mg/mL IgG with high purity. The synthesized pseudo-affinity adsorbents represented the abbility to purify immunoglobulin G in one-step process with high purity and efficiency.

1. Introduction

Because of the vital role of antibodies in the biotechnology, medicine, pharmaceutical, and food industries, isolation and purification of antibodies are very important and it has increased dramatically over the past decades [1–5]. Antibodies or immunoglobulins (Ig), are Y-shaped, unique and soluble glycoproteins that are secreted by lymphocyte B cells in the immune system to counteract foreign antigens. These specific antibody-antigen interactions have led to extensive applications of antibodies in various fields [5–9]. Immunoglobulin G (IgG) is one of the important and interesting antibodies of bovine milk and purification of this bioactive protein from bovine milk has attracted the researcher's attention during the last years [10–14]. It is the most common group of antibodies which is the smallest antibody among the other immunoglobulins and it can be stable throughout the purification process [15,16]. According to the important role of IgG for immunoaffinity chromatography, immunotherapy, drug delivery, and treatment of immune disorders like alloimmunization, rheumatoid arthritis, and cancer, it is very essential to isolate and purify it [17–22]. A large number of methods, including precipitation, electrophoresis, filtration, ion exchange, size exclusion, and affinity chromatography have been used for purification of antibodies [23,24]. Affinity chromatography is a kind of separation technique that employs the unique and reversible interactions between a protein and related ligand to isolate the target protein [25–29]. These binding properties that offered by affinity ligand are used for selective adsorption of target protein from a protein mixture [30,31]. Recently for simplifying the purification process and providing high selectivity, efficiency and easy recovery of protein, considerable efforts have been made. To recompensate these requirements, novel affinity methods have been developed by identifying and designing new ligands and matrices. The matrices can be employed in the affinity chromatography are divided into three groups: natural (Agarose, dextrose and cellulose beads), synthetic (acrylamide, polystyrene and polymethacralate derivatives) and inorganic type (porous silica and glass) [1,32–34]. Also, the most bispecific ligands that are extensively used for affinity separation of IgG, include protein A and G, textile dyes, histidine, thiophilic ligands and chelated metals [34–38]. Protein A chromatography is the most widely used method for purification of immunoglobulins, which has some disadvantages like high cost, ligand leakage, low stability, prone to degradation and loss of antibody activity under severe elution conditions [39–45]. On the other hand, pseudo-specific affinity chromatography can be extensively used to purify biomolecules, which at the same time compensates for the defects of protein A chromatography. In pseudo-specific affinity chromatography, the interactions among the immobilized ligand with the target protein, depending on the complementarity of their shape,

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charge and hydrophobicity [46–49]. The benefits and particular properties of histidine and imidazole as pseudo-affinity ligands have made them unique. Imidazole can be found in the structures of important biomolecules, including histidine, which has an important role in the location of protein binding [50–52]. Furthermore, they possess some specific properties such as good stability, mild hydrophobicity, a wide range of pK_a values, the asymmetric carbon atom and weak charge transfer due to the presence of imidazole moiety [46,53–57].

Herein we report the design and preparation of new adsorbents based on sepharose for purification of immunoglobulin G from bovine milk in one step and with high purity and efficiency. The sepharose beads activated by 1,2-ethanediol diglycidyl ether, 1,4-butanediol diglycidyl ether, and epichlorohydrin in different binding capacities and L-histidine and imidazole ligands were covalently attached to them. The performance of modified beads and effects of the binding capacity, ligand type and linker length on the purification of immunoglobulin G from bovine milk were evaluated.

2. Experimental

2.1. Materials

Sepharose CL-6B was obtained from Arg. Biotech. Co. (Tabriz, Iran). L-histidine (98%, Sigma-Aldrich), imidazole (99%, Sigma-Aldrich), 1,2-ethanediol (99%, Sigma-Aldrich), 1,4-butanediol (99%, Sigma-Aldrich), sodium borohydride (98%, Sigma-Aldrich), sodium sulfate (99%, Sigma-Aldrich), tetrabutylammonium chloride (97%, Sigma-Aldrich), acetic acid (99%, Sigma-Aldrich), sodium acetate (99%, Sigma-Aldrich) and sodium carbonate (99%, Dae-Jung) were used as received without further purifications. Sodium hydroxide (97%) and epichlorohydrin (99%) were obtained either from Merck (Darmstadt, Germany) and used as received. All the commercial solvents such as CH_2Cl_2 were distilled before use.

2.2. Instrumentation

The ^1H NMR spectra of synthesized compounds were recorded using a Bruker FT-400 MHz (Bruker Co., United States) spectrometer in deuterated chloroform ($CDCl_3$) as solvent at room temperature. The FT-IR spectra of synthesized compounds were recorded in/on pressed KBr plate on a Bruker-Tensor 270 (Bruker Co., United States) spectrometer. The morphology, microstructures and particle size distribution of the samples were analyzed by scanning electron microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDX) using an MIRA3 FE-SEM (TESCAN, Czech Republic) instrument. Fast protein liquid chromatography (GE healthcare Amersham AKTA FPLC UPC-900 P-920 INV-907 M-925 system) was used for purification of target protein from protein mixture. The electrophoresis analysis was carried out on an SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) page by a Mini-PROTEAN® 3 (Bio-Rad Co., United States) cell.

2.3. General method for synthesis of diglycidyl ether intermediates

Diglycidyl ether intermediates were prepared under phase-transfer catalytic condition: To the vigorously stirred mixture of epichlorohydrin (9.34 mL, 120 mmol), sodium hydroxide pellets (4.8 g, 120 mmol), water (0.5 mL, 28 mmol), and tetrabutylammonium chloride (0.28 g, 1 mmol), cooled Glycol (20 mmol, 1,2-ethanediol or 1,4-butanediol) was added dropwise. After the completion of the addition, the mixture was stirred for 45 min at 40 °C. The solid products that were formed alongside the reaction removed by filtration and washed with CH_2Cl_2 (30 mL × 3). The combined organic layers were dried over anhydrous Na_2SO_4 and additional dichloromethane and excess epichlorohydrin evaporated off by distillation to give a yellow oil. The resulting oil was purified by distillation under reduced pressure to give a pure and colorless product (89% yields).

2.3.1. 1,2-Ethanediol diglycidyl ether (EDDGE)

^1H NMR (400 MHz, $CDCl_3$): δ 3.59–3.71 (m, 4H, OCH_2CH_2), 3.77–3.80 (dd, 2H, OCH_2), 3.38–3.42 (dd, 2H, OCH_2), 3.13–3.16 (m, 2H, $CH_2(O)CH$), 2.58–2.60 (dd, 2H, $CH(O)CH_2$), 2.76–2.78 (dd, 2H, $CH(O)CH_2$).

2.3.2. 1,4-Butanediol diglycidyl ether (BDDGE)

^1H NMR (400 MHz, $CDCl_3$): δ 1.62–1.63 (m, 4H, $-CH_2-$), 3.45–3.50 (m, 4H, OCH_2CH_2), 2.55–2.56 (dd, 2H, $CH(O)CH_2$), 2.73–2.75 (dd, 2H, $CH(O)CH_2$), 3.09–3.11 (m, 2H, $CH_2(O)CH$), 3.30–3.35 (dd, 2H, OCH_2), 3.65–3.68 (dd, 2H, OCH_2).

2.4. Epoxy activation of sepharose

The sepharose CL-6B was activated with epichlorohydrin, 1,2-ethanediol diglycidyl ether, and 1,4-butanediol diglycidyl ether by procedures as previously described elsewhere with some modifications [58,59].

2.4.1. Activation of sepharose with epichlorohydrin (Sep-Epch)

In a round-bottom flask equipped with a magnetic stirrer, 1 g of washed and suction dried sepharose CL-6B was added into the aqueous solutions of 2 mL NaOH (0.5 M, 1 M, 2 M) containing 5 mg sodium borohydride. Epichlorohydrin (0.075, 0.15, 0.25 mL) was added to the mixture and shaken at 45 °C for 6 h. The activated gels were washed with an excess of distilled water and dried under vacuum conditions. Finally, epoxy activated sepharose gels with various amounts of epoxide groups (containing around 80, 120, 160 μmol of epoxide groups/mL gel) were obtained.

2.4.2. Activation of sepharose with EDDGE and BDDGE (Sep-EDDGE and Sep-BDDGE)

In a round-bottom flask equipped with a magnetic stirrer, 1 g of washed and suction dried sepharose CL-6B was added into the aqueous solution of 1 mL NaOH 1 M containing 2 mg sodium borohydride. Then 1 mL EDDGE or BDDGE was added to the reaction mixture and incubated overnight on a shaking at room temperature. The activated gels were washed with an excess of distilled water and vacuum dried.

2.5. Determination of epoxy groups

The determination of epoxy groups on the activated sepharose gels was carried out according to the method described by Sundberg and Porath [60]. 150 mg of modified gel, was added into the 15 mL of 1.3 M sodium thiosulfate solution and titrated with 0.1 M hydrochloric acid.

2.6. Immobilization of L-histidine and imidazole on epoxy activated sepharose (Sep-Epch-His and Sep-Epch-Im, Sep-EDDGE-Im and Sep-BDDGE-Im)

1 g of suction-dried epoxy activated sepharose with different epoxy content (80, 120 and 160 $\mu mol_{epoxide}/mL_{gel}$), EDDGE or BDDGE activated Sepharose, was transferred to 25 mL shaking flask containing 200–500 mg L-histidine or imidazole in 2 mL sodium carbonate (Na_2CO_3) 2 M and 5 mg sodium borohydride. The reaction mixture was stirred at 50 °C for 18 h. In the end, the modified sepharose gel was washed with plenty of distilled water and vacuum dried.

2.7. Chromatographic separation of IgG from skim milk

For preparation of the skim milk, the fresh bovine milk was centrifuged at 6000 rpm for 15 min at room temperature to remove the fat in the form of the upper cream layer. The pH of milk was adjusted in 4.6 with HCl 1 N and centrifuged again at 6000 rpm for 15 min to precipitate the casein. Afterward, the clear skim milk was collected and the pH raised to 7 by 1 N NaOH to protect immunoglobulins from

denaturation. The targeting antibody was purified from the pre-prepared skim milk by using an automated chromatography system (GE healthcare Amersham AKTA FPLC) and the column with the dimension of (6 cm × 1 cm i.d.) to give a bed volume of approximately 2 mL. The outlet of the column was connected to a UV monitor (UV monitor UPC-900 P-920) and to a fraction collector (Frac-950 fraction collector). The whole process of chromatography was performed at the flow rate of 1 mL/min at room temperature. The resulted modified sepharose gels were packed into the column and it was equilibrated with 25 mM acetate buffer, pH = 5 (equilibration buffer). 50 mL of skim milk containing 30 mg IgG was injected into the column and washed with equilibration buffer until any protein wasn't detected by absorption at 280 nm. Elution was carried out with the same buffer + 0.2 M NaCl and absorption measured at 280 nm. For regeneration of the column, the 50 mM NaOH solution, water, and equilibration buffer were used. Determination of Protein concentration was performed by Bradford's method [61].

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The obtained elution fractions from chromatographic runs were analyzed by SDS-PAGE to determine the purity of aim antibody using a Mini-PROTEAN® 3 system (Bio-Rad, USA) as described by Laemmli [62]. Acrylamide was used at 10.0% in the running gel and 4% in the stacking gel. Samples with pre-prepared sample buffer (5 μL of sample buffer + 15 μL of the sample) were placed in a boiling water bath at 95 °C for 5 min to denature the proteins. The samples were loaded onto a gel and run at a constant voltage of 30 V. Finally, the protein bands were stained with the Coomassie Brilliant Blue R-250 for 30 min and destained for 3–4 h [63].

3. Results and discussions

The pseudo-specific affinity adsorbents with a different type of linkers and ligands were used for the isolation and purification of immunoglobulin G from bovine milk. to prepare these pseudo-specific affinity adsorbents, the surface of the sepharose beads were activated in two different binding capacities (80 and 120 $\mu mol_{epoxide}/mL_{gel}$) using epichlorohydrin by means of nucleophilic substitution reaction. The L-histidine ligand was attached to the surface of activated sepharose beads via its amine group by the ring-opening base reaction. In the following, the surface of the sepharose beads was coated by epichlorohydrin using nucleophilic substitution reactions with three different binding capacities (80, 120 and 160 $\mu mol_{epoxide}/mL_{gel}$) and then imidazole ligand was immobilized onto the surface of activated sepharose beads (Fig. 1).

The two diglycidyl ether linkers, 1,2-ethanediol diglycidyl ether (EDDGE) and 1,4-butanediol diglycidyl ether (BDDGE), with different length were also synthesized by the reaction of 1,2-ethanediol and 1,4-butanediol with epichlorohydrin in the presence of sodium hydroxide and tetrabutylammonium chloride as a phase-transfer catalyst at room temperature (Fig. 2).

The sepharose beads were activated with synthesized diglycidyl ethers (EDDGE and BDDGE) to obtain Sep-EDDGE and Sep-BDDGE gels respectively. The imidazole was then immobilized on the activated sepharose beads surface (Sep-EDDGE and Sep-BDDGE) by the ring-opening base reaction to prepare Sep-EDDGE-Im and Sep-BDDGE-Im respectively (Fig. 3). The synthesized adsorbents are summarized in Table 1.

3.1. FT-IR study of adsorbents

The FT-IR spectrum of adsorbents Sep-Epch-His with binding capacities of 80 and 120 $\mu mol_{epoxide}/mL_{gel}$ is depicted in Fig. 4. As can be seen in Fig. 4A, the characteristic peak of O–H and N–H stretching vibrations are appeared at 3436 cm^{-1} as a strong and broad peak. The vibration peak at 2918 cm^{-1} is assigned to the aliphatic C–H. The characteristic peaks of C=C bond can be observed at 1465 cm^{-1}. The absorption peaks that are appeared at 1039 cm^{-1} and 1186 cm^{-1} associated to the C–N and C–O vibrations.

In the FT-IR spectrum of Sep-Epch-His (120 $\mu mol_{epoxide}/mL_{gel}$) (Fig. 4B), the vibration peaks of the O–H and N–H are appeared at 3434 cm^{-1}. The C–H aliphatic vibration gives a peak at 2921 cm^{-1}. The vibration peak of the carbonyl group of acid (COOH) due to the low concentration of the ligand compared to the sepharose appears as a very small peak at 1710 cm^{-1}. The vibration peak assigned to the C=C bond can be observed at 1466 and around 1519 cm^{-1}. The peaks are appeared at 1051 and 1178 cm^{-1} represent the C–O and C–N bonds.

The FT-IR spectra of Sep-Epch-Im (80, 120, 160 $\mu mol_{epoxide}/mL_{gel}$) are represented in Fig. 5. Fig. 5C is depicted the FT-IR spectrum of Sep-Epch-Im (80 $\mu mol_{epoxide}/mL_{gel}$). The peaks assigned to the stretching vibration of O–H and C–H aliphatic bonds are observed at 3432 cm^{-1} and 2901 cm^{-1} respectively.

The peak centered around 1500 cm^{-1} is represented the C=C bond and the peaks appeared at 1070 cm^{-1} and 1175 cm^{-1} are associated with vibrations of C–O and C–N bonds. The FT-IR spectrum is represented in Fig. 5D is belonged to the Sep-Epch-Im (120 $\mu mol_{epoxide}/mL_{gel}$). The observed peak at 3437 cm^{-1} and 2918 cm^{-1} are related to the O–H and C–H aliphatic bonds respectively. The characteristic peaks

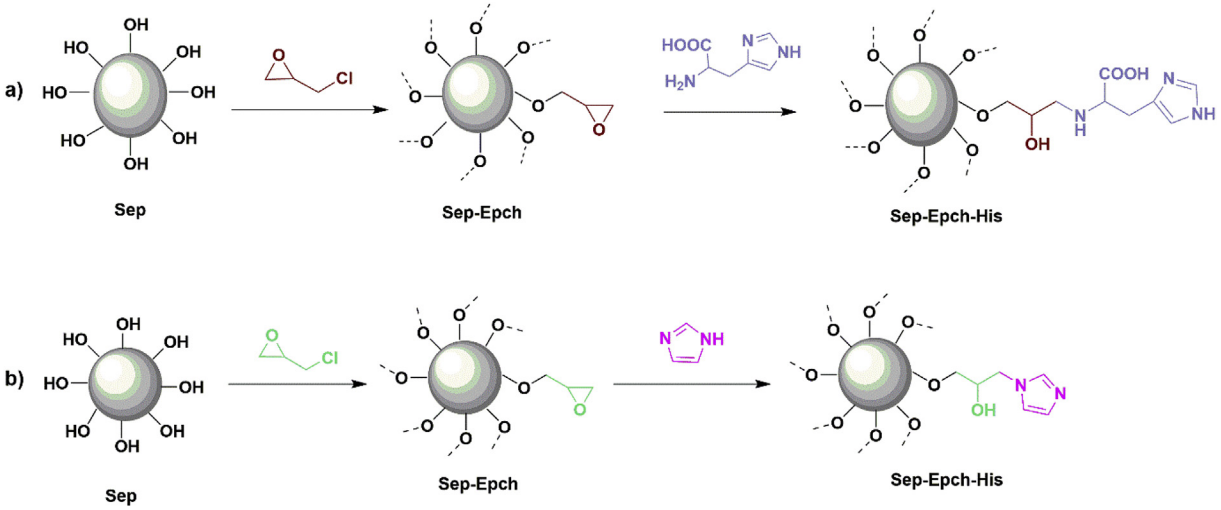

Fig. 1. Synthesis of (a) Sep-Epch-His and (b) Sep-Epch-Im.

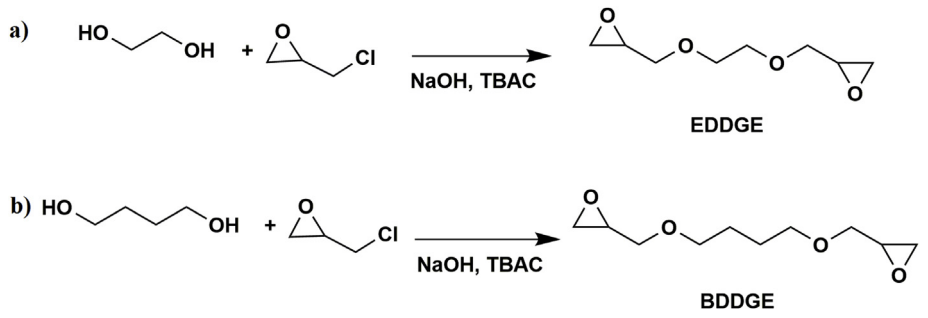

Fig. 2. Synthesis of (a) EDDGE and (b) BDDGE

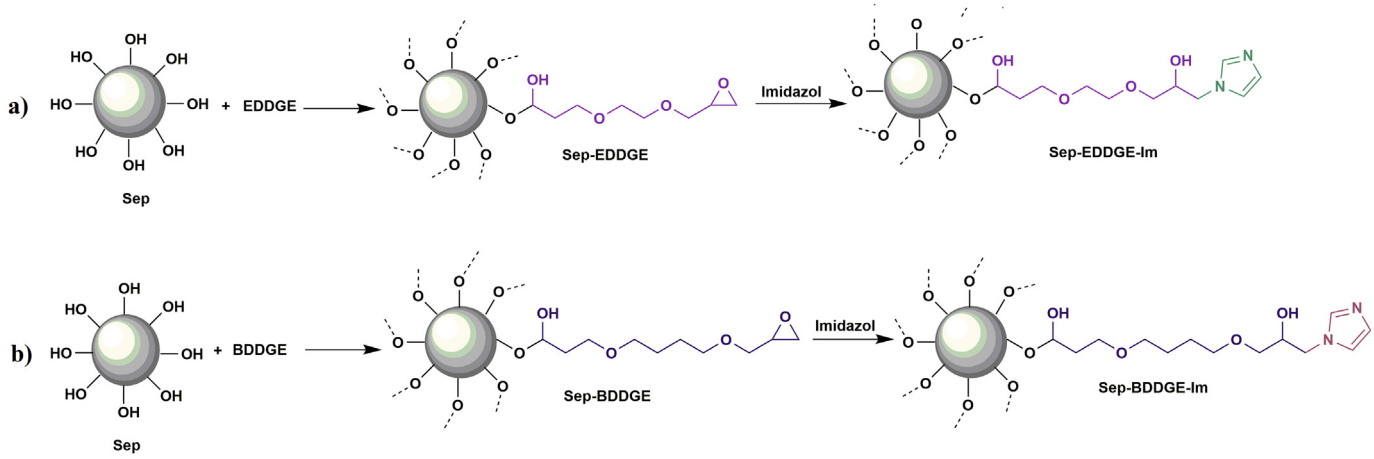

Fig. 3. Synthesis of (a) Sep-EDDGE-Im and (b) Sep-BDDGE-Im.

Table 1
Synthesized adsorbents with different linkers, ligands, and binding capacities.

Entry	Adsorbent		Binding capacity ($\mu mol_{epoxide}/mL_{gel}$)
1	Sep-Epch-His	**A**	80
2	Sep-Epch-His	**B**	120
3	Sep- Epch-Im	**C**	80
4	Sep- Epch-Im	**D**	120
5	Sep- Epch-Im	**E**	160
6	Sep-EDDGE-Im	**F**	–
7	Sep-BDDGE-Im	**G**	–

of C=C bond are appeared at 1463 and 1516 cm^{-1} and the vibrations of C–O and C–N bonds appeared at 1048 and 1183 cm^{-1}. The broad and sharp peak in comparison with spectrum 5C indicates a large amount of epoxy and loading of ligand groups in the Sep-Epch-Im (120 $\mu mol_{epoxide}/mL_{gel}$).

The FT-IR spectrum of 5E belongs to Sep-Epch-Im (160 $\mu mol_{epoxide}/mL_{gel}$). The O–H vibration appears at 3438 cm^{-1}, which in comparison to the spectra 5C and 5D has a wide and strong peak. The absorption peak at 2923 cm^{-1} represents C–H aliphatic and two peaks at 1464 and 1522 cm^{-1} represent C=C bond. The vibrations of C–O and C–N are observed at 1044 and 1187 cm^{-1}. Due to the high content of the epoxy groups and ligand, the peaks were appeared stronger and sharper than others (5C and 5D).

Fig. 6 represents the FT-IR spectra Sep-EDDGE-Im (6F) and Sep-BDDGE-Im (6G). The stretching vibrations of O–H and C–H aliphatic peak are observed at 3562 cm^{-1} and 2926 cm^{-1} respectively. The peak is related to the C=C bond observed at 1518 cm^{-1} and the peaks at 1030 and 1180 cm^{-1} represent the C–O and C–N bonds.

In Fig. 6G the stretching vibration of O–H and C–H aliphatic bonds appear at 3436 cm^{-1} and 2926 cm^{-1} respectively. The absorption peaks are appeared a 1463 cm^{-1} and 1516 cm^{-1} associated to the vibrations of C=C and the appeared peaks at 1053 and 1170 cm^{-1} associated to the vibrations of C–O and C–N bonds respectively.

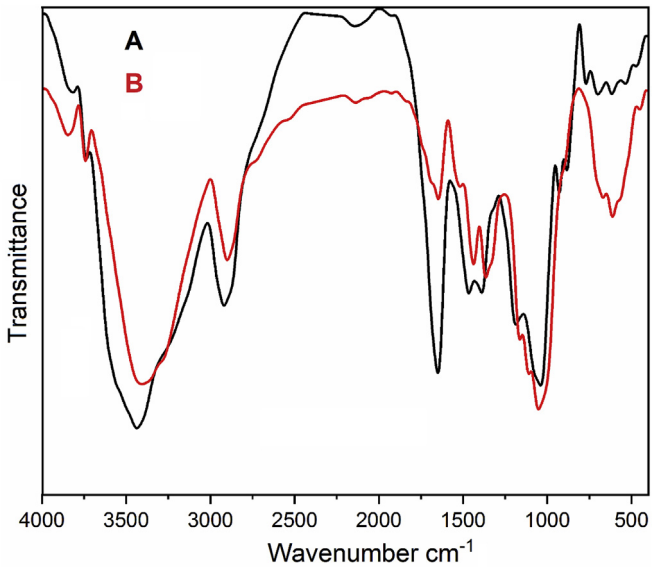

Fig. 4. FT-IR spectra of A) Sep-Epch-His (80 $\mu mol_{epoxide}/mL_{gel}$) and B) Sep-Epch-His (120 $\mu mol_{epoxide}/mL_{gel}$).

3.2. Energy dispersive X-ray spectroscopy (EDX)

For further investigation of the chemical composition of prepared pseudo-affinity adsorbents Energy-Dispersive X-ray (EDX) spectroscopy was carried out and shown in Fig. 7(a–h). The EDX analysis of sepharose shows only oxygen (O) and carbon (C) related to the chemical

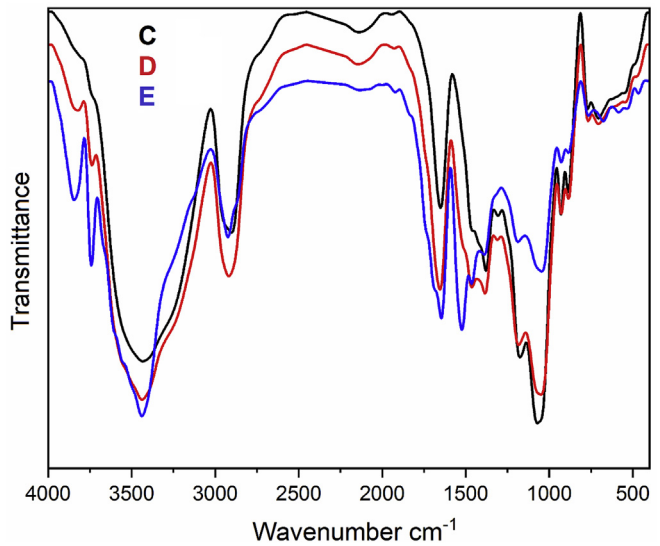

Fig. 5. FT-IR spectra of Sep-Epch-Im C) 80, D) 120 and E)160 $\mu mol_{epoxide}/mL_{gel}$.

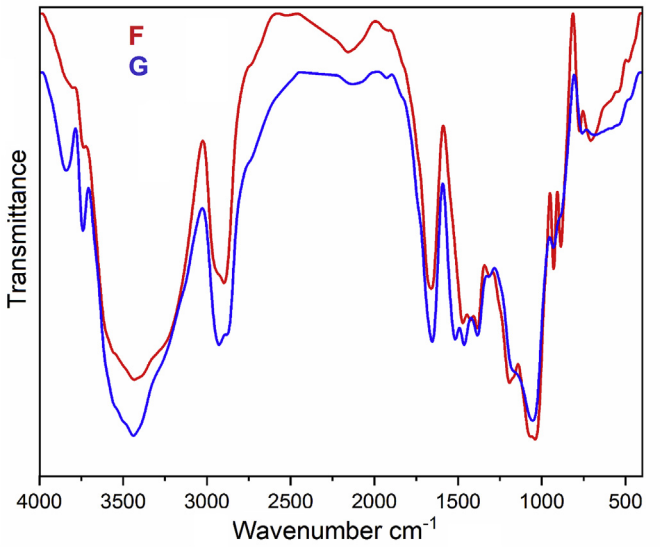

Fig. 6. FT-IR spectra of F) Sep-EDDGE-Im, G) Sep-BDDGE-Im.

composition of sepharose. Existence of the nitrogen (N), in Fig. 7(b–h) confirmed the immobilization of histidine and imidazole by means of ring-opening reaction on the sepharose surface.

3.3. Microscopic images

Microscopic images of the prepared adsorbents were performed by using an optical microscope and results shown in Fig. 8. As could be seen in Fig. 8(a–e), the spherical structures of particles are in micro size. The structure of the adsorbent particles after activating their surface with the linkers and ligands has remained spherical and no accumulation and aggregation detected in the structure of particles.

3.4. Scanning electron microscopy (SEM)

The morphologies of the prepared adsorbents and sepharose CL-6B were evaluated by scanning electron microscopy (SEM) with different magnification and depicted in Fig. 9. As could be seen in Fig. 9a, the sepharose has a spherical shape with a dimension of 44–177 μm. According to SEM images, the particle size and distribution in each adsorbent has approximately dimension with an average size of 44–177 μm, which in comparison with the size of the initial sepharose CL-6B particles there is no significant change in the particle size and distribution and there is no accumulation of particles after the surface modification.

3.5. IgG adsorption studies

The chromatographic results (Fig. 10-a) showed that both Sep-Epch-His adsorbents with a binding capacity of 80 (**A**) and 120 (**B**) $\mu mol_{epoxide}/mL_{gel}$ have the capability to attach to IgG and isolate it from bovine milk. Adsorbent **A** demonstrated more IgG adsorption efficiency in comparison with adsorbent **B**. It is due to the high amount of epoxy groups and the high concentrations of L-histidine ligand on the surface of the adsorbent **B**. It seems that there is a repulsive force between the free COOH groups and proteins, that leads to a reduction in the antibody recovery. The results of the chromatography and SDS-PAGE of adsorbents **A** and **B** are shown in Figure (10-I and 11). According to the obtained data, adsorbent **A** was able to purify target antibody with a yield of 46.66% and purity of > 80% from bovine milk. The results of the purification process are represented in Table 2.

The results obtained from Figure (10-II, 11) showed that all three adsorbents bearing imidazole (**C, D,** and **E)** have the capability to adsorb IgG from bovine milk with different efficiency and adsorption capacity. As can be seen, IgG with a molecular weight of 25 KDa (light chain) and 50 KDa (heavy chain) is observed as single bands in SDS-PAGE analysis with negligible impurities (Fig. 11). The adsorbent **E** demonstrated the best performance in adsorbing IgG due to the highest content of epoxy groups (160 $\mu mol_{epoxide}/mL_{gel}$) and the highest concentration of imidazole ligands (Fig. 10). According to obtained data adsorbent **E** purified IgG from the bovine milk with a yield of 88.33% and purity of > 85%. The results are summarized in Table 2.

To evaluate the linker length in the purification process, the sepharose activated by the synthesized linkers (EDDGE and BDDGE). The activated sepharose was modified with imidazole by the means of ring-opening base reaction to form adsorbent Sep-EDDGE-Im and Sep-BDDGE-Im. The obtained results from chromatography (Fig. 10-III) revealed that both adsorbents Sep-EDDGE-Im and Sep-BDDGE-Im had the ability to separate IgG from bovine milk. The interaction of both adsorbents was specific to IgG. It can be seen in the SDS-PAGE analysis that IgG was detected as a single bond without any further impurities (Fig. 11).

The adsorbent **F** shows better performance in adsorption of IgG than adsorbents G and it was able to purify IgG from the bovine milk with the yield of 41.66% and the purity of > 90% (Table 2).

Histidine is one of the amino acids that could be hold a positive charged in the side chain. Histidine has an isoelectric point (pI) of 7.59 and if the pH falls to 5, it can take a positive charge. The bovine immunoglobulin with pI value between 5.5 and 8.3 can became positively charged at a pH of 5. It seems that positive charge of ligand and biomolecule, resulted a repulsive force leads to the separation of IgG from the ligand [56].

3.6. Comparison with the related literature

There are a lot of reported articles about the different adsorbents with the various ligands and binding capacities for affinity purification of IgG in the literatures [47,64]. Premysl et al. [65] reported the purification of IgG using thiophilic ligands attached to crosslinked agarose bead with the yield of 0.29–0.32 mg IgG/mL and the purity of 74% and Protein G-Sepharose 4FF with binding capacity of 0.38–0.42 mg IgG/mL and purity of 81% from bovine milk. Denizli and Pishkin reported adsorption capacity of 24.0 mg HIgG/g with protein A-immobilized-poly(2-hydroxyethylmethacrylate) [66]. Muller-Shulte et al. [67] used several polymeric adsorbents made of different polymers and histidine as the ligand and obtained adsorption values of 0.05–0.23 mg IgG_1/mL

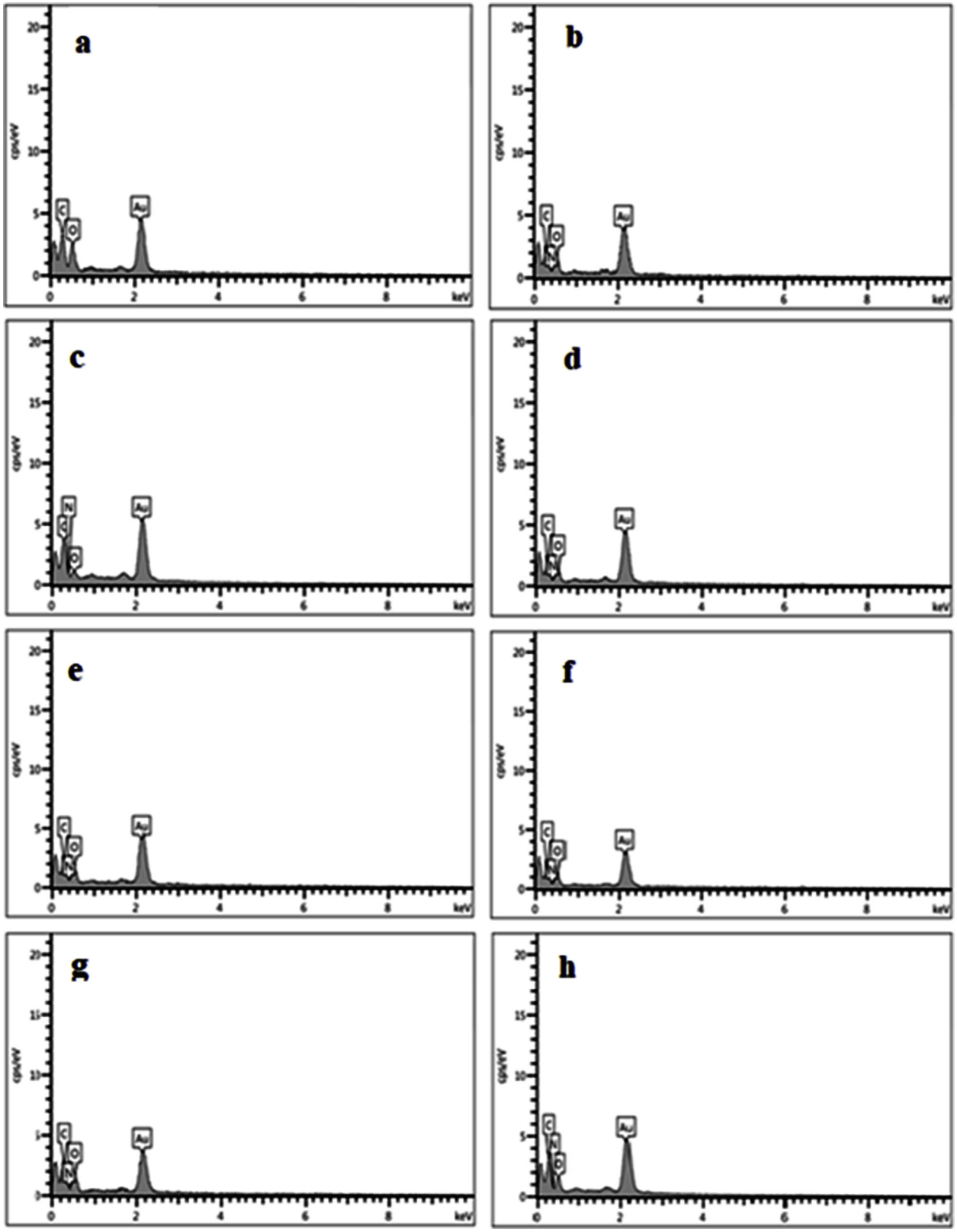

Fig. 7. EDX spectra of a) sepharose CL-6B b) Sep-Epch-His (80 $\mu mol_{epoxide}/mL_{gel}$) c) Sep-Epi-His (120 $\mu mol_{epoxide}/mL_{gel}$) d) Sep-Epch-Im (80 $\mu mol_{epoxide}/mL_{gel}$) e) Sep-Epch-Im (120 $\mu mol_{epoxide}/mL_{gel}$) f) Sep-Epch-Im (160 $\mu mol_{epoxide}/mL_{gel}$) g) Sep-EDDGE-Im h) Sep-BDDGE-Im.

of sorbent. The different commercial protein A affinity chromatography matrices including Affi-Gel, Eupergit, Ultrogel, Sepharose series, and Prosep A with adsorption capacities of 0.7–20.1 mg IgG_3/g were reported by Fuglistaller [45]. D. Muller-Schulte reported the purification of IgG with sepharose adsorbent and histidine ligand about 0.2 mg/mL [68]. Cu (II) metal ion affinity adsorbents were applied for purification of IgG with the yield of 97.4% with an 8-fold purification by Sulakshana Jain and Munishwar N. Gupta [37]. Stefano Menegatti et al. [35] were

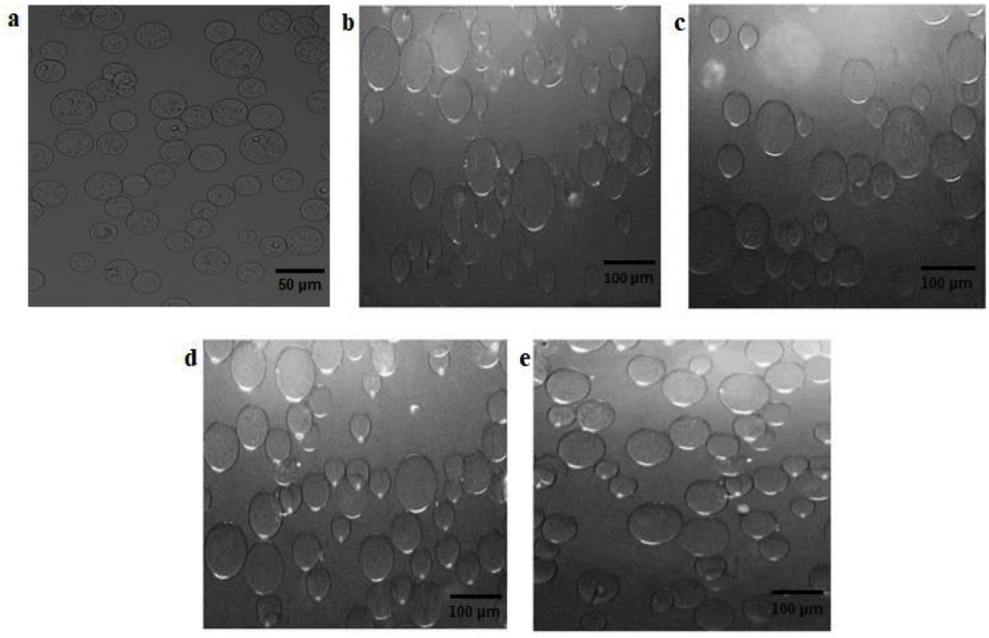

Fig. 8. Microscopic images of adsorbents: a) sepharose Cl–6B, b) Sep-Epch-His, c) Sep-Epch-Im, d) Sep-EDDGE-Im, e) Sep-BDDGE-Im.

recovered IgG from skim milk with 74% yield and 92% purity using a hexamer peptide ligand.

4. Conclusion

In summary, we designed and synthesized novel adsorbents based on sepharose with various content of epoxy groups and binding capacities, ligand type and linker length that showed good potential to purify immunoglobulin G from bovine milk. The sepharose surface has been activated by epichlorohydrin, 1,2-ethanediol diglycidyl ether and 1,4-butanediol diglycidyl ether. L-histidine and imidazole as pseudo-affinity ligands were then covalently immobilized on activated sepharose through ring-opening reaction. The adsorption behaviors of IgG on adsorbents bearing L-histidine demonstrated that the adsorbent with lower content of epoxy groups and ligand showed better performance with adsorption of 0.28 mg/mL IgG from bovine milk. A significant increase in IgG adsorption capacity was achieved by imidazole bearing adsorbent with the highest epoxy groups and ligand content up to 0.53 mg/mL. Besides, adsorbents with imidazole ligand separate immunoglobulin G with higher purity than histidine. The result represents that among the adsorbents with different linkers such as EDDGE and BDDGE, EDDGE showed better performance in adsorption of IgG. It purified the IgG about 0.25 mg/mL without any impurities. The results obtained from chromatography and SDS-PAGE analysis

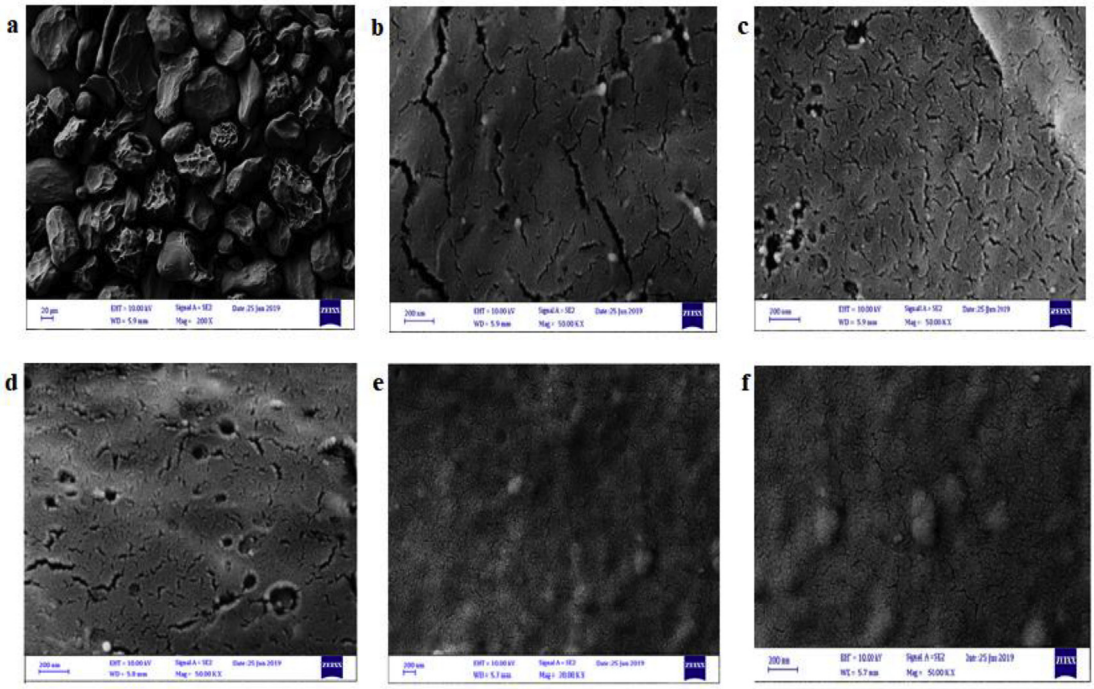

Fig. 9. SEM image of adsorbents: a, b) sepharose CL-6B c) Sep-Epch-His d) Sep-Epch-Im e) Sep-EDDGE-Im f) Sep-BDDGE-Im.

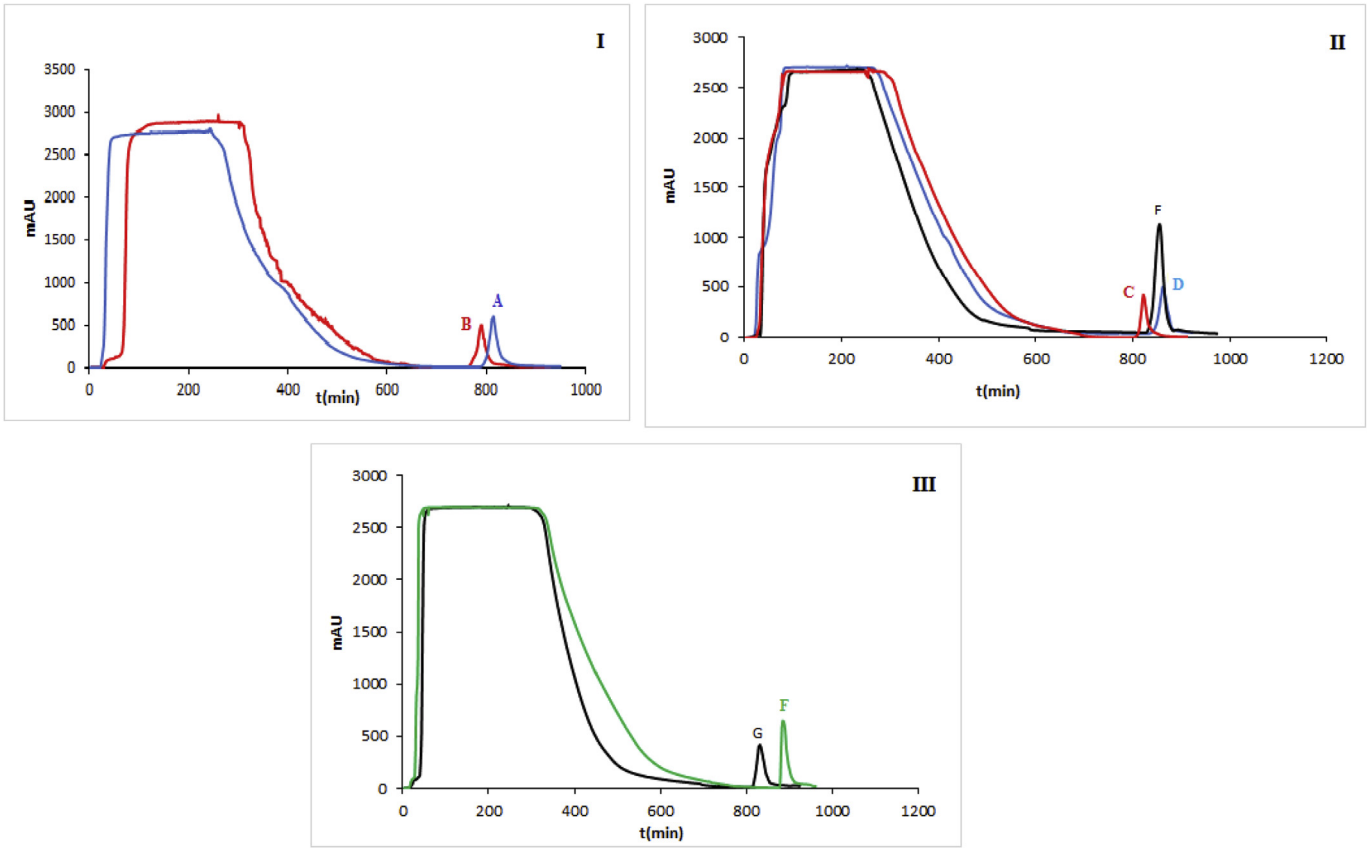

Fig. 10. Effect of the different ligands, linkers and binding capacities on the adsorption and elution of bovine IgG by affinity chromatography on adsorbents (A–G). conditions: bed volume, 2 mL; injection volume, 50 mL of skim milk containing 30 mg IgG; flow rate, 1 mL/min washing was carried out using 25 mM acetate buffer, pH = 5. Elution was carried out using 25 mM acetate buffer +0.2 M NaCl, pH = 5. Column regeneration was done with the 50 mM NaOH solution, water, and equilibration buffer.

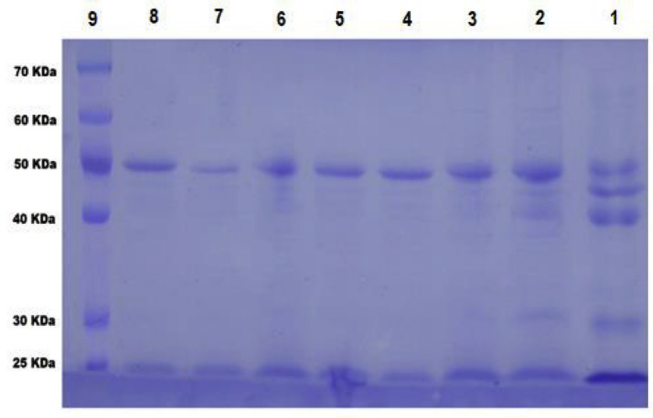

Fig. 11. The SDS-PAGE analysis of eluted fractions of skim milk on adsorbents A-G. Lane 1) skim milk, lane 2) fraction eluted from adsorbent A, lane 3) fraction eluted from adsorbent B, lane 4) fraction eluted from adsorbent C, lane 5) fraction eluted from adsorbent D, lane 6) fraction eluted from adsorbent E, lane 7) fraction eluted from adsorbent F, lane 8) fraction eluted from adsorbent G, lane 9) standard protein mark.

confirmed that the novel synthesized adsorbents are able to purify the IgG antibody in one step with high purity, and in the least time.

CRediT authorship contribution statement

Pariya Pourrostam-Ravadanaq: Visualization, Investigation, Writing - original draft, Software. **Kazem D. Safa:** Supervision. **Hassan Abbasi:** Writing - review & editing, Software.

Table 2
Results of IgG adsorption from bovine milk by adsorbents A-G.

Entry	adsorbent	Adsorbed IgG (mg/mL)	Yield (%)
1	**A**	0.28	46.66
2	**B**	0.15	25.00
3	**C**	0.17	28.33
4	**D**	0.19	31.66
5	**E**	0.53	88.33
6	**F**	0.25	41.66
7	**G**	0.16	26.66

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Appendix A. Supplementary data

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