

Size exclusion chromatography as a characterization tool for quality control of marine biopharmaceutical keyhole limpet hemocyanin

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ABSTRACT

Keyhole limpet hemocyanin is a large metalloprotein present in hemolymph of the mollusk *Megathura crenulata*. This protein is highly immunogenic, making it very attractive to the biopharmaceutical industry, where it is used as a carrier protein for haptens, an adjuvant, or as a stand-alone biologic for the treatment of various cancer diseases. The structural complexity of such immunogenic protein makes its characterization and quality control very challenging. Here, we developed, optimized and validated a size exclusion chromatography approach for quality control of keyhole limpet hemocyanin products. The separation procedure was performed on an Acquity UPLC BEH450 SEC column with pore size of 450 Å in a mobile phase composed of 25 mmol L⁻¹ phosphate buffer (pH 6.8) with 250 mmol L⁻¹ NaCl. These conditions led to appropriate elution of the analysed protein. The developed method showed very promising values of validation parameters, such as coefficient of determination ($R^2 = 0.9997$), sensitivity (limit of detection 10.21 µg mL⁻¹, limit of quantitation 31.25 µg mL⁻¹), precision (repeatability 0.3–2.5 %, intermediate precision 1.1–4.2 %), and accuracy (100.4–102.8 %). The developed method was used to determine the content of keyhole limpet hemocyanin in three commercially available samples obtained from various suppliers. The determined content ranged from 20.08 to 22.91 mg per vial.

Keywords: keyhole limpet hemocyanin, size exclusion chromatography, validation, carrier protein, quality control

INTRODUCTION

Biologics are characterized as a wide range of products – vaccines, blood and blood components, cells, gene therapies, tissues and recombinant therapeutic proteins. These products can be composed of sugars, proteins, nucleic acids or complex combinations of these moieties, or even living entities such as cells and tissues (1). Biologics represent very attractive molecules from the therapeutic and also economic perspectives. The global biologics market is expected to reach a value of nearly 800 billion USD by 2029 (2). In 2025, there were 12 new protein-based drugs approved by US FDA under the biologics license

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applications (3). These facts are also a reason for the revolutionization and fast growth of such kind of therapeutics in a broad spectrum of diseases (4). Novel trends in biologics are associated with the development of antibody drug conjugates (ADCs), therapeutic protein conjugates and vaccines (5–7) which could be effective in situations where the conventional drugs and therapeutic strategies are ineffective. In case of innovative immunotherapeutics, carrier proteins and protein-based adjuvants are becoming of great interest (8). Special attention is paid to the hemocyanins, large metalloglycoproteins present in hemolymph of mollusks (9). One of the crucial hemocyanins used in the development of immunotherapeutics against cancer (10), Alzheimer's disease and dementia (11–13) or opioids misuse (14, 15) is the keyhole limpet hemocyanin (KLH), which is obtained from the hemolymph of the giant keyhole limpet *Megathura crenulata*. KLH possesses a stable supramolecular structure with molecular mass in the MDa range (16). However, KLH dedicated for clinical purposes is typically used as either native high molecular mass substance or purified subunit, *i.e.*, KLH1 ($M_r = 390$ kDa) or KLH2 ($M_r = 350$ kDa) (17). Its characterization and quality control are very challenging, therefore, there is a need to dispose with appropriate and accurate analytical methods (18).

Size exclusion chromatography (SEC) in combination with UV or photodiode array (PDA) detection represents the major method used for the characterization of proteins and their aggregates owing to its simplicity, low cost, minimal sample pretreatment requirements, ease of use, high speed and high sample throughput capability (19). SEC is robust and easy to validate and commonly used for lot release testing as well as in-process monitoring of soluble aggregates (20). This technique offers the possibility to identify and monitor the presence of fragments and aggregates which may occur during the production, formulation and/or storage of the biologics. The product-related impurities (*i.e.*, fragments and aggregates) may be associated with altered pharmacokinetics and pharmacodynamics properties of the original biologics (21–25). According to the properties, simplicity and information offered, SEC represents the unambiguous choice for protein biopharmaceuticals characterization in routine quality control laboratories (26). In SEC, the mechanism of the separation of high molecular mass species (HMWS) and/or low molecular mass species (LMWS) is achieved based on the difference in hydrodynamic volumes and the filtration of the different species through a column packed with particles having well-defined pore size (27). The main drawback of SEC is that it does not enable accurate determination of the molecular mass of an analyte. However, that issue can be solved by implementation of a multi-angle light scattering (MALS) detector into the instrumentation setup (26). Recently, the improvements in SEC of therapeutic proteins are oriented towards new stationary phases which can be used in the ultra-high-performance liquid chromatography setup, and clarification of the separation mechanisms and interaction of proteins with such SEC stationary phases (28–37).

Herein, we introduce a simple UHPLC SEC approach with UV detection to characterize a high-molecular mass protein carrier/biopharmaceutical, KLH. A stepwise optimization procedure of the method was realized including stationary phase selection, mobile phase selection, and investigation of the increasing salt concentration on the separation of highly molecular substances. The optimized SEC-UV approach was validated and finally, it was applied to determine the content of KLH in three commercially available products.

EXPERIMENTAL

Reagents and samples

Waters BEH450 SEC protein standard mix (Waters, USA), a mixture composed of five substances (thyroglobulin – $M_r = 667$ kDa, immunoglobulin G (IgG) – $M_r = 150$ kDa, bovine serum albumin (BSA) – $M_r = 66.4$ kDa, myoglobin – $M_r = 17$ kDa, uracil – $M_r = 112$ Da).

KLH preparation VACMUNE® in liquid form (GMP grade and Research and development grade) was purchased from biosyn Arzneimittel GmbH (Germany), hemocyanin from *Megathura crenulata* (giant keyhole limpet) as a lyophilized powder was obtained from Sigma-Aldrich (Germany), and product Thermo Scientific™ Inject™ mcKLH (lyophilized powder) from Thermo Fisher Scientific (USA).

All reagents, NaCl, NaH_2PO_4 , Na_2HPO_4 , citric acid, sodium citrate, 2-(*N*-morpholino) ethanesulfonic acid – MES, sodium acetate, ammonium formate, 2-bis(2-hydroxyethyl) amino-2-(hydroxymethyl)-1,3-propanediol – BIS-TRIS, NaOH, of *p.a.*, LC or LC-MS grade were obtained from Sigma-Aldrich, namely, Merck KGaA (Germany). A water purification system Milli-Q (Millipore, France) was used to obtain deionized (MPW) water with a resistivity of 18.2 MΩ cm at room temperature (25 °C).

Samples preparation

All samples, *i.e.*, Waters BEH450 SEC protein standard mix and KLH samples were reconstituted or diluted from the concentrated commercial solutions with the use of 25 mmol L⁻¹ phosphate buffer (pH 6.8) as described in the section *Size exclusion chromatography*.

The final concentration of individual components in Waters BEH450 SEC protein standard mix after the dilution procedure (500 μL of 100 mmol L⁻¹ sodium phosphate buffer at pH 6.8 was added to the 1.5-mL vial containing the lyophilized Waters BEH450 SEC protein standard mix) was as follows: thyroglobulin dimer ($M_r = 1340$ kDa), $c = 0.1$ mg mL⁻¹; thyroglobulin ($M_r = 667$ kDa); $c = 3$ mg mL⁻¹, IgG ($M_r = 150$ kDa), $c = 2$ mg mL⁻¹; BSA ($M_r = 66$ kDa), $c = 5$ mg mL⁻¹; 5 – myoglobin ($M_r = 17$ kDa), $c = 2$ mg mL⁻¹; uracil ($M_r = 112$ Da), $c = 0.1$ mg mL⁻¹. During the storage, handling, and reconstitution of the protein mix standard, thyroglobulin easily forms a dimer, therefore the presence of this new component in final sample is observed.

Commercially available samples containing KLH obtained from three suppliers were prepared by their appropriate dilution (with the 25 mmol L⁻¹ phosphate buffer, pH 6.8) to the concentration level of 2 mg mL⁻¹ (final volume of the sample was 100 μL) and further centrifugation of the liquid samples at 30000 *g* for 15 minutes. The supernatant of the centrifuged samples was transferred to the LC vials and directly injected into the UHPLC apparatus.

For the validation procedure, seven calibration solutions at the concentration levels ranging from 31.25 to 2000.0 μg mL⁻¹ (31.25, 62.5, 125.0, 250.0, 500.0, 1000.0 and 2000.0 μg mL⁻¹) were prepared in triplicates by appropriate dilution of the concentrated KLH solution (the GMP grade VACMUNE® liquid solution with declared concentration of 20 mg mL⁻¹ KLH). For the validation procedure (recovery test), three quality control (QC) samples at following concentration levels – 31.25 μg mL⁻¹ (QC1), 250 μg mL⁻¹ (QC2), and 2000 μg mL⁻¹ (QC3) were prepared by appropriate dilution of the concentrated KLH solution. Testing of stability

was performed by incubation of the commercially available KLH samples (VACMUNE® diluted to the final concentration of 2 mg mL⁻¹ with the 25 mmol L⁻¹ phosphate buffer, pH 6.8) in 2-mL Protein LoBind® tubes (Eppendorf, Germany) under the room temperature (25 °C) for one week. Before the analysis, all of the samples were centrifuged at 30000 g for 15 min, and the supernatant was then transferred into the LC vials.

Size exclusion chromatography

SEC experiments were performed using an Acquity H-class UPLC system (Waters) equipped with a quaternary solvent delivery pump, an autosampler and an UV detector set at 280 nm wavelength. Data acquisition and instrument control were performed by Empower Pro 3 software (Waters). The SEC separation was realized in the 25 mmol L⁻¹ phosphate buffer (pH = 6.8) containing 250 mmol L⁻¹ NaCl (SEC buffer), which was prepared automatically during the analysis by mixing appropriate amounts of four solvents located in individual lines of the UPLC system. The lines were filled with following solutions: line A – 100 mmol L⁻¹ dibasic sodium phosphate, line B – 100 mmol L⁻¹ monobasic sodium phosphate, line C – 1 mol L⁻¹ sodium chloride, and line D – MPW. The mobile phase was delivered at the flow rate of 0.4 mL min⁻¹. The injection volume was 5 µL.

Three different SEC columns were tested: Acquity UPLC BEH450 SEC (4.6 × 300 mm, 2.5 µm) with pore size 450 Å (Waters) coupled to Acquity UPLC BEH450 SEC guard column (4.6 × 30 mm, 2.5 µm, 450 Å), ULTRAHYDROGEL 2000 (7.8 × 300 mm, 12 µm) with pore size 2000 Å (Waters), and PSS PROTEEMA Micro (4.6 × 250 mm, 5 µm) with pore size 1000 Å (PSS Polymer Standards, Germany). The analyses were realized under the stable temperature of 25 °C.

PAGE electrophoresis

Protein samples were separated by electrophoresis on 5 % Tris-acetate polyacrylamide gels. Prior to sample loading, 4×SDS sample loading buffer was supplemented with dithiothreitol (DTT) and mixed with the protein sample. Samples were subsequently denatured, boiled for 5 min at 95 °C, and 2 µg per lane was loaded onto the gel for electrophoretic separation. For the protein staining, we used Coomassie dye (SimplyBlue™ SafeStain, Invitrogen, USA).

RESULTS AND DISCUSSION

Optimization of SEC-UV method was accompanied with testing of multiple factors, including stationary phase, mobile phase composition, column temperature and the presence of additives. The optimization procedure was realized in a simple stepwise manner.

Stationary phase optimization

SEC analysis of high molecular mass substances, such as KLH and/or its subunits demands the use of an appropriate separation column, which is able to characterize proteins at the demanded molecular mass range. As shown by the PAGE electrophoresis experiment, in case of KLH, the presence of molecular mass species at the levels higher

than 400 kDa are expected (Fig. 1). Therefore, columns with relatively large pore size are preferred. Here, three various SEC columns were tested, *i.e.*, Acquity UPLC BEH450 SEC (pore size 450 Å), PROTEEMA Micro (pore size 1000 Å) and ULTRAHYDROGEL 2000 (pore size 2000 Å). The optimization procedure was performed with the use of the SEC buffer (defined in the section EXPERIMENTAL, *Size exclusion chromatography*).

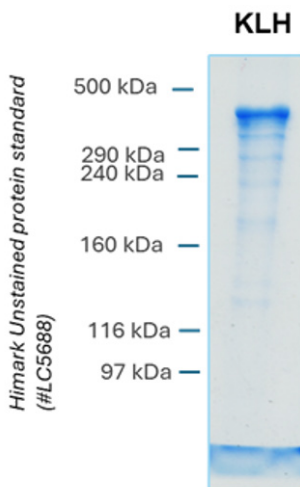


Fig. 1. Analysis of KLH sample (VACMUNE®) by PAGE.

The optimization procedure was realized with the use of three sample types, *i.e.*, a commercial BEH450 SEC protein standard mix containing five substances with various molecular masses at defined concentration levels, a KLH sample at the concentration level of 2 mg mL⁻¹, and a KLH sample at the same concentration level incubated for one week at 25 °C. The results obtained from the analysis of the defined protein standard mix showed, that only the Acquity UPLC BEH450 SEC column with pore size of 450 Å was able to separate and partially resolve all of the components at an appropriate level (Fig. 2a, upper trace). During the SEC analysis of the protein standard mix the presence of a thyroglobulin dimer was observed (M_r = 1340 kDa) what was in good agreement with the specifications of the manufacturer. The other two SEC columns, *i.e.*, PROTEEMA Micro with pore size of 1000 Å and ULTRAHYDROGEL 2000 with pore size of 2000 Å were not able to resolve the components of the protein standard mixture (Figs. 2b,c, upper traces). However, all of the tested separation columns showed appropriate elution profiles of KLH samples (Fig. 2, lower traces) and similar separation characteristics (Table I). Moreover, in the case of ULTRAHYDROGEL 2000 column, the time of analysis was much longer (30 min) compared to Acquity UPLC BEH450 SEC and PROTEEMA Micro column (16 min).

To clearly compare the applicability of the SEC columns, a KLH sample incubated for one week at room temperature was analysed. It is expected, that the stressed KLH sample will contain aggregates with very high molecular mass. As can be seen from the chromatograms presented in Fig. 3, the presence of and separation of such aggregates was obtained

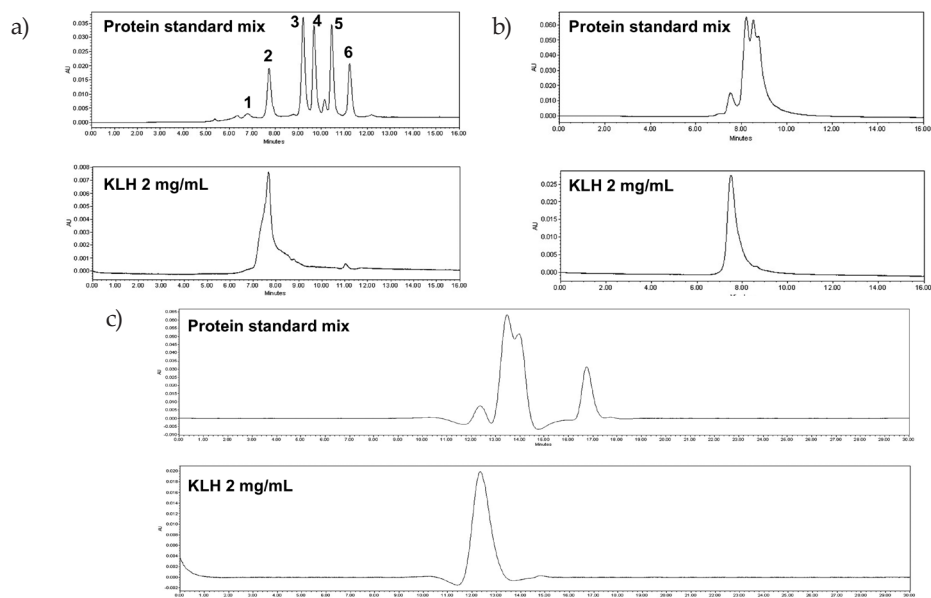


Fig. 2. Separation of protein mixture and KLH sample on three tested SEC columns: a) column Acuity UPLC BEH450 SEC; b) column PROTEEMA Micro; c) column ULTRAHYDROGEL 2000. Mobile phase: 25 mmol L⁻¹ phosphate buffer (pH 6.8) + 250 mmol L⁻¹ NaCl. Key to the peaks: BEH450 SEC protein standard mix: 1 – thyroglobulin dimer ($M_r = 1340$ kDa), $c = 0.1$ mg mL⁻¹, 2 – thyroglobulin ($M_r = 667$ kDa), $c = 3$ mg mL⁻¹, 3 – IgG ($M_r = 150$ kDa), $c = 2$ mg mL⁻¹, 4 – BSA ($M_r = 66$ kDa), $c = 5$ mg mL⁻¹, 5 – myoglobin ($M_r = 17$ kDa), $c = 2$ mg mL⁻¹, 6 – uracil ($M_r = 112$ Da), $c = 0.1$ mg mL⁻¹.

Table I. Comparison of tested SEC columns

	Acuity UPLC BEH450 SEC	PSS PROTEEMA Micro	ULTRAHYDROGEL 2000
Pore size (Å)	450	1000	2000
Elution time (min) ^a	7.57	7.61	12.35
Peak area (RSD, %) ($n = 3$) ^a	0.29	0.41	0.36
Column efficiency (N) ^{a,b}	1999	1896	1462

^a KLH (2 mg mL⁻¹); ^b Column efficiency (N) as number of theoretical plates: $N = 5.545 (t_R/w_{1/2})^2$, where t_R is the elution time and $w_{1/2}$ is the peak width at half of the peak's height.

only when the Acuity UPLC BEH450 SEC column was used (Fig. 3a). In case of PROTEEMA Micro column and ULTRAHYDROGEL 2000 column, the presence of aggregates was not evident. According to these results, further optimization procedure and measurements were realized with the Acuity UPLC BEH450 SEC column. These findings are in good

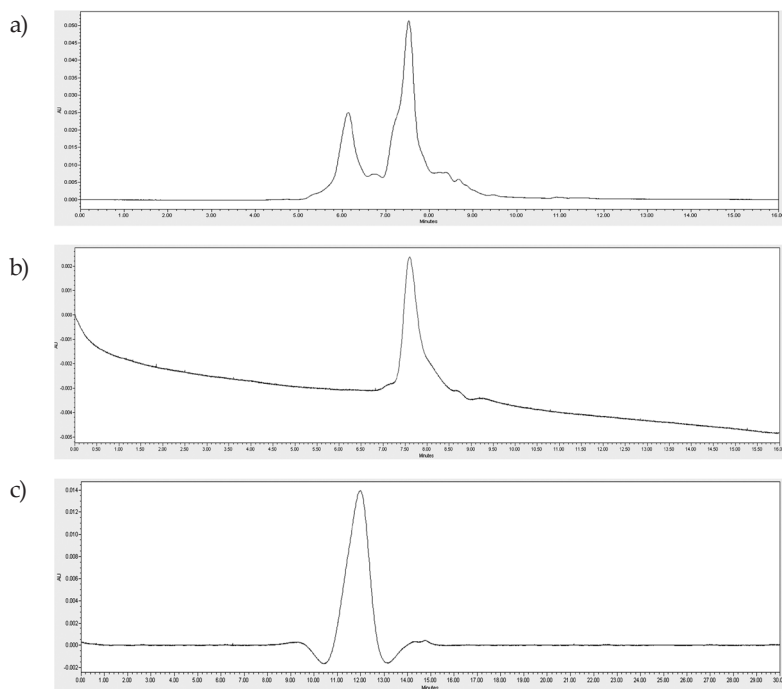


Fig. 3. Chromatograms obtained from the SEC analysis of KLH sample at the concentration level of 2 mg mL^{-1} incubated for a week at the room temperature (25°C). Separation performed on the separation column: a) acquity UPLC BEH450 SEC; b) PROTEOMA Micro; c) ULTRAHYDROGEL 2000. Mobile phase: 25 mmol L^{-1} phosphate buffer (pH 6.8) + 250 mmol L^{-1} NaCl.

agreement with the recent knowledge about modern $150 \times 4.6 \text{ mm}$ SEC columns packed with sub- $3\text{-}\mu\text{m}$ particles accompanied with effective separation efficiency, shorter time of analysis what significantly facilitates their implementation into analysis of biologics (36).

Effect of mobile phase composition on SEC analysis

Further, the effect of various mobile phases on the separation of protein standard mix (Waters BEH450 SEC protein standard mix) was investigated. The following separation buffers were investigated: 25 mmol L^{-1} and 50 mmol L^{-1} ammonium acetate (pH 7.4), 25 and 50 mmol L^{-1} sodium acetate (pH 7.4), 100 mmol L^{-1} ammonium formate (pH 7.4), 25 and 50 mmol L^{-1} MES (pH 6.5), 50 mmol L^{-1} BIS-TRIS (pH 7.2), 100 mmol L^{-1} citrate buffer (pH 7.4), or 100 mmol L^{-1} sodium citrate solution (pH 7.4). As can be seen from Fig. 4, the mobile phases composed of 25 mmol L^{-1} NH_4Ac with addition of 250 mmol L^{-1} NaCl and 25 mmol L^{-1} sodium acetate with addition of 250 mmol L^{-1} NaCl were able to offer similar results to the ones obtained with the primary selected SEC buffer, *i.e.*, 25 mmol L^{-1} phosphate buffer with addition of 250 mmol L^{-1} NaCl (see Fig. 2a, upper trace). Higher concentration of ammonium acetate or sodium acetate (*i.e.*, 50 mmol L^{-1}) led to improved peak

shape of the protein mix standard component with the lowest M_r – uracil (112 Da) (Figs. 4a-d). With the use of mobile phases composed of 25 or 50 mmol L⁻¹ MES with addition of 250 mmol L⁻¹ NaCl, it was not possible to resolve the peaks (Figs. 4f,g). Similar separation behaviour of the protein mix standard was observed using the mobile phase composed of 100 mmol L⁻¹ ammonium formate (Fig. 4h) and 50 mmol L⁻¹ BIS-TRIS (Fig. 4i). The mobile

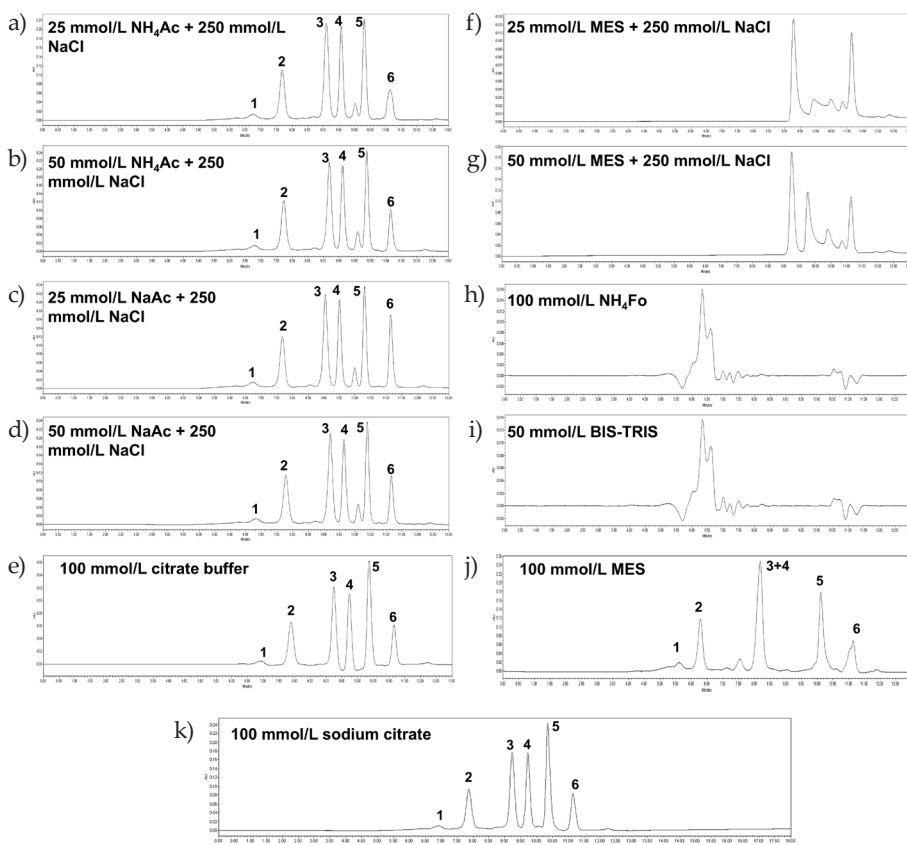


Fig. 4. Optimization of mobile phase composition. Illustrative SEC chromatograms obtained from the analysis of protein standard mix under the following composition of the mobile phase: a) 25 mmol L⁻¹ NH₄Ac + 250 mmol L⁻¹ NaCl; b) 50 mmol L⁻¹ NH₄Ac + 250 mmol L⁻¹ NaCl; c) 25 mmol L⁻¹ NaAc + 250 mmol L⁻¹ NaCl; d) 50 mmol L⁻¹ NaAc + 250 mmol L⁻¹ NaCl; e) 100 mmol L⁻¹ citrate buffer; f) 25 mmol L⁻¹ MES + 250 mmol L⁻¹ NaCl; g) 50 mmol L⁻¹ NH₄Ac + 250 mmol L⁻¹ NaCl; h) 100 mmol L⁻¹ NH₄Fo; i) 50 mmol L⁻¹ BIS-TRIS; j) 100 mmol L⁻¹ MES; k) 100 mmol L⁻¹ sodium citrate. Key to the peaks: BEH450 SEC protein standard mix: 1 – thyroglobulin dimer (M_r = 1340 kDa), c = 0.1 mg mL⁻¹, 2 – thyroglobulin (M_r = 667 kDa), c = 3 mg mL⁻¹, 3 – IgG (M_r = 150 kDa), c = 2 mg mL⁻¹, 4 – BSA (M_r = 66 kDa), c = 5 mg mL⁻¹, 5 – myoglobin (M_r = 17 kDa), c = 2 mg mL⁻¹, 6 – uracil (M_r = 112 Da), c = 0.1 mg mL⁻¹. Ac – acetate, BIS-TRIS – 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol, Fo – formate, MES – 2-(*N*-morpholino)ethanesulfonic acid.

phase containing only 100 mmol L⁻¹ MES provided just one peak of two components of the protein mixture standard – IgG and BSA (Fig. 4j). The solutions containing citrate were able to offer similar results to the ones obtained when using ammonium acetate or sodium acetate (Figs. 4e,k) or the starting SEC buffer (25 mmol L⁻¹ phosphate buffer with 250 mmol L⁻¹ NaCl). However, the citrate solutions were not able to distinguish impurities present in protein mix standard which eluted between BSA and myoglobin (see the small peak before peak 5 present in chromatograms in Figs. 4a-d). In case of KLH solutions, citrate buffer may act as sufficient stabilizer due to its chelating properties (it can bind free or exposed metal ions), however the preparations containing KLH are typically stabilized by presence of Mg²⁺ and Ca²⁺ ions. The citrate buffers may therefore remove or sequester these ions from the solution, and so affect the stability of KLH in solution. The addition of Mg²⁺ and Ca²⁺ ions into the tested mobile phases was omitted due to the fact that in case of phosphate buffer, creation of poorly soluble Mg²⁺ and Ca²⁺ salts may occur. In case of formate and acetate buffers, it may also come to creation of microprecipitates during the analysis when the buffer pH is too low.

The ability of the mobile phases to resolve all six components of the protein standard mixture is summarized in Table II. Accordingly, 25 mmol L⁻¹ phosphate buffer with the addition of 250 mmol L⁻¹ NaCl seems to be the most suitable mobile phase for further experiments.

Effect of column temperature and salt addition in the mobile phase

The column temperatures in the range of 25–55 °C were investigated. The experiments were realized with the use of protein mix standard and also with the KLH sample VACMUNE® (Fig. 5). In the case of protein standard mix, no significant changes were

Table II. Effect of mobile phase composition on resolution of the components of the protein mix standard

	Resolution (R _s)						
	Buffer 1	Buffer 2	Buffer 3	Buffer 4	Buffer 5	Buffer 6	Buffer 7
Thyroglobulin dimer	1.38	1.21	1.47	1.43	1.42	1.60	1.56
Thyroglobulin	2.33	2.21	2.22	2.17	2.32	2.24	2.24
IgG	0.89	0.80	0.83	0.84	0.86	0.87	0.89
BSA	1.44	1.51	1.51	1.61	1.66	1.20	1.16
Myoglobin	1.40	2.13	1.49	1.38	1.34	1.52	1.37
Uracil							

Buffer 1 – 25 mmol L⁻¹ phosphate buffer + 250 mmol L⁻¹ NaCl (pH 6.8), buffer 2 – 25 mmol L⁻¹ ammonium acetate + 250 mmol L⁻¹ NaCl (pH 7.4), buffer 3 – 50 mmol L⁻¹ ammonium acetate + 250 mmol L⁻¹ NaCl (pH 7.4), buffer 4 – 25 mmol L⁻¹ sodium acetate + 250 mmol L⁻¹ NaCl (pH 7.4), buffer 5 – 50 mmol L⁻¹ sodium acetate + 250 mmol L⁻¹ NaCl (pH 7.4), buffer 6 – 100 mmol L⁻¹ citrate buffer (pH 7.4), buffer 7 – 100 mmol L⁻¹ sodium citrate solution (pH 7.4). Resolution: $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where t_2 and t_1 is the elution time of the latter and the former eluting substance, resp., w_1 and w_2 indicating base width of each peak.

observed in peak shape, height or area (Fig. 5a, upper trace). Differences were observed during the investigation of KLH sample (Fig. 5a, lower trace). Higher temperatures led to decreased peak area of KLH. However, the total recovered area remained the same during the whole experimental set. The remaining peak area which does not represent the defined integrated interval of KLH peak belongs to the low molecular species eluting after the defined KLH peak. In case of peak height, the column temperature of 40 °C was associated with decreased peak intensity. However, further increase of the column temperature led to the increase of the peak height. According to the obtained results summarized in Table III, the column temperature of 25 °C was selected as the optimal one.

Table III. Effect of column temperature on the separation of KLH^a

Temperature (°C)	Elution time (min)	Average peak area ^b	RSD peak area (%) ^b	Average peak height ^b	RSD peak area (%) ^b
25	7.682	483815	1.5	12490	0.2
30	7.687	470226	1.9	12263	0.3
40	7.649	421555	1.8	9639	0.3
50	7.504	350668	2.5	11239	0.4
55	7.474	327864	2.8	11652	0.4

^a KLH sample VACMUNE® at the concentration level of 2 mg mL⁻¹; ^b *n* = 3

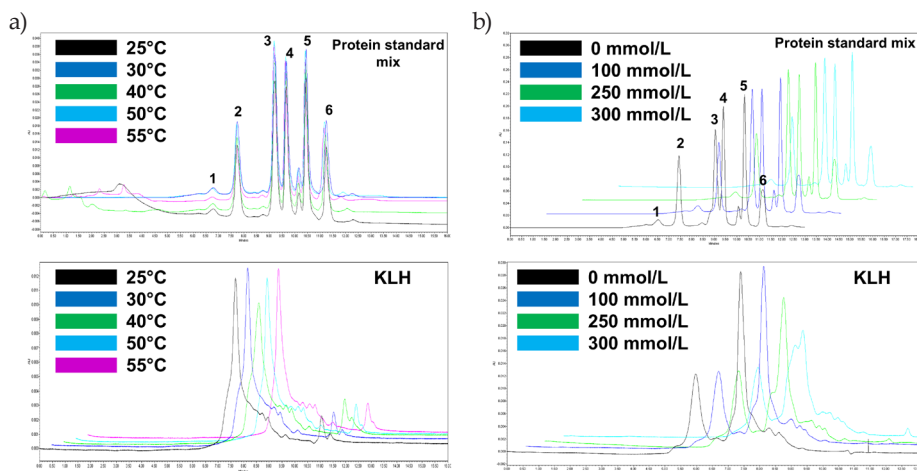


Fig. 5. Effect of: a) column temperature; b) NaCl concentration added to the mobile phase (25 mmol L⁻¹ phosphate buffer (pH 6.8), on the separation of protein standard mix (upper trace) and KLH sample (2 mg mL⁻¹, lower trace). Key to the peaks: BEH450 SEC protein standard mix: 1 – thyroglobulin dimer ($M_r = 1340$ kDa), $c = 0.1$ mg mL⁻¹, 2 – thyroglobulin ($M_r = 667$ kDa), $c = 3$ mg mL⁻¹, 3 – IgG ($M_r = 150$ kDa), $c = 2$ mg mL⁻¹, 4 – BSA ($M_r = 66$ kDa), $c = 5$ mg mL⁻¹, 5 – myoglobin ($M_r = 17$ kDa), $c = 2$ mg mL⁻¹, 6 – uracil ($M_r = 112$ Da), $c = 0.1$ mg mL⁻¹.

Table IV. Effect of salt concentration on resolution of the components of the protein mix standard

	Resolution (R_s)			
	NaCl concentration (mmol L ⁻¹)			
	0	100	250	300
Thyroglobulin dimer	1.06	1.07	1.38	1.13
Thyroglobulin	2.07	1.98	2.33	2.08
IgG	0.57	0.80	0.89	0.95
BSA	1.56	1.51	1.44	1.47
Myoglobin	1.07	1.12	1.40	1.40
Uracil				

Resolution: $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where t_2 and t_1 is the elution time of the latter and the former eluting substance, resp., w_1 and w_2 indicating base width of each peak.

The effect of salt addition to the mobile phase was represented by phosphate buffer. As can be seen in the Fig. 5b (upper trace), addition of NaCl resulted in improved resolution between the investigated components of the protein mix standard. Higher salt concentration (higher ionic strength) was accompanied with better resolution, however, the concentration of 250 mmol L⁻¹ NaCl was able to offer the best results in terms of resolution of the high molecular mass components (Table IV).

To obtain better information about the effect of salt addition on the SEC elution and separation properties of the investigated analyte, a sample of KLH of 2 mg mL⁻¹ which was incubated for one week at room temperature (25 °C) was investigated. Such type of sample was selected according to the expected formation of high molecular mass aggregates what could offer more complex and comprehensive information for the optimization procedure. The obtained results are presented in Fig. 5b (lower trace). Under all tested separation conditions, the presence of additional peak representing high molecular mass aggregate(s) of KLH was observed. The results from these experiments are summarized in Table V. Higher formation of early eluting components when using 300 mmol L⁻¹ NaCl (higher ionic strength) may be caused by progressive suppression of electrostatic secondary interactions with the diol surface. Such elution conditions alter the chromatographic resolution and recovery. This effect was observed in the case of the high molecular components of the protein mix standard, when higher ionic strength of the buffer system might be responsible for somewhat improved resolution between thyroglobulin dimer and thyroglobulin, and between thyroglobulin and IgG.

As can be seen in the Table V, the sum of percentages of the investigated substances is ~80 %. The remaining percentage belongs to the high molecular and low molecular species present in the samples out of the integration windows for peak of KLH and KLH aggregate. The total recovered area remains conserved under the stress conditions.

According to the results presented in Table IV and V, the addition of salt at the concentration level of 250 mmol L⁻¹ was finally selected as the optimal one.

Table V. Effect of salt concentration on SEC analysis of short term stressed KLH sample

NaCl concentration (mmol L ⁻¹)	Ionic strength (mmol L ⁻¹)	KLH aggregate		KLH	
		Elution time (min)	Mean area ± SD (%) ^a	Elution time (min)	Mean area ± SD (%) ^a
0	39	6.113	27.5 ± 0.2	7.531	52.5 ± 0.3
100	139	6.112	27.6 ± 0.2	7.531	52.6 ± 0.3
250	289	6.113	27.2 ± 0.3	7.532	52.7 ± 0.5
300	339	6.113	29.4 ± 0.2	7.532	47.8 ± 0.2

^a *n* = 3

Method validation

The developed and optimized SEC-UV method was partially validated according to the demands of the ICH Q2(R2) guideline (38).

Selectivity of the method was demonstrated by the analysis of blank samples which indicated no presence of any peak at the elution time of KLH (Fig. 6a).

Linearity of the developed SEC-UV method was established across the working range from 31.25 to 2000 µg mL⁻¹, with coefficient of determination higher than 0.99 ($R^2 = 0.9997$).

Sensitivity of the method was evaluated according to the parameters such as limit of detection (*LOD*) and limit of quantitation (*LOQ*). *LOD* represents the lowest concentration

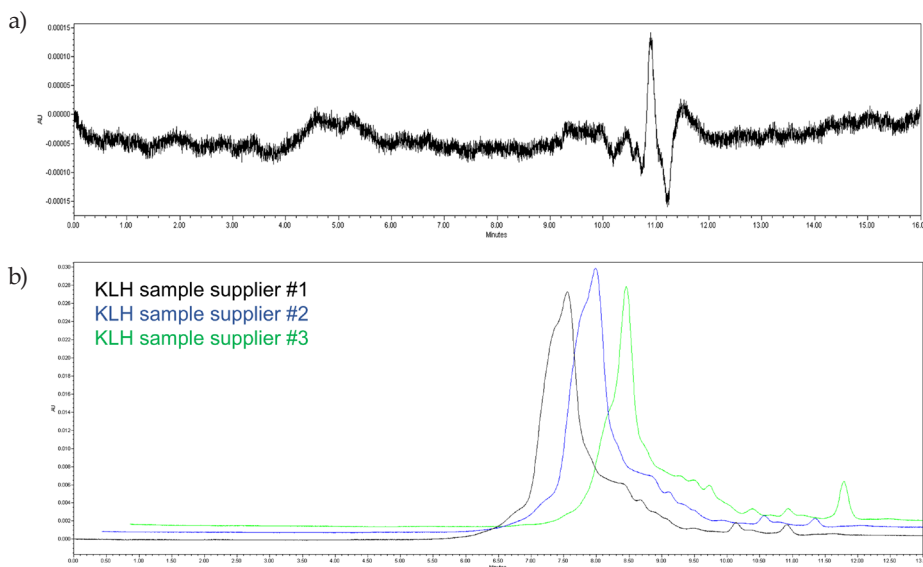


Fig. 6. Selectivity of SEC method – analysis of: a) blank sample; b) SEC analysis of three various KLH samples obtained from different suppliers.

of the analyte which can be reliably detected and *LOQ* represents the lowest concentration of the analyte which can be reliably quantified by the analytical method. These parameters were calculated according to the signal-to-noise ratio (*S/N*): *LOD* = 3(*S/N*), and *LOQ* = 10(*S/N*). The obtained results are summarized in Table VI and indicate *LOD* (10.21 µg mL⁻¹) and *LOQ* (31.25 µg mL⁻¹) values, which were suitable for detection and quantification purposes of the proposed method.

Table VI. SEC method validation

Validation parameter	
Linearity range (µg mL ⁻¹)	31.25–2000.0
Regression line equation	$y = 840.38x - 15897$
Standard deviation of slope	± 3.53
Standard deviation of intercept	± 3081.86
Coefficient of determination (<i>R</i> ²)	0.9997
Limit of detection (µg mL ⁻¹)	10.21
Limit of quantitation (µg mL ⁻¹)	31.25
Repeatability (<i>n</i> = 6, RSD, %)	2.5 (QC 1), 2.5 (QC 2), 0.3 (QC 3)
Intermediate precision (<i>n</i> = 18, RSD, %)	4.2 (QC 1), 4.4 (QC 2), 1.1 (QC 3)
QC 1 (<i>n</i> = 3, mean recovery, %)	102.8
QC 2 (<i>n</i> = 3, mean recovery, %)	100.9
QC 3 (<i>n</i> = 3, mean recovery, %)	100.4

Precision of the method was established as repeatability and intermediate precision. The repeatability was evaluated by measurement of a sample at the *LOQ* concentration level, *i.e.*, 31.25 µg mL⁻¹, within the same day in six replicates. The intermediate precision was realized with the use of a sample at the same concentration level on three consecutive days and by another analyst. The obtained values summarized in Table VI clearly present precision level of the developed SEC-UV approach, repeatability – RSD ≤ 2.5 % and intermediate precision – RSD ≤ 4.4 %.

Accuracy was evaluated as recovery at three concentration levels, *i.e.*, 31.25 µg mL⁻¹ (QC 1), 250 µg mL⁻¹ (QC 2), and 2000 µg mL⁻¹ (QC 3). Recovery value ranged from 100.4–102.8 %.

Robustness of the method represents its ability to remain unaffected by small, deliberate variations in operation parameters. Here, the mobile phase flow rate changes (± 0.1 mL min⁻¹) and temperature shifts (± 0.1 °C) led to negligible changes in peak area of the investigated analyte/analytes (% peak area ≤ 0.7 %).

Analysis of KLH samples

Finally, the developed SEC method was applied to search for characteristic profiles of three commercially available KLH samples and to investigate the concentration of such

products. The characteristic SEC profiles of the investigated samples are illustrated in Fig. 6b, and the determined content of KLH in each sample is presented in Table VII. As can be seen in Fig. 6, slight differences were observed among the samples. All of the samples disposed the same elution time maxima, at approx. 7.6 min. The differences in elution profiles may be caused by differences in original composition of the samples (presence of buffering agents, additives, stabilizers, preservatives, *etc.* – such as sodium phosphate buffer (pH 7.4), NaCl (0.46 mol L⁻¹), PVP (2 %), or sucrose (41 mmol L⁻¹) or their formulation form (*i.e.*, liquid – supplier #3, *vs.* lyophilized – supplier #1 and supplier #2) in which the KLH product is supplied. The determined content of KLH in the investigated samples is summarized in Table VII. The obtained results are in good agreement with the declared information by the manufacturer, where the content of KLH should be in the range from 20 to 26 mg per vial.

Table VII. Determination of KLH in commercially available samples

Sample	Average conc. (mg mL ⁻¹)	RSD (%) ^a	Content per vial (mg)
KLH sample supplier #1	2.18	2.27	21.84
KLH sample supplier #2	2.29	1.54	22.91
KLH sample supplier #3	2.01	1.14	20.08

^a *n* = 3

CONCLUSIONS

This research describes a univariate experimental optimization strategy of SEC for analysis of strong immunogenic protein KLH. The strategy included investigation of the effect of mobile phase composition, type of stationary phase, column temperature, and additives on elution profile (separation) of protein mix standard and KLH samples. The proposed and developed SEC method was validated, and finally applied to determine the content of KLH in three commercially available samples. The presented results create a good starting point for further investigation and could be adapted into the field of in-process quality control during the development of the biologics, and also into the quality control procedures of the final drug products. However, there are still some important questions which need to be answered including long-term stability studies and detail characterization of the KLH molecule and its aggregates with the modern advanced analytical approaches such as high-resolution mass spectrometry.

Acronyms, abbreviations, symbols. – ADC – antibody drug conjugate; BIS-TRIS – 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol; BSA – bovine serum albumin; DTT – dithiothreitol; HMWS – high molecular mass species; ICH – The International Council for Harmonisation of Technical Requirements for Pharmaceutical for Human Use; IgG – immunoglobulin G; KLH – keyhole limpet hemocyanin; LMWS – low molecular mass species; LOD – limit of detection; LOQ – limit of quantitation; MALS – multi-angle light scattering; MES – 2-(*N*-morpholino)ethanesulfonic acid; MPW – Millipore water (deionized water); PAGE – polyacrylamide gel electrophoresis; QC – quality control; S/N – signal-to-noise ratio; SEC – size exclusion chromatography; US FDA – United States Food and Drug Administration.

Data availability statement. – Data available on request from the authors.

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Conflict of interest. – The authors declare no conflict of interest.

Author's contribution. – Conceptualization A.K. and J.P.; investigation, P.C. and J.P.; data curation, P.C. and J.P., formal analysis P.M. and A.K.; visualisation J.P.; writing, original draft, P.C.; writing, review and editing P.M., A.K., and J.P. All authors have read and agreed to the published version of the manuscript.

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