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Porous polymer monoliths with complementary retention mechanisms for online solid-phase extraction liquid chromatography to determine lysozyme in egg white

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ABSTRACT

This work demonstrates the determination of lysozyme in egg-white samples after enrichment and cleanup by weak cation exchange (WCX) following separation by reversed-phase liquid chromatography (RPLC). The WCX column was prepared from glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) and functionalized with iminodiacetate (IDA). Reversed-phase columns were prepared using butyl methacrylate (BMA) and EDMA. Photopolymerization formed the poly(GMA-co-EDMA) column inside vinylized polypropylene tubes whereas poly(BMA-co-EDMA) used thermal polymerization inside functionalized Silcosteel® tubes. The preparation of poly(GMA-co-EDMA) was fast (about 2 h), from preparing the polypropylene tube to washing the formed monolith with acetonitrile (ACN), but functionalization demanded an overnight period of pumping IDA through the column immersed in a water bath thermostated at 80 °C. Preparation of the poly(BMA-co-EDMA) also demanded overnight heating at 60 °C, with subsequent washing of the formed monolith with ACN. Egg-white samples diluted at a 1:10 m v^{-1} ratio in phosphate buffer (pH 7.0) were injected first through IDA@poly(GMA-co-EDMA) to retain lysozyme (pI 11.4) and remove the proteins with a pI < 7.0. Elution of the lysozyme from the cation exchange column was made with 5% (v v^{-1}) acetonitrile in 0.1% (v v^{-1}) TFA. RPLC then analyzed the eluate with a gradient from 5 to 50% ACN in 0.1% TFA. The limits of detection and quantification were 0.07 and 0.23 mg mL^{-1}, respectively. Egg-white lysozyme concentrations varied between 2.26 ± 0.06 and 4.41 ± 0.08 mg g^{-1}, and spiking/recovery experiments at two concentration levels (0.25 and 0.50 mg mL^{-1}) resulted in recoveries from 94 to 115%, thus demonstrating the columns working with orthogonal selectivity provided enrichment of less abundant lysozyme and accurate results, provided by an efficient cleanup of the sample matrix.

1. Introduction

Solid phase extraction (SPE) was introduced in the mid-1970s aiming to overcome the liquid-liquid extraction (LLE) limitations such as the use of cumbersome glassware, toxic organic solvents, analyst exposure, and difficult automation [1–4]. SPE consists of four steps: (i) column conditioning, (ii) sample loading, (iii) column washing, and (iv) elution. These operations can be made manually, but they are easily automated by coupling online the SPE column to analyzers, eliminating the sample handling between the enrichment and separation steps, provided pumps and valves are available. This hyphenation minimizes the risks of losses by evaporation and sample contamination. Another advantage of online over offline SPE is that the totality of the extracted analyte is injected into the analyzer, substantially decreasing the volume of the sample that has to be processed before the analysis [5,6].

If coupled to liquid chromatography, the analysis of complex samples benefits from better selectivity using an analytical column with a retention mode complementary to that of the SPE [7,8]. SPE methods use commercially available packed columns and cartridges, which are expensive and can be used only a few times. Thus, low-cost alternative materials are in a continuous search by analysts.

Porous organic monolithic columns have been used for chromatographic separations and sample preparations since the 1990s [9,10]. Due to their morphology constituted by a network of interconnected macropores and a consequent high permeability to the sample solution, polymeric monolithic columns serve as a means of fast extraction, enrichment and separation, especially of macromolecules [11–14]. Functional groups that cover the pore surfaces control the retention mode. For instance, butyl-, lauryl- and stearyl-methacrylate monomers provide hydrophobic retention modes useful for reversed-phase (RP) or

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hydrophobic interactions [13,15,16]. Glycidyl methacrylate (GMA) is a versatile monomer since there is a wide variety of reagents able to functionalize its epoxy group conferring ion exchange and affinity retention modes [13,17,18]. The versatility of fine-tuning the retention mode makes the polymer monoliths potential candidates for developing solid phase extraction (SPE) and liquid chromatography methods [19,20] using complementary retention modes.

Egg white is a model of a complex sample rich in a variety of proteins having a wide range of concentrations (ovomucoid, 9.5–11%; ovalbumin, 54–66%; ovotransferrin, 12–13%; and lysozyme, 2.3–4.5%) [21]. Among the available proteins, lysozyme (14.3 kDa; 129 amino acids) is of interest to different areas due to its antibacterial, anti-microbial, antiviral, and antitumor properties. It can also be a food preservative [22–24]. Due to these characteristics and the low lysozyme content in egg whites, the search for new and efficient technologies for their separation, quantification and purification is of great interest [24–26].

This paper describes the determination of lysozyme in egg white samples after enrichment and cleanup via cation exchange followed by separation in reversed-phase mode. We demonstrated the potential orthogonality of monolithic polymeric columns, which were synthesized in a simple and fast way in the laboratory (with low waste generation), thus serving as a low-cost alternative to commercial chromatographic columns and cartridges.

2. Materials and methods

2.1. Reagents

The monomers butyl methacrylate (BMA), glycidyl methacrylate (GMA), and ethylene glycol dimethacrylate (EDMA) were purchased from Sigma-Aldrich St. Louis, MO, USA) and passed through a basic aluminum oxide column to remove polymerization inhibitors. N-propanol and 1,4-butanediol were obtained from Sigma-Aldrich (used as received) and served as porogenic solvents. Azobisisobutyronitrile (AIBN), 2,2-Dimethoxy-2-phenyl-acetophenone (DMPAP) and benzophenone (BP) were the radical initiators. HPLC-grade methanol, acetone, acetonitrile, trifluoroacetic acid (TFA), and ethyl alcohol were from Merck KGaA (Darmstadt, Germany). Iminodiacetatic acid (IDA) was used to produce a weak cation exchanger and was used as received from Sigma-Aldrich. Phosphate buffer solutions (PBS) were prepared from analytical grade KH_2PO_4 and K_2HPO_4 salts from Merck. Silcosteel® tubing with 1.59 mm o.d. and 1.016 mm i.d. were purchased from Restek (Bellefonte, PA, USA). Polypropylene tubes with 2.0 mm i.d. were obtained from ink pens.

2.2. Instrumentation

Photografting of the ink-pen inner wall tubes and monolithic photopolymerization were made in a Specrolinker XL-1000 UV-crosslinker from Spectronics Corporation (Westbury, New York, USA) provided with five 8-watt lamps (254 nm).

Separation experiments were made in a Dionex Ultimate 3000 Dual Micro LC system (Dionex Softron GmbH, ThermoFisher Scientific, Germany) using dual micro DGP-3600 RS pumps with an SRD-3600 in-line degasser, a WPS-3000SL automatic sampler with a sampling loop for volumes between 1.0 and 100 μL, a TCC 3000SD column compartment and an MWD-3000 UV/Vis detector coupled to a 2.5 μ semi-micro flow cell. Control of the instrument, data acquisition and processing were made with the software Chromeleon® 6.8. Online SPE used a 2-way 6-port injection valve commanded by the Chromeleon® software. Connections of the Silcosteel® column to the chromatographic systems were made with P-742 PEEK ZDV unions for 1/16 inch o.d., whereas the ink pen SPE column was fitted to the injection valve with P-702 PEEK unions, XP-335 PEEK nuts and P-300 ETFE, all from IDEX Health and Science (Oak Harbor, WA, USA).

2.3. Functionalization of the inner wall of the polypropylene (PP) tubes

One hundred to 110 mm long PP tubes from ink pens were washed with ethanol and acetone (five times with 1 mL aliquots of each solvent) and dried under N_2. Then, the tubes filled with 5 wt% BP in methanol, formerly sonicated (10 min), and purged with N_2 (10 min), were closed on both ends and irradiated for 20 min under 254 nm at 120 mJ cm^{-2}. After this first step, the tubes were washed with methanol and dried with N_2. Then, they were filled with a sonicated (10 min) and deaerated (10 min) 15 wt% EDMA solution in methanol and irradiated for 10 min under 254 nm at 120 mJ cm^{-2}. Finally, the tubes were washed with methanol and dried under a flow of N_2. In all experiments, the tubes were positioned 2.5 cm apart from the UV lamps [27–30].

2.4. Functionalization of the inner wall of the Silcosteel® tubes

The inner wall was sequentially washed with ethanol, water, 0.2 mol L^{-1} NaOH (60 min at 5 μ s^{-1}), water, 0.2 mol L^{-1} HCl (60 min at 5 μ s^{-1}), water and ethanol. Next, the tube was filled with 20 wt% 3-(trimethoxysilyl)propyl methacrylate in 95% (v^{-1}) ethanol (apparent pH adjusted to 5.0), previously sonicated for 5 min [31]. The ends of the tube were closed with pieces Pharmed® peristaltic pump tubes sealed with solid PTFE tubes. The system was heated overnight at 60 °C inside the oven of a gas chromatograph. Finally, the tube was washed with acetone, dried under a flow of N_2 and cut into pieces of approximately 15 cm.

2.5. Preparation of poly(glycidyl methacrylate-co-ethylene dimethacrylate) and poly(butyl methacrylate-co-ethylene dimethacrylate) columns

A polymerization mixture containing 24 wt% GMA, 16 wt% EDMA, 45,5 wt% 1-propanol, and 14,5 wt% 1,4 butanediol was prepared in a 2 mL amber vial in the presence of 1.0 wt% DMPAP (concerning the monomers) [32]. The mixture was sonicated (10 min), purged with N_2 (10 min), and used to fill the activated PP tubes, which were closed on both ends, vertically positioned 2.5 cm apart from the UV-lamps, and irradiated for 20 min under 254 nm at 120 mJ cm^{-2}. Next, the columns were flushed with ACN at 500 μ min^{-1} using an HPLC pump until a constant pressure and then with water to remove ACN.

The poly(GMA-co-EDMA) columns were heated at 80 ºC (immersed in a water bath) and flushed (8 μL min^{-1}) with 10 mL of 0.75 mol L^{-1} IDA solution in 0.34 mol L^{-1} sodium chloride neutralized to pH 10 using a KD Scientific infusion pump. The columns were washed with deionized water following the modification procedure to remove the excess reagent.

The preparation of the poly(BMA-co-EDMA) column followed the protocol proposed by Coufal et al. [33] using 18 wt% BMA, 22 wt% EDMA, 36 wt% n-propanol and 18 wt% 1,4 butanediol in the presence of 1 wt% AIBN (concerning the monomers). The sonicated (10 min) and N_2-purged (10 min) mixture filled the Silcosteel® tube, which was closed at both ends and heated at 60 °C for 20 h. The column was flushed with ACN to remove the unreacted monomers, solvent and initiator until reaching a constant pressure. It was further used without any other modification.

2.6. Samples

Standard white and brown, free-range, and organic chicken eggs were purchased in local food stores. Egg white samples were diluted 1:10 (m v^{-1}) in 20 mmol L^{-1} phosphate buffer, pH 7.0, and filtered in 0.22 μm syringe filters before analysis [25].

2.7. Online WCX-SPE-RPLC procedure

The dual gradient chromatographic system assembled according to Fig. 1 consisted of two pumps (Pumps 1 and 2), an autosampler, and one

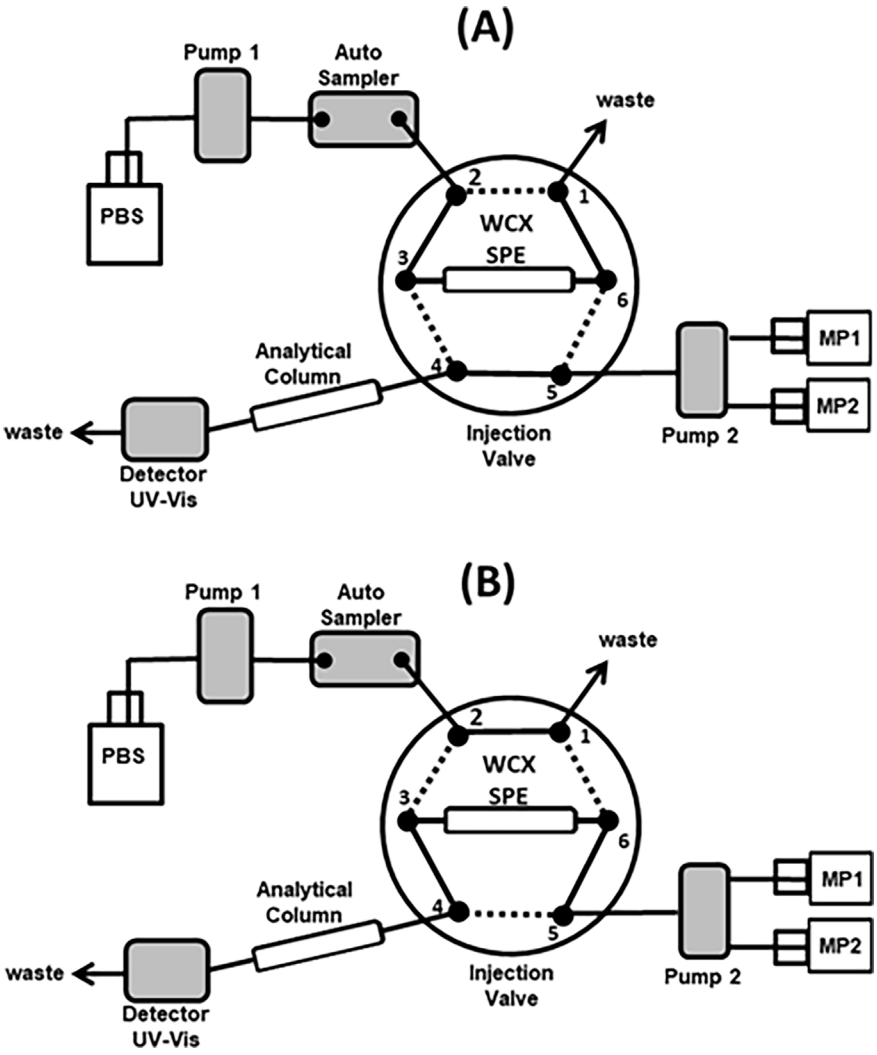

Fig. 1. Chromatographic system for online WCX-SPE-RPLC in (A) loading and (B) injection positions. PBS = 20 mmol L^{-1} phosphate buffer (pH 7.0), MP1 = 0.1% TFA in water, MP2 = 0.1% TFA in ACN, UV-Detection at 205 nm.

2-way, 6-port injection valve for SPE. Pump 1 performed the SPE column conditioning, sample loading (2 μL), and washing (1.5 min) using 20 mmol L^{-1} phosphate buffer solution (PBS, pH 7.0) at 0.50 mL min^{-1}. Simultaneously, Pump 2 is conditioning the analytical column with 5% ACN in 0.1% TFA at 0.50 mL min^{-1} (Fig 1A). After loading and washing SPE steps, the SPE valve commutes to the injection position so that the 5% ACN in 0.1% TFA solution from the Pump 2 transfer (for 2 min) lysozyme and other retained proteins from the WCX column to the RP analytical column (Fig 1B). As the transfer time ends, the SPE valve commutes back to the load position for reconditioning with 20 mmol L^{-1} PBS from Pump 1 while Pump 2 separates the proteins parked in the analytical column with a gradient from 5 to 50% ACN in 0.1% TFA from 3.5 to 8 min (all% referring to volumetric ratios). The flow rate of the Pump 2 was constant at 0.50 mL min^{-1}.

3. Results and discussion

3.1. Columns characterization

Both the poly(BMA-co-EDMA) in Silcosteel® and IDA@poly(GMA-co-EDMA) in polypropylene tubes were described in previous papers [19,34,35]. In short, the poly(BMA-co-EDMA) column exhibited a permeability of (5.82 ± 0.15) × 10^{-14} m^2 with a morphology characterized by globules measuring around 1 μm with homogeneous and regular spherical shapes arranged in 3 to 4 μm clusters separated by large flow-though pores. The poly(GMA-co-EDMA) parent monolith is structured in globules measuring about 2.0 ± 0.5 μm interconnected in cauliflower-like clusters measuring 4 to 10 μm, separated by large flow-through pores exhibiting diverse geometries and sizes around 6 μm. For the unmodified column, the permeability was (3.31 ± 0.09) × 10^{-13} m^2 ($n = 3$) the functionalization with IDA did not alter the permeability of the columns, which was (3.34 ± 0.06) × 10^{-13} m^2 ($n = 3$). Elemental analysis suggests incorporation of 0.33 ± 0.05 wt% of N in the IDA@poly(GMA-co-EDMA)-IDA. Pumping Cu(II) continuously to the columns suggests a breakthrough after loading the column with 55 ± 5 μmol g^{-1} [34].

3.2. WCX-SPE-RPLC method development

The principle of this sample preparation method relies on the weak cation exchange properties of the IDA@poly(GMA-co-EDMA) column. This monolith is prepared by amination of the epoxy ring, leaving two free carboxylate moles per each mole of immobilized IDA. Conditioning the column with PBS at pH 7.0 produces a negatively charged pore surface that can be neutralized by trifluoroacetic acid. Lysozyme is a less abundant protein in egg white samples (3.4 dry wt%) having an isoelectric point of 11.4, thus positively charged at pH 7.0, whereas the most abundant albumins such as ovalbumin (54 dry wt%) and conalbumin (12 dry wt%) are negatively charged since their pI are 4.5 and 6.1, respectively. Other proteins in chicken egg whites are ovomucoid (11.0 dry wt%), ovomucin (3.5 dry wt%) and globulins G2 and G3 (4 dry wt% each), but their pI are < 5.8. Another positively charged protein at pH 7.0 is the minor avidin (0.05 dry w%), with pI 10.0 [33].

Proteins such as lysozyme and avidin retain electrostatically onto the IDA@poly(GMA-co-EDMA) SPE column at pH 7.0, whereas the phos-

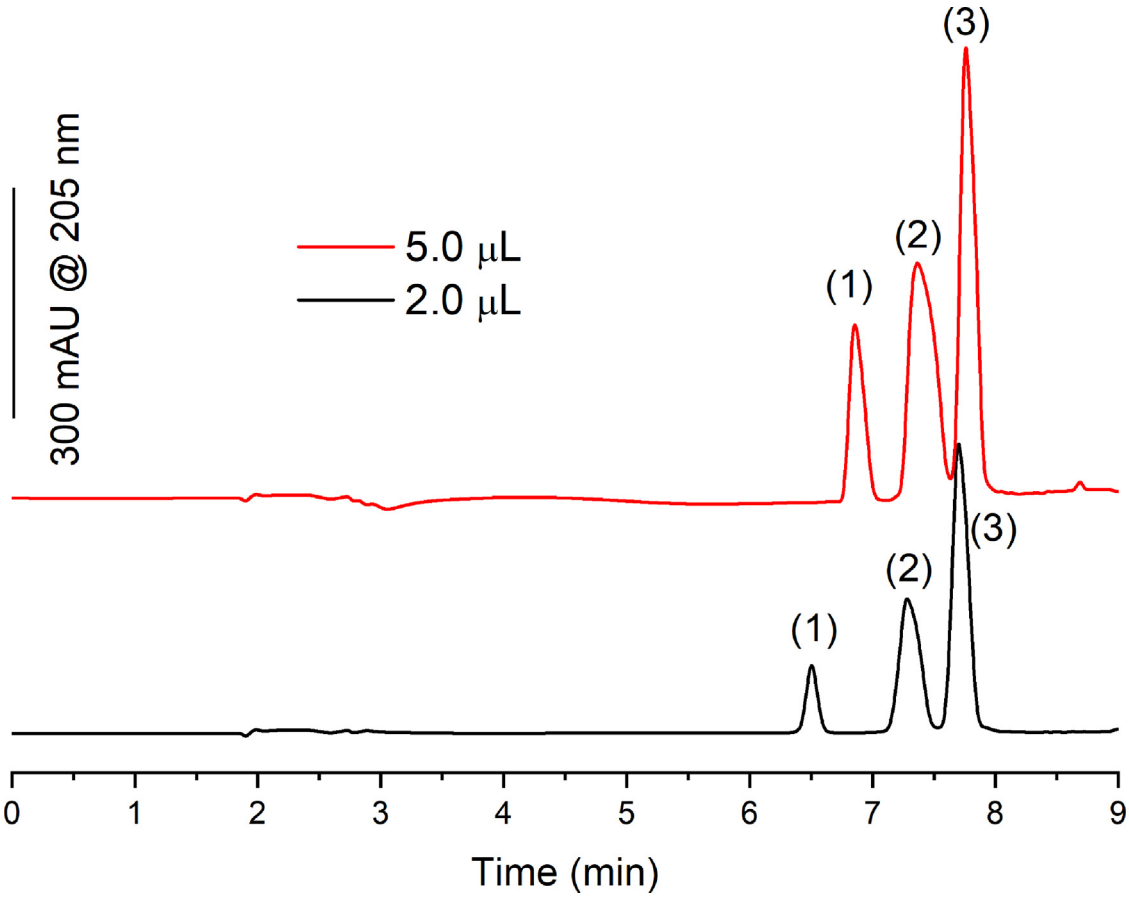

Fig. 2. Reversed-phase blank subtracted chromatograms of a mixture of ovalbumin, (1) ribonuclease A, (2) cytochrome C, and (3) lysozyme (all at 1.0 mg mL^{-1}) obtained after injecting 2.0 or 5.0 μL of the mix in the WCX column (0.50 mL min^{-1}) using 2 min of transfer time from the WCX to the RP columns. Separation was achieved by 5 to 50% gradient of ACN in 0.1% TFA from 3.5 to 8.0 min. Oven column temperature = 60 °C.

phate buffer carries ovalbumin, conalbumin, ovomucoid, ovomucin and globulins to the waste during the loading and washing steps [25]. By commuting the SPE rotary valve to the injection position for 2 min, Pump 2 (Fig 1) drives the mobile phase composed of 5% (v v^{-1}) ACN in 0.1% (v^{-1}) TFA through the WCX column, protonating the carboxylate functionalities and breaking the electrostatic interactions that retained lysozyme and other proteins with pI > 7. The proteins were thus back-eluted to the poly(BMA-co-EDMA) reversed-phase column for chromatographic separation by a linear gradient of ACN from 5 to 50% (v v^{-1}) in 0.1% (v v^{-1}) TFA from 3.5 to 8 min at a flow rate of 0.50 mL min^{-1}, monitoring the absorbance at 205 nm. While Pump 2 works on protein separations, Pump 1 reconditions the IDA@poly(GMA-co-EDMA) column with the 20 mmol L^{-1} PBS (pH 7.0).

A mixture of ovalbumin, ribonuclease A, cytochrome C and lysozyme (all at 1.0 mg mL^{-1}) was analyzed by the procedure described in item 2.7. The resulting chromatogram exhibited only three peaks corresponding to ribonuclease A (pI 9.6), cytochrome C (pI 10–10.5) and lysozyme (pI 11.4), an elution order consistent with previous RP separations of the protein mixture by the poly(BMA-co-EDMA) column (Fig 2) [35]. Ovalbumin was not detected because it passed unretained through the WCX column buffered at pH 7.0.

The transfer time (the time the SPE valve remains in the injection position) was evaluated for 1, 2 and 3 min aiming to optimize the lysozyme transference between the columns. A transfer time of 2 min was adopted in the methodology because 1 min was not enough to quantitatively carry lysozyme from the WCX to the RP column, leading to a reduced lysozyme peak in the RPLC, whereas 3 min lead to a peak having a similar area as the one observed for 2 min.

After confirming the orthogonal WCX and RP retention mechanisms and a suitable transfer time, the effect of sample volume was evaluated by injecting 2 and 5 μL of mixed solution. The peak heights increased proportionally with the sample volume, but worsened the chromatographic resolution, especially between the cytochrome C and lysozyme peaks (Fig 2). Thus, a sample volume of 2 μL was adopted to provide a suitable sensitivity without overloading the microbore analytical column.

3.3. Application to egg-white sample

Reversed-phase chromatogram of the diluted egg white sample exhibited six peaks (Fig 3A). Peaks 3 and 6 can be assigned to lysozyme and ovalbumin, respectively, because their retention times coincide with those observed in chromatograms obtained for these two isolated proteins. Lysozyme peak appears with retention times close to two adjacent peaks that can lead to quantification errors due to resolution < 1.5 ($Rs_{2,3}$ = 1.07 and $Rs_{3,4}$ = 1.43). The online SPE cleanup provided by retaining lysozyme by WCX enhanced the measurements' selectivity of the reversed-phase separation in the analytical column, as evidenced in Fig 3B since the only observed peak was that for lysozyme, confirmed by injecting an isolated lysozyme solution. The sensitivity was also significantly enhanced, with a rough 2.5-fold enrichment factor (considering peak 3 heights).

3.4. Quantification of lysozyme in egg white

Quantification was made by external calibration using lysozyme concentrations between 0.25 and 1.50 mg mL^{-1}. Peak areas (*A*) in-

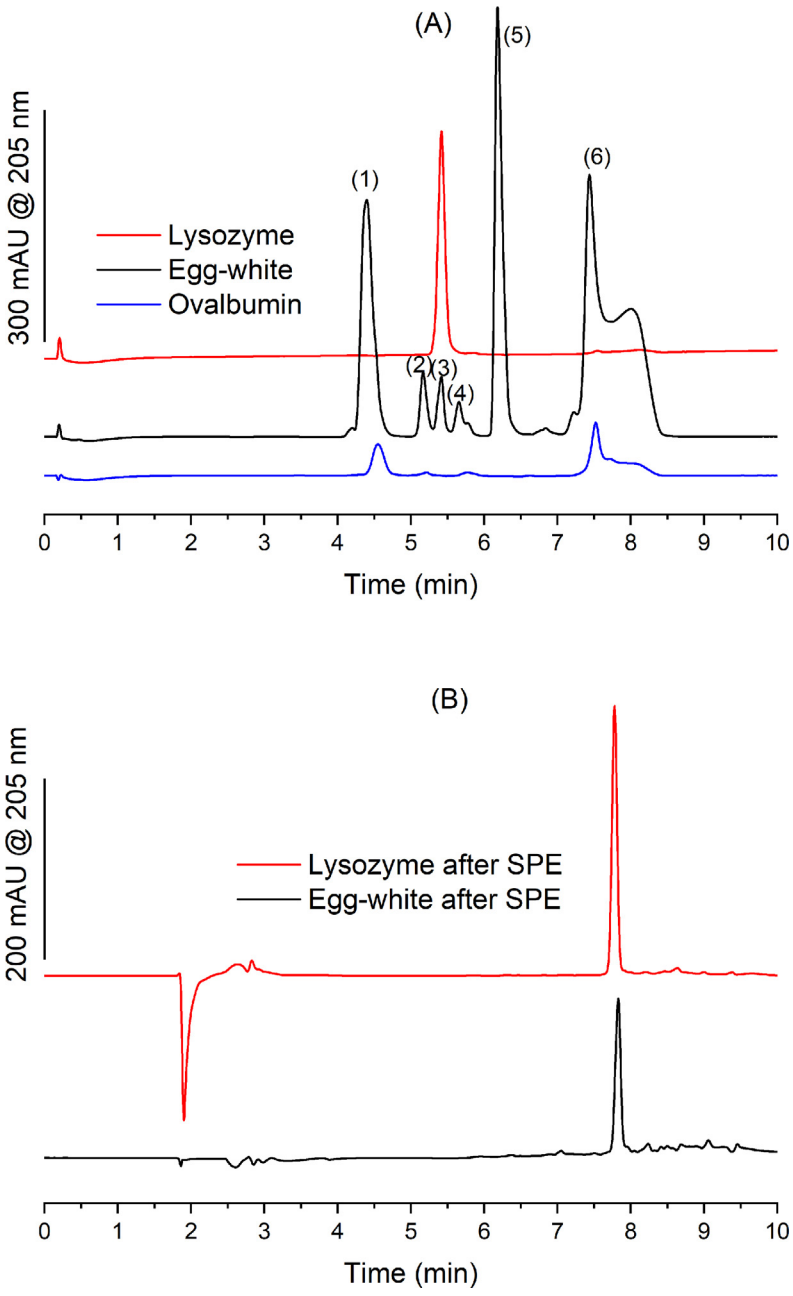

Fig. 3. (A) Blank-subtracted reversed-phase chromatograms of an egg-white sample superposed to chromatograms of isolated lysozyme (peak 3) and ovalbumin (peak 6) obtained with a gradient from 5 to 50% ACN in 0.1% TFA in 8 min (all% referring to volumetric ratios). (B) Blank-subtracted reversed-phase chromatograms of an egg-white sample superposed to chromatograms of isolated lysozyme after SPE in the WCX column and 2 min transfer (with 5% ACN in 0.1% TFA) to the RP analytical column, performing the separation with a gradient from 5 to 50% ACN in 0.1% TFA from 3.5 to 8 min. All flow rates = 0.50 mL min^{-1}. Column oven = 60 °C.

creased linearly with the concentrations (C_{lys}) fitting the equation: $A = (66 \pm 1)C_{lys} - (2 \pm 2)$ with $R^2 = 0.9994$ and $S_{y/x}$ (the standard deviation of the y-residuals) = 1.51. The LOD and LOQ were computed using Eqs. (1) and (2), respectively, where m is the slope of the calibration curve, resulting in $LOD = 0.07$ mg mL^{-1} and $LOQ = 0.23$ mg mL^{-1} [36].

$$LOD = {}^{3S_{y/x}}/_{m} \quad (1)$$

$$LOD = {}^{10S_{y/x}}/_{m} \quad (2)$$

Egg-white lysozyme concentrations varied between 2.26 ± 0.06 and 4.41 ± 0.08 mg g^{-1} (Table 1), a concentration range similar to that reported by Kiyota et al. [37], between 2.1 and 3.5 mg g^{-1}, consistent with the mean 2.3 – 4.5% concentrations found in egg-white samples. Spiking/recovery experiments at two concentration levels (0.25 and 0.50 mg mL^{-1}) resulted in recoveries from 94 to 115%, showing good accuracy for the proposed methodology.

Table 1
Lysozyme concentrations in different egg white samples by WCX-SPE-RPLC and WCX SIC. The results are means of triplicates for each sample.

Sample	Lysozyme concentration (mg g^{-1})		F	*t*
	WCX-SPE-RPLC	WCX-SIC		
Standard white	2.70 ± 0.07	2.9 ± 0.2	8.16	−1.63
Standard white-large	2.26 ± 0.06	2.2 ± 0.1	2.77	0.89
Free range	3.97 ± 0.02	4.1 ± 0.2	25	−1.10
Organic	4.41 ± 0.08	4.2 ± 0.3	14	1.17
Brown	2.7 ± 0.2	2.9 ± 0.2	1	−1.22

Because no other chromatographic peak was observed in RPLC, the quantification of lysozyme could be made directly by WCX in a simpler instrumental setup such as the Sequential Injection Chromatograph [38], shown in Fig S1. The SIC procedure, detailed in SI, was based on injecting 50 μL of the egg white sample buffered in 20 mmol L^{-1} PBS (pH 7.0), followed by washing with the same PBS buffer to eliminate the

proteins with pI < 7.0 (Fig S2). Finally, lysozyme elution to the detector is made by injecting 500 μL of PBS buffer in 0.20 mol L^{-1} NaCl. WCX-SIC was about 3-fold faster than WCX-SPE-RPLC but its use was only possible because the concentration of avidin and other proteins with pI > 7 were significantly lower than that of lysozyme in the samples. Otherwise coeluting proteins would severely affect the accuracy of the lysozyme determination by the WCX-SIC method.

A comparison of the results obtained by online WCX-SPE-RPLC and WCX-SIC (Table 1) shows that at a P=0.05, there is no evidence of significant differences in the precision of both methods because the $F_{2,2}$ values were less than the critical F value for the two-tailed test ($F_{2,2}$ = 39). Using the one-tailed test ($F_{2,2}$ = 19.00), only for the sample of free-range eggs the online WCX-SPE-RPLC provided a statistically better precision [36]. Comparing the two experimental concentration means of each sample by the *t*-test (Table 1) suggests the absence of systematic errors because the computed *t*-values were less than the critical value for 4 degrees of freedom and P=0.05 (t=2.78). A comparison between the concentration means using the paired *t*-test [36] shows that the $|t|$ value was 0.718, which is less than the critical value of 2.78 for 4 degrees of freedom at P=0.05, thus confirming that there are no significant differences between the lysozyme concentrations obtained by online WCX-SPE-RPLC and WCX-SIC.

4. Conclusions

This paper adds a piece of knowledge on the potential applications of polymer monolithic columns with orthogonal retention mechanisms to perform online sample cleanup and protein quantification in complex matrices. The columns were easily prepared inside different tube housings (polypropylene and Silcosteel®) by either thermal- or photopolymerization. While the WCX SPE column retained lysozyme, all the other major proteins having isoelectric points < 7.0 were washed up to the waste by PBS at pH 7.0. Trifluoroacetic acid protonated the carboxylate groups of the WCX column to transfer lysozyme to the RPLC column to complete the separation. The straightforward online approach enabled automated quantitative analyte transfer from the SPE to the analytical column, thus requiring significantly lower sample amounts per analysis, minimizing errors associated with sample handling. The versatility of the monolithic columns, especially the wide variety of chemistries available to tailor their retention mechanisms has a vast field of application for online sample preparation involving complex matrices such as biological fluids, natural and processed food, and environmental samples besides proteomic research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2023.100069.

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