



Simultaneous separation of the four major allergens of hen egg white

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ABSTRACT

Hen egg is a worldwide top-consumed food that has attracted public health concerns because it can induce allergic reactions in sensitized individuals. Food allergy investigations need highly purified egg allergens. However, a limited number of purification methods have been described for the combined separation of more than two egg allergens and only few of them have evaluated the immunological activity of these purified proteins. The aim of this work was to develop a chromatographic method for the separation of the four major egg allergens (ovomucoid, ovalbumin, ovotransferrin, and lysozyme) with a demonstrated immunological activity. After a pre-processing step for ovomucin precipitation and pH adjustment, remaining egg white proteins were loaded onto CM-Sepharose column and major egg allergens were separated using cation-exchange chromatography. Yield of ovomucoid, ovalbumin, ovotransferrin, and lysozyme was 60.0%, 52.1%, 29.6%, and 90.2%, respectively. Purified allergens were compared with their commercial standards, showing a high purity as well as a maintained antigenicity. The protocol described in this work is simple, quick, low-cost, and suitable for the study of the immunological properties of these allergens. For higher ovalbumin demand in the lab, 2.1 g ovalbumin can be produced in a single process with high purity.

1. Introduction

Hen egg is a worldwide daily consumed food considered as an optimal source of protein, vitamins, and minerals. Egg-derived components are also highly used as ingredients in food industry based on their nutritional and functional properties [1]. However, egg proteins are able to induce allergic reactions in sensitized individuals, especially in children. Reported prevalence of egg allergy has increased in recent years, reaching from 1% to 2% of the general population [2–4]. In addition, egg allergy has been associated with an augmented risk of developing other food allergies and asthma [5,6]. Allergy to egg impacts on the normal growth during the childhood and deeply affects the quality of life of patients and their families.

Both egg white and yolk contained potentially allergenic proteins, and cross-reactivity has been reported between them [7]. However, egg white proteins have been described as the most important allergens of the egg. Although egg white contains more than 40 different proteins, ovomucoid, ovalbumin, ovotransferrin, and lysozyme have been recognized as the four major egg white allergens (Table 1).

The purified commercial formats of these four allergens have been largely used for the establishment of their allergenic properties. Nevertheless, some of the commercially available egg white proteins

are contaminated with endotoxin, which modifies the immunogenic characteristics of these allergens and hampers the study of their immunological properties [15].

To exclude the effect of endotoxin, some researchers re-purified the commercial egg allergens [16]. Alternatively, egg allergens can also be directly purified from hen egg white in the laboratory by using chromatographic method [17]. Most of the published studies in this field targeted at a single specific allergen and only few reports described the combined separation of more than two allergens of egg white. Vachier et al. purified lysozyme, ovotransferrin, and ovalbumin using a Q Sepharose Fast Flow (FF) column [18]. Shibusawa et al. separated the same egg white proteins by counter-current chromatography [19], and Dileep et al. used anion- and cation-exchange chromatography for the separation of these allergens [20]. Wang et al. separated ovomucoid, ovalbumin and ovotransferrin by free-flow isoelectric focusing recently [21]. In addition, a strategy based on anion-exchange chromatography has been reported for the purification of lysozyme, ovotransferrin, ovalbumin, and ovoflavoprotein from egg white [22]. On the other hand, a combined strategy based on precipitation steps followed by a cation-exchange chromatography has been applied for the separation of lysozyme, ovomucin, ovotransferrin, and ovalbumin [23]. The citations above could not separate all the four major allergens in hen egg white.

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Table 1
Molecular and biological properties of the four major egg allergens.

	% of total protein (w/w)	Molecular weight (kDa)	Isoelectric point (pI)	Biological function (s)	Reference
Ovomucoid (Gal d 1)	11	28	4.1	serine protease inhibition activity	[8,9]
Ovalbumin (Gal d 2)	54	45	4.5	Not clear	[10]
Ovotransferrin (Gal d 3)	12	77	6.5	iron binding, antimicrobial activity	[11,12]
Lysozyme (Gal d 4)	3.4	14.3	10.7	antibacterial activity	[13,14]

In this regard, Tankrathok et al. purified the four major egg allergens by using a combined chromatographic strategy divided in two sequential steps: firstly an anion-exchange chromatography (Q-Sepharose FF) followed by a cation-exchange chromatography (Carboxymethyl-Toyopearl 650 M) [24]. However, protein solution needed to be processed between the two steps and some of the allergens could be easily denatured during the separation processes, losing their immunological characteristics.

Since the separation of the four major egg allergens has never been performed in one single step, the aim of this study was to develop a simple, economical, and one-step chromatographic method to separate ovomucoid, ovalbumin, ovotransferrin and lysozyme with high purity and maintained immunological properties.

2. Materials and methods

2.1. Materials

Fresh hen eggs were from a local chicken farm. CM-Sepharose FF column was from GE Healthcare (Uppsala, Sweden). Ultrafiltration centrifugal tube was from Millipore (Darmstadt, Germany). Complete and incomplete Freund's adjuvant as well as ovomucoid, ovalbumin (grade V), ovotransferrin, and lysozyme standards were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of "egg white loading solution"

Undesired proteins, including ovomucin and globulins, were firstly removed from egg white according to Tong et al. [25]. Briefly, egg white, after separating from egg yolk and chalazae, was added to an equal volume of distilled water. The solution was adjusted to pH 6.0 with 2 M hydrochloric acid and then gently stirred at 4 °C for 3 h. The gelatinous precipitate was removed by centrifugation at 3000g for 10 min (4 °C) and the collected supernatant was adjusted to pH 3.8 with 2 M hydrochloric acid, gently stirred at 4 °C for 3 h, and then centrifuged as above. The resulting egg white protein solution was frozen at −20 °C for its further cation-exchange chromatography application.

2.3. Purification of the four allergens by CM-Sepharose FF chromatography

5 mL of the "egg white loading solution" was loaded onto a CM-Sepharose FF column (1.6 cm × 20 cm) previously equilibrated with 0.02 M acetate buffer (pH 4.0). The elution was kept at 1.5 mL/min. After the flow-through fraction, the bound fraction was eluted as following: (1) Acetate buffer (pH 4.3) containing gradient elution (0–0.2 M) of NaCl was used for the elution of ovomucoid and ovalbumin; (2) Acetate buffer (pH 5.5) containing gradient elution (0.1–0.2 M) of NaCl allowed the separation of ovotransferrin; (3) Finally, phosphate buffer (pH 7.4) containing gradient elution (0–0.4 M) of NaCl was used for the elution of ovotransferrin and lysozyme. Scheme of the elution pattern is presented in Fig. 1. Fractions were collected, concentrated with PEG 20,000, and centrifuged in ultrafiltration centrifugal tube to remove salts. The ultrafiltration centrifugal tube sizes were 30 kDa for ovalbumin and ovotransferrin, and 3 kDa for ovomucoid and lysozyme. Ovalbumin was directly concentrated by ultrafiltration without PEG. Desalted protein concentrates

were lyophilized and stored at −20 °C until further analysis.

2.4. Gram-scale purification of ovalbumin using CM-Sepharose FF chromatography

80 mL of the "egg white loading solution" was loaded onto a CM-Sepharose FF column (2.5 cm × 40 cm) previously equilibrated with 0.02 M acetate buffer (pH 4.0). The elution was kept at 2.5 mL/min. Separation of ovalbumin was achieved by washing with acetate buffer (pH 5.5). The collected fraction was filtered and preserved as indicated above.

2.5. Purity determination using SDS-PAGE

The fractions collected after elution as well as the concentrated samples and commercial standards prepared at 5 mg/mL were mixed at 3:1 (v/v) with a commercial sample buffer (Beyotime, Shanghai, China). Samples were heated at 95 °C for 10 min and 12 µL of each solution were loaded into the wells of a 12% SDS-PAGE gel. Electrophoretic separation was performed at 100 V. Finally, gel was stained with Coomassie R-250.

2.6. Yield analysis

The obtained yields of ovomucoid, ovalbumin ovotransferrin and lysozyme were calculated by comparing the amount of separated proteins after lyophilization with their proportion in egg white, 11%, 54%, 12%, and 3.4% respectively. Protein content in egg white was determined by the Kjeldahl method.

2.7. Endotoxin content quantification

The endotoxin level of the chromatographically purified proteins and their corresponding commercial standards was quantified by using the Bacteria ET ELISA Kit (Jiancheng Bioengineering institute, Nanjing, China), according to the instructions of the manufacturer.

2.8. Immunoreactivity analysis

Immunoreactivity of the purified allergens was compared with that of their commercial standards by using inhibition ELISA. Rabbit polyclonal antibodies were produced following an established methodology [26] with minor modifications. Eight -week-old New Zealand rabbits (permission number SCXK(Yu) 2017-0010) were immunized and boosted with 1 mg of the respective protein.

IgG-binding capacity was assessed by inhibition ELISA as reported [27]. Briefly, 96-well microtiter plate was pre-coated with the chromatographically purified allergens. The competitive antigens used in this assay were both purified and commercial proteins. The corresponding rabbit antiserum was used as primary antibody and goat anti-rabbit IgG as secondary antibody. Color was developed by OPD and read at 490 nm. IC50 was analyzed by SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) and statistical analyses were assessed using Student's *t*-test. $p < 0.05$ was considered statistically significant.

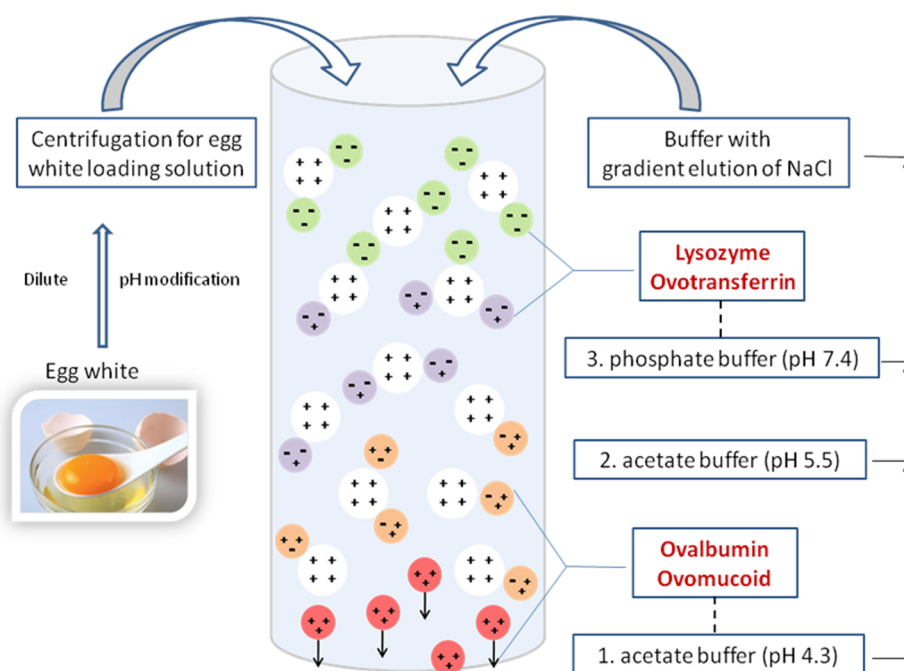


Fig. 1. A schematic representation of the cation-exchange chromatographic method developed.

3. Results and discussion

3.1. Cation-exchange chromatographic method for simultaneous purification of the four major egg allergens

The egg white loading solution was adjusted to pH 3.8, a pH value chosen based on the titration curve of CM-Sepharose FF column and on the isoelectric points (pIs) of the four targeted egg allergens. At this pH value, allergenic proteins of interest are positively charged and bound to the solid phase of the cation-exchange column after loading. The chromatographic media, previously equilibrated with acetate buffer (pH 4.0), was eluted with the same buffer after egg white solution loading for the obtention of flow-through fraction. The whole separation process used acetate buffer (pH 4.3) containing gradient elution of NaCl (0–0.2 M), acetate buffer (pH 5.5) containing gradient elution of NaCl (0.1–0.2 M) and phosphate buffer (pH 7.4) containing gradient elution of NaCl (0–0.4 M). The obtained chromatogram using this gradient showed that targeted proteins were eluted at retention times of 75, 139, 280, 344, and 441 min (Fig. 2a). Elution order of the four allergens was in concordance with their specific pIs. Indeed, SDS-PAGE analysis of the collected fractions revealed that ovomucoid was eluted in the second fraction (F2, collected from 60 to 88 min), ovalbumin in F3 (collected from 108 to 204 min), ovotransferrin in F4 (collected from 264 to 296 min) and F5 (collected from 336 to 360 min), and lysozyme in F6 (collected from 412 to 452 min), as shown in Fig. 2b. For the collection of optimal pure ovotransferrin, F5 was selected because F4 resulted in ovotransferrin contaminated with other proteins. Purified ovomucoid was eluted in F2. The diffuse band observed on SDS-PAGE (lane 2 of Fig. 2b) corresponds to ovomucoid fraction due to its high glycosylation rate [9].

3.2. Purity of collected allergens compared with their commercial standards

We compared the purity of the chromatographically purified and lyophilized allergens with their corresponding commercial standards (Fig. 3, lanes 1–8). The analysis of the isolated proteins not only revealed that obtained ovomucoid and lysozyme reached a similar purity with their commercial standards, but also that ovotransferrin and ovalbumin showed an enriched purity pattern compared to their

commercial standards. Additionally, lyophilized ovalbumin (Lane 8 in Fig. 3) also showed a higher purity than the collected fraction (Lane 3 in Fig. 2) probably due to ultrafiltration performed before lyophilization of the sample.

3.3. Yield and endotoxin content of the purified allergens

The yield of the four allergens was calculated by comparing weight of purified protein after lyophilization to its proportion in the whole egg white used as protein source. Based on the Kjeldahl method, 11.38% of whole egg white (including water) was considered protein. Table 2 shows the yields of the four allergens purified in this protocol compared with previously reported methods that separated more than two egg proteins. Yields of ovomucoid and lysozyme was much higher than the combined chromatographic method (21% and 55%, respectively), while the yields obtained for ovalbumin and ovotransferrin showed similar values (21% and 54%, respectively) [24]. Although some studies have reported a higher yield for individual egg allergen, our chromatographic method has shown better recovery results for the simultaneous separation of several egg proteins using a single protocol.

Regarding the endotoxin content, the values obtained for the four purified allergens were below the detection limit of the commercial kit used, while for commercial ovalbumin the content is about 400 EU/mg.

3.4. Immunoreactivity of the chromatographically purified allergens

After immunization with egg allergens, specific titer levels found in rabbit sera were higher than 100,000. Immunoreactivity of the purified allergens was assessed by inhibition ELISA and obtained values were compared with their commercial standards (Table 3). Results showed that chromatographically obtained ovomucoid, ovotransferrin, and lysozyme had similar IC50 values than the corresponding commercial standards used, indicating a parallel antigenicity. However, purified ovalbumin showed a significantly lower IC50 value than its commercial standard ($p < 0.01$). Thus, ovalbumin obtained by our developed chromatographic method is likely to exert a higher immunogenic effect than its commercial standard.

Ovomucoid has been considered as the immune-dominant allergen of hen's egg white, and a major obstacle to produce hypoallergenic egg-

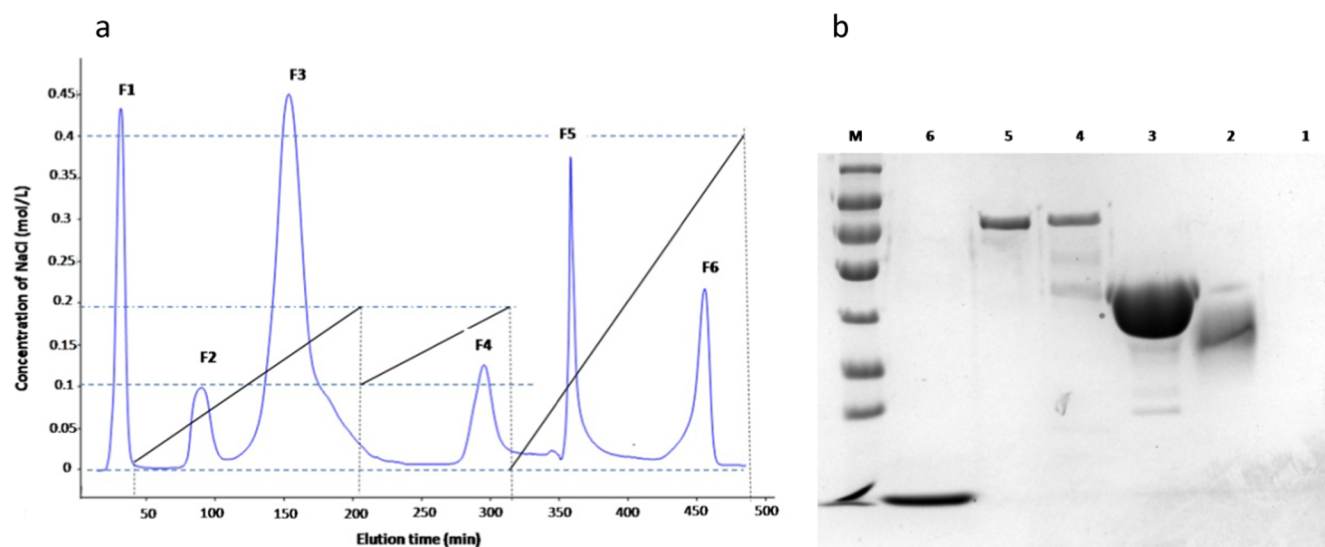


Fig. 2. Chromatographic pattern of the four major egg allergens. (a) Elution profile of the cation-exchange chromatographic method and (b) SDS-PAGE analysis of fraction collected. F1-F6 in (a) correspond to lane1-6 in (b).

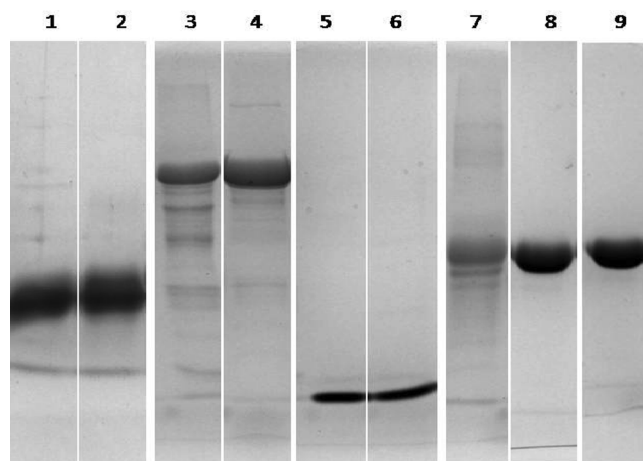


Fig. 3. Purity of the four major egg allergens obtained by the chromatographic method developed and their commercial standards (45 µg of protein loaded per well). (1) standard ovomucoid; (2) purified ovomucoid; (3) standard ovotransferrin; (4) purified ovotransferrin; (5) standard lysozyme; (6) purified lysozyme; (7) standard ovalbumin; (8) purified ovalbumin; (9) purified ovalbumin by the gram-scale separation step.

derived products due to its heat and digestive stability. Several efforts have been made to reduce its allergenicity, although the purification of intact ovomucoid is usually needed for this aim. The most common method applied to obtain purified ovomucoid is precipitation using organic chemical [28,29]. However, this is the first time that immunogenic activity of purified ovomucoid is established. In fact,

Table 2

Yields of the four major allergens purified in this work compared with previously reported chromatographic methods.

Method	Yield (%)				Reference
	Ovomucoid	Ovalbumin	Ovotransferrin	Lysozyme	
CM-Sepharose FF chromatography	60.0	52.1	29.6	90.2	This work
Q Sepharose FF chromatography	–	83	100	60	[18]
Q Sepharose FF and SP Sepharose FF chromatography	–	44.63	17.48	2.71	[20]
PEG precipitation followed by Q Sepharose FF chromatography steps	–	88.64	77.75	30.1	[22]
Amberlite FPC 3500 ion exchange resin and ammonium sulfate and citric acid combinations	–	98.54	83.39	89.72	[23]
Q Sepharose FF and CM-Toyopearl 650 M chromatography	21	54	21	55	[24]

Table 3

IC₅₀ values of the chromatographically purified allergens compared with their commercial standards.

	Purified	Commercial standard
Ovomucoid	3.23 ± 0.25	3.61 ± 0.86
Ovalbumin	1.24 ± 0.09	2.63 ± 0.30*
Ovotransferrin	2.22 ± 0.24	2.84 ± 0.80
Lysozyme	0.42 ± 0.01	0.58 ± 0.19

* $P < 0.01$.

Abeyrathne et al. [28] tried to test its activity, but no antibody for this protein was available. In this study, this challenge has been solved by the production of anti-ovomucoid sera, and results showed that ovomucoid purified following our cation-exchange chromatography protocol exhibited a similar immunological activity than its commercial standard.

3.5. Gram-scale separation of ovalbumin using cation-exchange chromatography

Ovalbumin has been largely used in experimental models of allergy [30–33], being usually consumed in gram-scale for studies *in vivo*. In order to obtain large amounts of ovalbumin from egg white, we decided to use our optimized chromatographic method to purify in a bigger quantity of this protein, using limited experimental apparatus in the lab. 80 mL of egg white solution were loaded onto the CM-Sepharose FF column and we obtained 2.1 g of purified ovalbumin in a single purification process. The elution curve as well as the SDS-PAGE result is shown in Fig. 4. The purity of gram-scaled ovalbumin obtained using

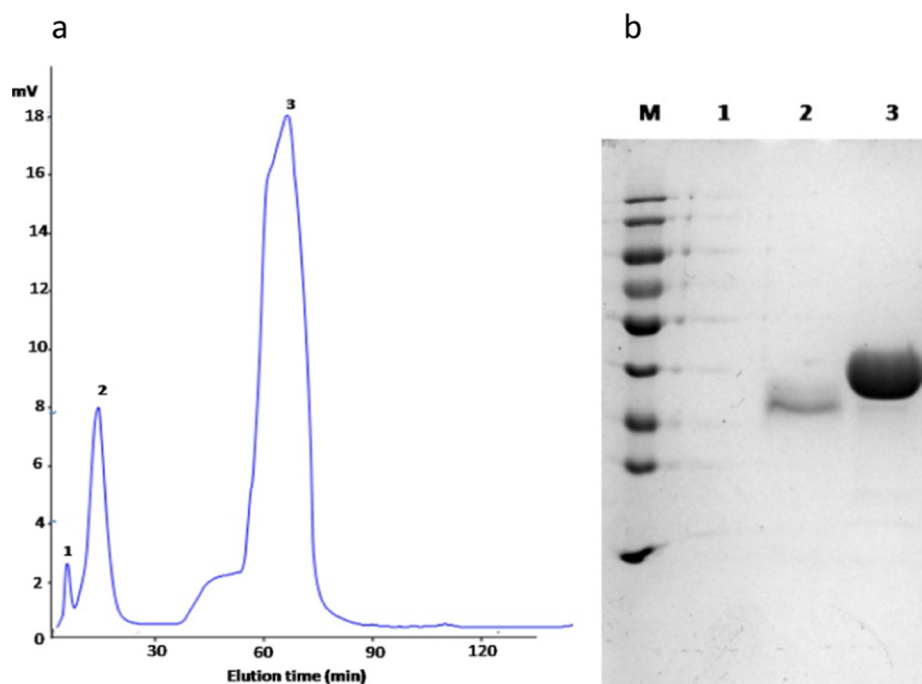


Fig. 4. Chromatographic pattern of the ovalbumin gram-scale purification. (a) Elution profile of the cation-exchange chromatographic method and (b) SDS-PAGE analysis of fraction collected. Fractions 1–3 in (a) correspond to lane 1–3 in (b).

this system was also higher than commercial ovalbumin (Fig. 3, lanes 7 and 9).

4. Conclusion

Simultaneous separation of ovomucoid, ovalbumin, ovotransferrin, and lysozyme was achieved by using a CM-Sepharose FF chromatography. After a process including egg white pre-treatment, chromatographic separation, fraction concentration, desalting, and lyophilization, all the four egg allergens showed high purity and immunoreactivity, comparing with their corresponding commercial standards. This paper provides an easy and low-cost method to get the four egg allergens for laboratory usage, with high purity and immunological activity.

CCRediT authorship contribution statement

Xiaojuan Ma: Conceptualization, Writing - original draft. : **Rui Liang:** Investigation. **Xiaotong Yang:** Investigation. **Jingkun Gou:** Formal analysis. **Yan Li:** Supervision. **Daniel Lozano-Ojalvo:** Writing - review & editing.

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jchromb.2020.122231>.

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