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# Hen egg white fractionation by ion-exchange chromatography

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**Abstract**

Major hen egg white proteins have been widely studied for their functional properties but these studies still are unable to explain, alone, all of the biological properties of hen egg white. Hence, it is still interesting to produce pure and non-altered proteins to improve our knowledge on the biological properties of hen egg white. Presently, identification and characterization of both bioactive peptides and minor proteins from hen egg white is essential work for progressing in the understanding of hen egg white biological properties. With this objective in mind, a new process for a complete "mucin free" hen egg white fractionation based on ion exchange chromatography is proposed. "Mucin free" egg white is fractionated into six different fractions. Four of them are high-recovery yield purified fractions of lysozyme, ovotransferrin, ovalbumin and flavoprotein. The two other fractions are enriched in recently detected minor proteins in hen egg white.


*Keywords:* Hen egg white fractionation; Ion exchange chromatography; Electrophoresis; Reverse-phase chromatography; LC–MS–MS; Ovalbumin; Ovotransferrin; Lysozyme; Flavoprotein; Ovalbumin gene X; Ovalbumin gene Y; Ovoglycoprotein; Ovomucoid

## 1. Introduction

Hen egg white represents an essential ingredient, which has been used for many years by the food industry because of its excellent technological properties. Moreover, hen egg white possesses many biologically active proteins that could offer a better valorisation for hen egg white: lysozyme as anti-microbial, antiviral, antiphlogistic and antalgic agent [1–4], ovotransferrin as an anti-microbial agent [4–6], avidin as a vitamin carrier and antimicrobial agent [7], flavoprotein as a vitamin stabilizer, ovokinin from ovalbumin as an anti-hypertensive agent [8,9] and ovomucin as a source of glycopeptides with antiviral activities, anti-tumor and immunomodulating effects [10–13].

Many procedures for purifying these proteins were developed to study, and in some case to use, the biological activity of hen egg white proteins. Purifications were mostly performed on liquid chromatography because of the absence of protein denaturation and its high selectivity. Presently, lysozyme (3.5% of egg white proteins) and avidin (0.05%) are the main egg white proteins extracted for commercial applications; lysozyme is extracted on an industrial scale by a combination of chromatography and salting out precipitation techniques [14,15] whereas avidin is purified by affinity chromatography [16]. With a pI of 4.5, ovalbumin (54% of egg white proteins) was mainly purified by anion exchange chromatography [17–23]. Ovotransferrin (13%) has been purified either by cation exchange chromatography [24,25] or anion exchange chromatography [26–30]. Ovomucin has been precipitated at low ionic strength and acidic pH. Its purity rate was increased through different water or salt washings [31,32]. Moreover, some authors proposed to hydrolyse ovomucin to increase its solubility [33–37]. Flavoprotein was isolated by different methods involving several separation steps such as salt precipitation, anion exchange chromatography and gel filtration chromatography [38–40].

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A single step procedure based on anion exchange chromatography was also proposed [41].

In spite of having a wide spectrum of action, these proteins are unable to explain, alone, all the biological activities of hen egg white. Identification and characterization of both bioactive peptides from main egg white proteins and minor egg white proteins is an important research axis to understand the biological properties of hen egg white. From a practical point of view, minor egg white protein identification needs the clarification of egg white from its quantitatively major egg white proteins, i.e. ovalbumin and ovotransferrin. Moreover, the characterization of their biological activity needs the extraction of significant quantities of unaltered proteins. The aim of this study was to develop an easy procedure for fractionating the whole egg white for further biological activity studies. Using three successive steps on ion exchange chromatography, egg white was fractionated into six fractions. Four of them are high-recovery yield purified fractions of the well-known egg white proteins: ovalbumin, ovotransferrin, lysozyme and flavoprotein. They represent well-characterized fractions for bioactive peptide identification from major hen egg white. The two other fractions are enriched out in recently detected minor proteins in hen egg white.

## 2. Experiments

### 2.1. Preparation of mucin-free egg white solution

Hen eggs were purchased from a local market (10-day-old refrigerated eggs). Albumen from each egg was manually separated from the yolk and pooled. One hundred and ninety milliliters of egg white was diluted with 570 mL of distilled water and the mixture was adjusted to pH 6 with HCl 1 M. The solution was stirred overnight at 2 °C enabling ovomucin precipitation. The mixture developed a white, gelatinous precipitate, which was removed by 5 min centrifugation at $3000 \times g$ (4 °C). Prior to cation exchange chromatography, the mixture was adjusted to pH 8 with NaOH 1 M, and then centrifuged at $24{,}000 \times g$ for 30 min at 4 °C in order to remove insoluble material. The supernatant, called "mucin-free" egg white ("mucin-free" EW) was used in the following steps. "Mucin-free" EW protein concentration was 21 g $L^{-1}$.

### 2.2. Preparative ion exchange chromatography

Preparative chromatographies were performed with 100 mL (5 cm × 5 cm i.d.) of S Ceramic Hyper DF (cation exchanger from Biosepra, Cergy Saint-Christophe, France) and 250 mL (12.7 cm × 5 cm i.d.) of Q Sepharose Fast Flow (anion exchanger from Amersham Biosciences, Uppsala, Sweden). Resins were packed into two XK 50/20 columns Pharmacia Biotech AB (Saclay, France). The columns were connected successively to the same Biopilot TM system (Pharmacia Biotech AB) equipped with 280 nm UV, conductivity and pH detectors. Lysozyme and ovotransferrin were extracted in two successive steps by cation exchange chromatography on S Hyper DF. The co-product was then used as starting material for anion exchange chromatography. A fraction ($F_A$), ovalbumin fraction, B fraction ($F_B$) and flavoprotein fraction were extracted by single step anion exchange chromatography on Q Sepharose FF. The complete "mucin-free" EW fractionation procedure is described in Fig. 1. All egg white protein fractions were desalted by dialysis against deionised water, freeze-dried and lyophilised.

### 2.3. Nitrogen determination

Protein quantities were determined using the Kjeldahl method. The conversion coefficient was 6.35.

### 2.4. Analytical chromatography

Reverse-phase (RP) chromatography was performed on HPLC Spectra Physics (Series P200) on a Vydac C4 214 TP (5 cm × 0.21 cm i.d., particle size 5 μm) column (Touzart et Matignon, Vitry $^{s}$/Seine, France). HPLC-grade acetonitrile (ACN) (Carlo Erba, Nanterre, France) containing 0.025% trifluoroacetic acid was used as the eluent under gradient elution conditions. The linear gradient elution increased from 7 to 70% ACN in 17 min, at a flow-rate of 0.8 mL $min^{-1}$, at room temperature. Detection was carried out at 280 nm with a UV–vis detector (Spectra Physics UV 100). The chromatograms were processed with Azur V2.0 software (Datalys, France).

### 2.5. Polyacrylamide gel electrophoresis

#### 2.5.1. SDS–PAGE

SDS polyacrylamide gel electrophoresis was conducted using 12.5% acrylamide separating gel and 4% stacking gel containing 0.1% SDS, with a Biorad Mini Protean II system [42]. SDS-protein samples were heated at 95 °C for 3 min. Electrophoresis was carried out at 75 V in stacking gel and 150 V in separating gel for 1 h30 using an electrophoretic buffer of Tris-Glycine containing 0.1% SDS. The gel was stained with 0.05% Coomassie Blue R250, 49.95% water, 40% ethanol and 10% acetic acid for 1 h and subsequently destained with 50% water, 40% ethanol and 10% acetic acid.

#### 2.5.2. Isoelectric focusing

Conventional isoelectric focusing (IEF) in ampholyte carrier buffers was performed using 7.5% acrylamide, 10% glycerol, and 3% ampholytes. Samples were diluted in 50% glycerol, 2% ampholyte and 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS). The cathode solution contained 20 mM lysine/20 mM arginine and the anode solution was 10 mM $H_3PO_4$. Migration was performed using a Biorad Mini Protean II system and running conditions were 1 h at 100 V, 1 h at 250 V and 30 min at 500 V.

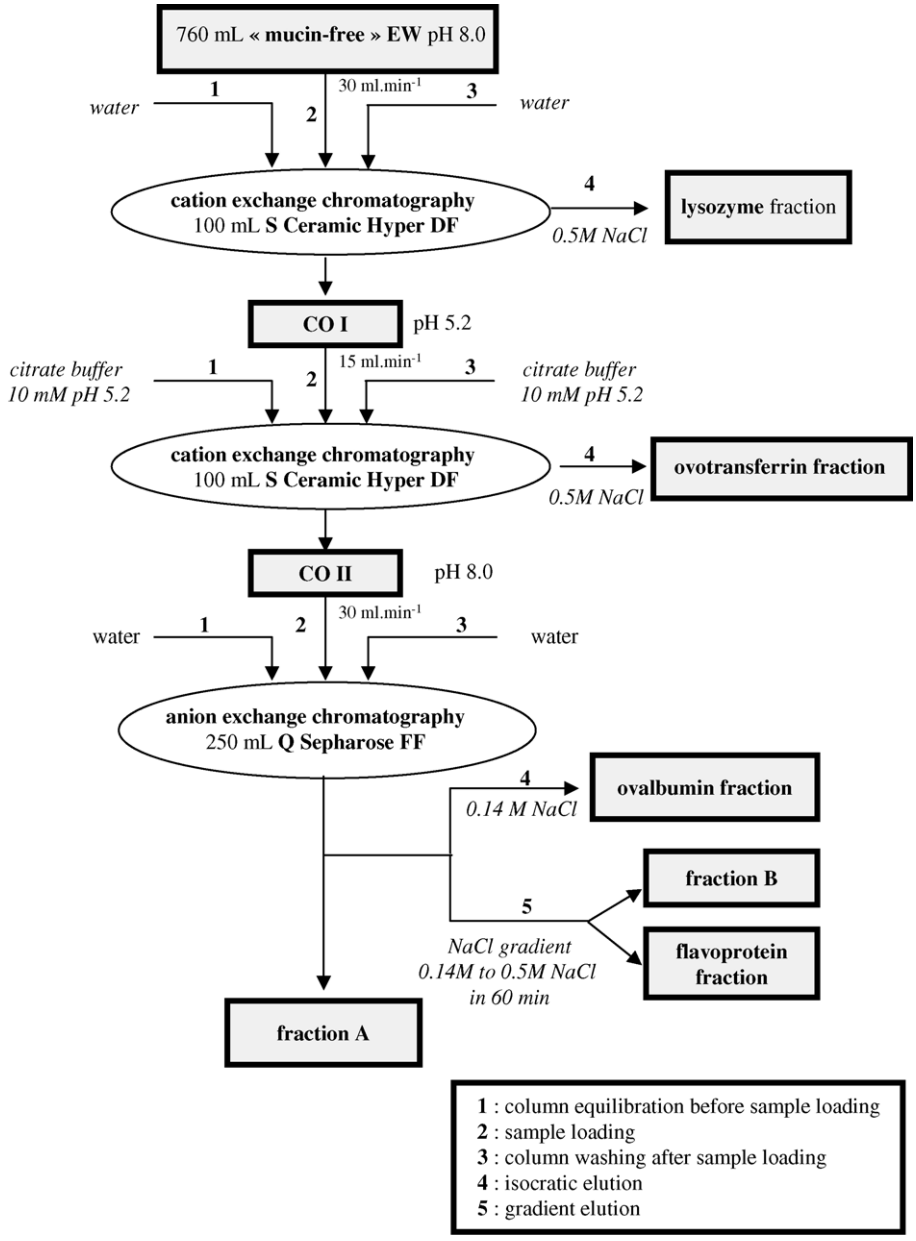


Fig. 1. Hen egg white fractionation process based on ion exchange chromatography. "mucin-free" EW was "mucin-free" egg white protein; COI and COII, were co-product I and II, respectively.

### 2.6. Peptide mass analysis

To progress in fraction characterization, SDS bands or RP–HPLC peaks (Vydac C4 214 TP) were analysed by mass spectrometry after enzymatic digestion.

#### 2.6.1. Protein enzymatic digestion

*SDS–PAGE bands.* Gel pieces were excised from the gel and washed with acetonitrile, $NH_4HCO_3$, acetonitrile and dried in a Speed-vac. Before enzymatic digestion, reduction and alkylation were achieved with DTT and iodoacetamide, respectively. The gel pieces were washed with ultra pure water, acetonitrile, $NH_4HCO_3$ and dried in a Speed-vac. Each sample was hydrolysed with trypsin overnight at 37 °C. The resulting peptides were extracted from the supernatant after successive washing steps of the gel pieces with $NH_4HCO_3$, acetonitrile, and finally acetonitrile/HCOOH. The supernatants containing peptides were then dried in a Speed-vac and maintained at −20 °C until mass spectrometry analysis (MALDI-TOF).

*RP–HPLC peaks.* 1 ml of $F_A$ and $F_B$ fractions (1 mg/mL) were injected on the C4 column VYDAC (RP–HPLC) and uncharacterised peaks were collected and concentrated in a Speed-vac. Before enzymatic digestion, reduction and alkylation were achieved with DTT and iodoacetamide, respectively. Samples were dried in a Speed-vac and hydrolysed

overnight with trypsin solution at 37 °C. Once hydrolysed, samples were frozen at −20 °C until mass spectrometry analysis (LC–MS–MS).

*2.6.2. Mass spectrometry analysis*

*MALDI-TOF analysis.* The tryptic digests resulting from SDS–PAGE separation were analysed by MALDI-TOF mass spectrometry on a Voyager DE STR spectrometer (Applied Biosystems, Framingham, CA) equipped with a nitrogen laser (337 nm, 20 Hz). Spectra were acquired in the reflector mode (positive mode) with a 130 ns extraction delay. An external calibration was firstly performed in the range 900–3000 Da. An internal calibration was then performed by using trypsin peptides.

*LC–MS–MS analysis.* The tryptic digests resulting from uncharacterized RP–HPLC peaks were analysed by liquid chromatography/electrospray ionisation mass spectrometry. Peptide separation was performed with a home made column (180 μm i.d. × 70 mm) packed with reverse phase Symmetry C18 resin (Waters, Milliford, MA). Peptide molecular masses were obtained using an API-III Plus triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada), fitted with an atmospheric-pressure ionization source. Ion detection was performed in positive mode and mass calculation with Biomultiview 1.3.1 (Sciex software package) from scan mass to charge *m/z*.

*2.6.3. Protein databank interrogation*

Protein identification was performed by interrogating the Swiss-Prot and Trembl protein databanks available on Expasy (http://us.expasy.org/). Mascot Search (http://www.matrixscience.com) software was employed for peptide sequencing and peptide mass fingerprinting data analysis. The average mass lists or monoisotopic mass lists were used for LC–MS–MS and MALDI-TOF analysis, respectively. The mass accuracy was lower than 0.5 ppm. The carbamidomethylation of cysteins, methionine oxidation and one missed cleavage were considered during the interrogation.

## 3. Results and discussion

### *3.1. Optimisation of fractionation procedure*

Ion exchange chromatography has been widely used at laboratory scale for egg white protein extraction because of the low resulting denaturation and the release of a non-altered by-product; in addition, ion exchange chromatography is easily scaled up to an industrial level. Presently, in procedures described in the literature, most of the major egg white proteins are obtained by ion exchange chromatography with high purity as the main objective for biological, biochemical or biophysical studies. Moreover, most of these procedures are developed for only one-target protein. Unlike many of the previous studies, we fractionated a "mucin-free" EW into homogeneous, well-characterized fractions with the aim of doing further biological activity analysis.

"Mucin-free" EW was used as starting material for ion exchange chromatography fractionation because of the ability of ovomucin to precipitate into the column during fractionation steps. Moreover, ovomucin fractionation, with further purifications by successive water or KCl washings and gel permeation chromatography in order to remove co-precipitated proteins such as lysozyme and ovalbumin, has been widely studied [31,32,35–37]. The procedure we used did not give any new insight: ovomucin was removed from egg white with a 1/3 (v/v) egg white/water dilution together with a pH reduction to 6.0 in order to enable ovomucin precipitation. After dilution and ovomucin removing, the "mucin-free" EW protein content was 2.1%. This latter was fractionated by ion exchange chromatography taking into account the physicochemical characteristics of egg white proteins [43]. An alkaline fraction containing lysozyme ("lysozyme fraction") and a neutral fraction containing ovotransferrin ("ovotransferrin fraction") were obtained by cation exchange chromatography while four acidic fractions ("A fraction", "ovalbumin fraction", "B fraction" and "flavoprotein fraction") were obtained from the anion exchange chromatography (Fig. 1).

*3.1.1. Cation exchange chromatography (CEC)*

"Mucin-free" EW (760 mL) adjusted to pH 8.0 was loaded onto the column containing 100 mL of cationic exchanger. The "mucin-free" EW chromatographic profile on RP–HPLC is given in Fig. 2. At pH 8, lysozyme and avidin are the only proteins among egg white proteins that are positively charged (pI of 10.7 and 10, respectively; [43]) and able to bind to the cationic exchanger. Other egg white proteins were not retained by the cationic exchanger and were recovered as coproduct I (COI, Fig. 1). COI RP–HPLC profile (Fig. 2) clearly shows the complete and specific retention of lysozyme onto the column. The "lysozyme fraction" that probably also contains avidin, which accounts for roughly 0.05% of egg white proteins, was obtained with an isocratic elution at 0.5 M NaCl. A further separation of lysozyme and avidin from "lysozyme fraction" could be obtained by the direct crystallisation of lysozyme at pH 9.5 in the presence of 5% sodium chloride [14]. This additional step was not performed in this study.

In COI, the egg white proteins, except ovotransferrin (pI 6.5), have a pI which is lower than 5.7 [43]. The "ovotransferrin fraction" was extracted from COI at pH 5.2 with the same cationic column as for the "lysozyme fraction" preparation. Proteins that were not retained by the cationic exchanger were eluted as coproduct II (COII). The recovery yield of ovotransferrin was estimated at 78% by COII RP–HPLC analysis (Fig. 2). The "ovotransferrin fraction" elution from the column was performed with an isocratic elution at 0.5 M NaCl.

pH values ranging from 5.0 to 6.2 were also tested for ovotransferrin fractionation (data not shown). However, when COI pH was adjusted to lower than 5.2, the ovotransferrin

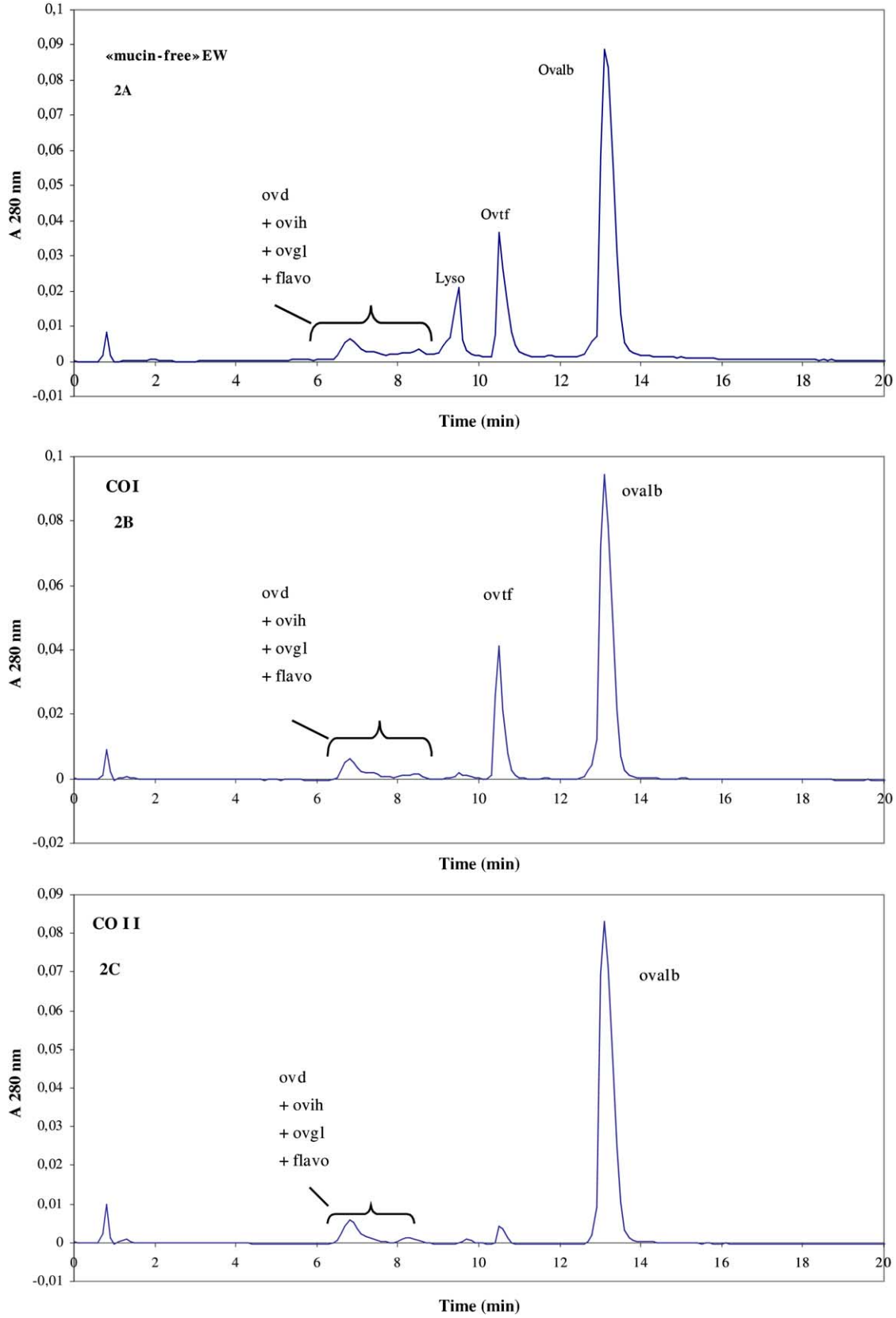


Fig. 2. Reverse-phase chromatography on Vydac C4 214 TP (5 cm × 0.21 cm i.d.) of "mucin-free" EW, COI and COII produced by extraction procedure presented in Fig. 1:A "mucin-free" EW; B, co-product I (COI); C, co-product II (COII). Lyso: lysozyme; Flavo: flavoprotein; Ovalb: ovalbumin; Ovd: ovomucoid; Ovgl: ovoglobulines; Ovih: ovoinhibitor; Ovtf: ovotransferrin.

purity rate decreased because of an increased contamination by more acidic proteins that could bind to the exchanger together with ovotransferrin. When the pH of COI was higher than 5.2, ovotransferrin purity rate increased but the recovery yield decreased because of the decreased gap between pH and ovotransferrin pI. Moreover, COII was contaminated by an increased quantity of ovotransferrin.

A one-step chromatographic procedure on cationic exchanger for lysozyme and ovotransferrin fractionation from a "mucin-free" EW was also tested at pH 5.2. The volume of exchanger used in this study (100 mL) enabled the retention of lysozyme while ovotransferrin started to be largely eluted from the column when more than 500 mL of "mucin-free" EW were loaded onto the column. The Fig. 3 shows the elution of ovotransferrin according to the volume of "mucin-free" EW loaded onto the column. Ovalbumin which is not retained on the cationic exchanger was used as internal standard. In "mucin-free" EW the ratio of ovotransferrin to ovalbumin chromatographic peak area is 0.3. It may be possible that the large excess of positive charges on lysozyme surface at pH 5.2 could drive out from the column the proteins with lower affinity for the exchanger, such as ovotransferrin. However many other factors drive a column-based separation such as bed height, fluid linear velocity and differences between the diffusion coefficients of two proteins in the mobile and stationary phases. In the following, lysozyme and ovotransferrin fractionations were realised in two successive steps using the same cationic exchanger.

### 3.1.2. Anion exchange chromatography

For anion exchange chromatography, COII was adjusted to pH 8.0. At this pH value, egg proteins in COII are negatively charged and should bind to the anionic exchanger since ovotransferrin and proteins with higher pI were already removed. The volume of anionic exchanger used to fractionate the acidic proteins of egg white was determined experimentally in order to have a saturation of the exchanger-binding sites

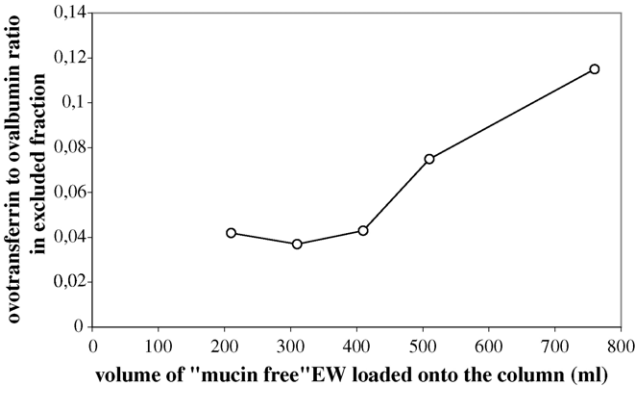


Fig. 3. Elution of ovotransferrin versus volume of "mucin-free" EW loaded onto 100 mL S Ceramic Hyper DF. In this scheme, lysozyme and ovotransferrin are extracted in one step by cationic chromatographic procedure. Ovalbumin, which is not retained by the column, was used as internal standard.

with ovalbumin and proteins with higher affinity for anionic exchanger contained in COII [23]. Hence, during loading, all proteins were bound to the column until the dynamic resin capacity in our conditions of fractionation was reached. Then, ovalbumin and the more acidic proteins displaced proteins with a lower affinity for the resin (pI higher than that of ovalbumin). The fraction eluted from the column during COII saturation loading, called "A fraction", was constituted of proteins with an affinity for ion exchangers between ovalbumin and ovotransferrin. The characterization of this fraction will be presented below.

A NaCl gradient was used to elute tightly bound proteins from the column. Firstly, the "ovalbumin fraction" was obtained with an isocratic elution at 0.14 M NaCl as previously described [23]. Ovalbumin was recovered until the absorbance at 280 nm reached the baseline. Then, a linear NaCl gradient from 0.14 M to 0.5 M was applied to the column. It enabled the elution of remaining proteins bound to the anionic exchanger into two well-defined chromatographic peaks that were recovered separately. The first peak corresponded to the "B fraction" and will be characterized in the next section, while the other one was the "flavoprotein fraction".

## 3.2. Characterization of protein fractions

An extraction procedure was performed using 760 ml of "mucin-free" EW, which represented 16 g of proteins. After ion exchange chromatography, dialysis and lyophilisation, 13 g of proteins were recovered which represented a global recovery yield of 82% (Table 1).

The six protein fractions obtained from the semi-preparative chromatographies ("lysozyme fraction", "ovotransferrin fraction", "A fraction", "ovalbumin fraction", "B fraction" and "flavoprotein fraction") were analysed by both RP–HPLC (Fig. 4) and SDS–PAGE (Fig. 5). In some cases, IEF electrophoresis or mass spectrometry was necessary for an unambiguous characterization of the fraction content.

### 3.2.1. Major protein fractions: lysozyme, ovostransferrin, ovalbumin and flavoprotein fractions

In accordance with the litterature [44], lysozyme, ovotransferrin and ovalbumin were easily detected in "mucin-free" EW by RP–HPLC (Fig. 2). On the other hand, this method did not enable the separation of ovomucoid, ovoinhibitor, ovoglobulins and flavoprotein. These proteins were detected as small badly resolved peaks before lysozyme chromatographic peak.

Lysozyme, ovalbumin and flavoprotein fractions appeared like homogeneous fractions by RP–HPLC (Fig. 4A, D and F, respectively) and by SDS–PAGE (Fig. 5). Purity was calculated from the integrated chromatograms and was estimated at 95, 91 and 100%, respectively (Table 1). The recovery yields were estimated by calculating the ratio (lyophilised protein weight/theoretical protein amount in EW) × 100. Recovery yields of 100, 80 and 50% were obtained for

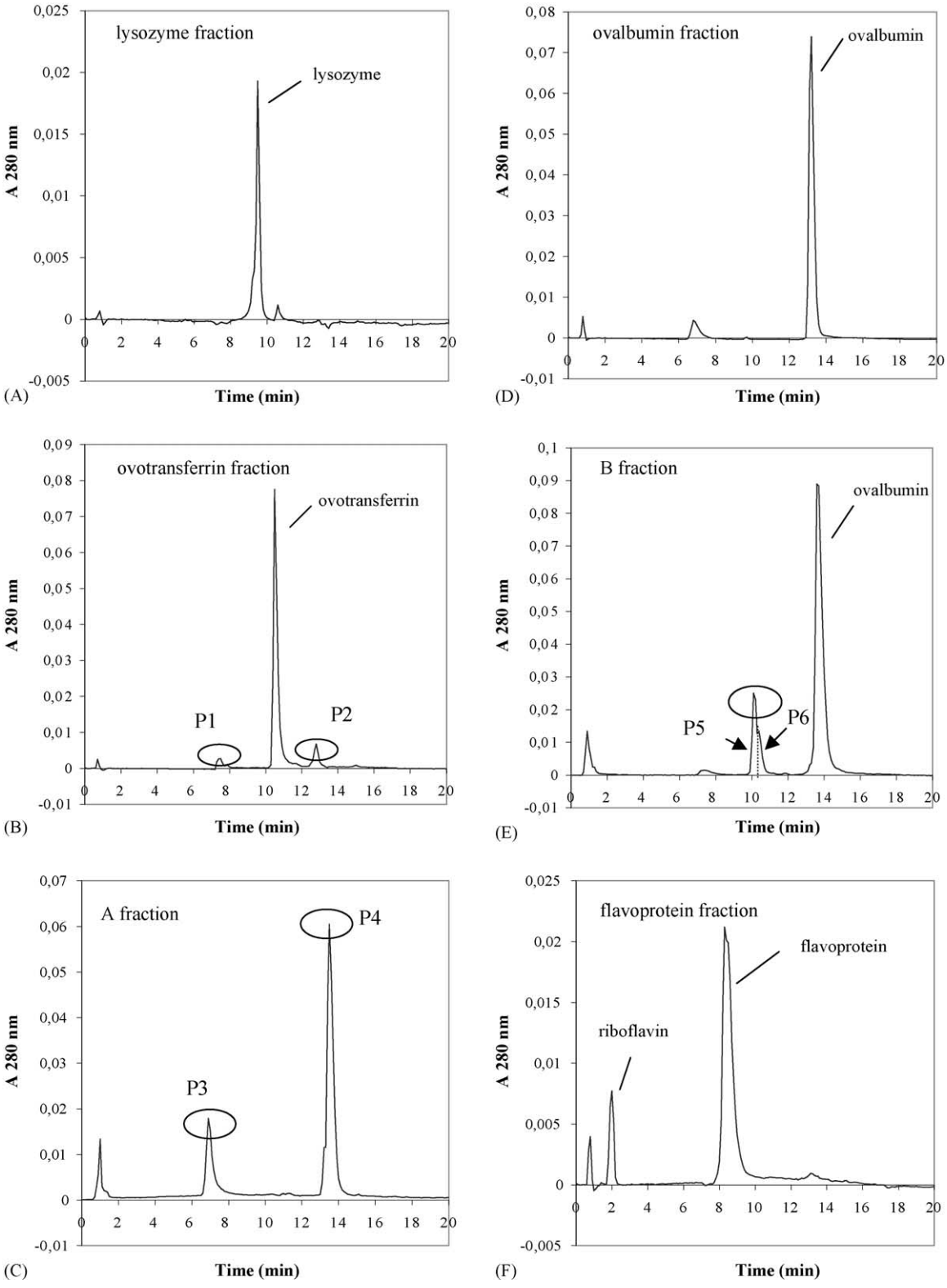


Fig. 4. Reverse-phase chromatography on Vydac C4 214 TP (5 cm × 0.21 cm i.d.) of the protein fractions produced by extraction procedure presented in Fig. 1;A, lysozyme fraction; B, ovotransferrin fraction; C, "A fraction", $F_A$; D, ovalbumin fraction; E, "B fraction" $F_B$; F, flavoprotein fraction.

Table 1
Report of global extraction procedure from 760 ml of "mucin-free" EW (or 16 g of total protein)

| Fraction | Weight fraction (dry matter, g) | Theoretical quantity (g) | Composition and purity rate | Recovered yield |
|---|---|---|---|---|
| Lysozyme fraction | 0.70 | 3.5% (0.56) | Lysozyme 95% | 100% |
| Ovotransferrin fraction | 1.83 | 13% (2.08) | Ovotransferrin 89%<br>Ovoinhibitor<br>Ovalbumin X | 78% |
| A fraction | 2.38 | 11% (1.76) | Ovomucoïd<br>Ovalbumin Y<br>Ovalbumin | |
| Ovalbumin fraction | 7.50 | 54% (8.64) | Ovalbumin 91% | 80% |
| B fraction | 0.63 | – | Ovalbumin<br>Ovoglycoprotein<br>– | |
| Flavoprotein fraction | 0.04 | 0.5% (0.08) | Flavoprotein 100% | 50% |
| Total protein recovered | ≈13.00 | | – | |
| Global recovered yield | ≈82% | | – | |

Purity rates were automatically calculated from the integrated chromatograms ((protein peak area/total peak area) × 100). Recovery yields were calculated by the ratio: (lyophilisated protein fraction weight × purity rate)/theoretical protein amount in EW × 100.

lysozyme, ovalbumin and flavoprotein, respectively. Flavoprotein recovery yield was low (50%) but could be explained by the small quantity of "mucin-free" EW applied to the column. The loss of a few mg of proteins in the different steps leading to flavoprotein, including dialysis or lyophilisation steps, could explain the low flavoprotein recovery yield. Actually, during other different flavoprotein extraction assays, using larger quantities of "mucin-free" EW as starting material, better flavoprotein recovery yields were obtained (>85%).

RP–HPLC of "ovotransferrin fraction" revealed one major ovotransferrin peak that constituted 89% of the fraction protein content and 2 smaller peaks at retention time 7.5 min (P1) and 12.8 min (P2) (Fig. 4B). The SDS–PAGE pattern presented an intense band at the molecular weight (77.7 kDa)

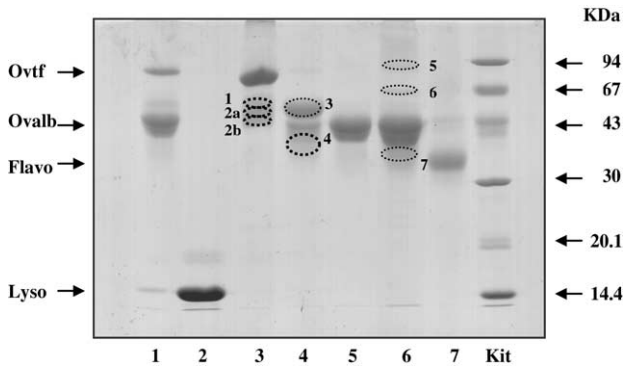


Fig. 5. SDS–PAGE analysis of protein fractions on 12.5% acrylamide gel stained with Coomassie Blue R250. Ten to thirty-five micrograms of lyophilised fraction protein were loaded onto gel (lane 1, "mucin-free" EW; lane 2, lysozyme fraction; lane 3, ovotransferrin fraction; lane 4, "A fraction", $F_A$, lane 5, ovalbumin fraction; lane 6, "B fraction", $F_B$; lane 7, flavoprotein fraction.

corresponding to ovotransferrin, as previously described [45] and three other weak bands B1, B2a, B2b (Fig. 5) were impossible to identify by simple comparison with already known EW protein electrophoretic mobilities. So components corresponding to these bands were submitted to peptide mass fingerprinting in order to progress in EW protein characterization.

B1 was identified as ovoinhibitor (9 matching peptides, sequence coverage 11%, MOWSE Score 2.72e + 004). Ovoinhibitor, which represents only 0.5–1.5% of albumen protein, has an apparent pI of 5.1 [43]. Its presence in the "ovotransferrin fraction" is not surprising as the pH used for ovotransferrin extraction was adjusted to 5.2. Small quantities of ovoinhibitor could be able to bind to the exchanger.

B2a and B2b were identified as the same protein and attributed to ovalbumin gene X (10 and 6 matching peptides, sequence coverage 27 and 21% for B2a and B2b, MOWSE Score 1.77e + 005 and 1.3e + 003, respectively). Ovalbumin gene X (accession number in Swiss-Prot databank: P01013) was described in 1980 by Heilig et al. [46]; like the ovalbumin gene, this gene was expressed in oviduct under steroid hormonal control. The ovalbumin gene X sequence was shorter than the ovalbumin one: 232 amino acids for ovalbumin gene X compared with 385 amino acids for the ovalbumin sequence. To our knowledge, this was the first time that ovalbumin gene X had been detected in albumen.

Following mass spectrometry analysis, and according to Nau et al. [44], all peaks detected in the "ovotransferrin fraction" RP–HPLC profile could be attributed. The peak P1 (retention time 7.5 min) was unambiguously attributed to ovoinhibitor because its retention time on RP–HPLC was close to the retention time previously described by Nau et al. [44] for ovoinhibitor. Therefore, the peak P2 (retention time 12.8 min) was attributed to ovalbumin gene X.

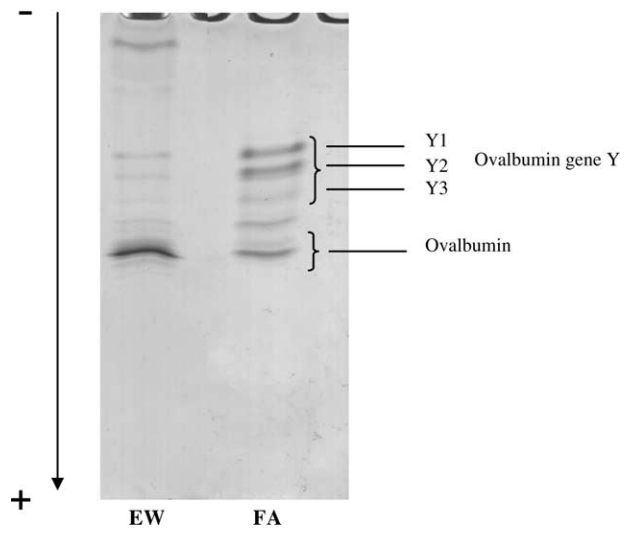


Fig. 6. Isoelectric focusing (IEF) analysis of "A fraction" ($F_A$) and egg white (EW).

*3.2.2. Enriched fractions into minor proteins: $F_A$ and $F_B$*

*$F_A$ characterization.* The $F_A$ fraction presented two intense peaks on RP–HPLC chromatogram (peaks P3 and P4 – Fig. 4C). The P4 peak had a retention time, which was very close to the ovalbumin one. However, the SDS–PAGE profile clearly showed an enrichment of minor egg white proteins whose electrophoretic bands were identified as band 3 and band 4 in Fig. 5. Between these two bands (B3 and B4) a weak band corresponding to ovalbumin [45] was also identified but its quantity in $F_A$ seem to be very low.

To progress in EW characterization, $F_A$ isoelectric focusing (IEF) was performed and its profile is shown in Fig. 6. In comparison with EW, the IEF profile showed that $F_A$ was highly enriched in ovalbumin gene Y. The three bands Y1, Y2, Y3 detected in the $F_A$ IEF profile were the same as those detected in the EW IEF profile and recently attributed to the ovalbumin gene Y [47]. In addition, some ovalbumin was detected on the IEF profile confirming SDS–PAGE analysis.

This result was also confirmed by LC–MS–MS peptide sequencing after $F_A$ fraction RP–HPLC peak collection. The peak P4 was attributed to a mixture of ovalbumin and ovalbumin gene Y. Ovalbumin attribution was based on 6 sequenced peptides (85–98, 127–140, 143–156, 323–336, 340–353, 370–381) matching with ovalbumin sequence. Ovalbumin gene Y attribution was based on 6 sequenced peptides (124–136, 125–136, 144–152, 354–362, 363–372, 373–384) matching with the ovalbumin gene Y sequence. It was not surprising to detect a trace of ovalbumin in this fraction since the "A fraction" was displaced from the column by ovalbumin and proteins with higher affinity to the anionic exchanger. Ovalbumin gene Y has a lower affinity for the anionic exchanger than ovalbumin and this method is interesting to produce an enriched ovalbumin gene Y fraction.

The peak P3 was also submitted to LC–MS–MS sequencing and was attributed to ovomucoid, as one sequenced peptide matched with the ovomucoid sequence (137–146). Ovomucoïd has a theoretical pI of 4.1 [43]. It seems to have a slightly lower affinity for the anionic exchanger than ovalbumin. Ovomucoid is usually difficult to visualize by SDS–PAGE with the usual protein staining methods (Coomassie Brilliant Blue or silver staining) probably because of its high glycosylation rate. Desert et al. [45] identified ovomucoid as a diffuse band in SDS–PAGE just under the ovalbumin one, which would correspond to band 4 in our electrophoretic pattern. Its high glycosylation rate could also explain the low quantity of tryptic peptides we obtained for ovomucoid identification. In addition, the quantity of ovomucoid estimated from the EW RP–HPLC profile either with a 214-nm or a 280-nm UV detection is far lower than the quantity expected from EW. Ovomucoïd represented 11% of the total protein content in EW corresponding to 1.76 g in the EW sample we used in this experiment. In addition, it was not detected in any other collected fractions. In $F_A$, we recovered 2.38 g of proteins. Since ovalbumin gene Y is a minor egg white protein and the ovalbumin gene Y quantity in $F_A$ is higher than the ovalbumin one (SDS–PAGE pattern, Fig. 5), there is evidence that ovomucoid is the most abundant protein in $F_A$ and most of the ovomucoid was concentrated in this fraction.

*$F_B$ characterization.* The "B fraction" is eluted between ovalbumin and flavoprotein fractions on anion exchange chromatography. On RP–HPLC chromatogram, three peaks at retention time 10.2 min, 10.6 min and 14 min were detected (Fig. 4E). Presently, the peaks at retention time 10.2 min (peak P5) and 10.6 min (peak P6), were unidentified on RP–HPLC, while the other one (main peak) was unambiguously attributed to ovalbumin. This latter was confirmed by LC–MS–MS peptide sequencing as 4 sequenced peptides (127–140, 143–156, 323–336, 370–381) matched with the ovalbumin sequence. It was not surprising to have ovalbumin in "B fraction" since ovalbumin molecules previously retained on the anionic exchanger were probably not completely eluted using 0.14 M NaCl. On the other hand, the P5 and P6 peaks were collected separately, hydrolysed by trypsin and the peptides were submitted to sequencing by LC–MS–MS. These two peaks were attributed to ovoglycoprotein. In the two fractions, ovoglycoprotein attribution was based on the same three sequenced peptides (139–148, 155–165, 171–180) matching with the ovoglycoprotein sequence. Ovoglycoprotein is a quantitatively minor protein in EW (0.5–1% total protein). It is a glycoprotein of 24.4 kDa [43] containing 30% glycanes with an apparent pI of 3.9. The "B fraction" could be an interesting fraction for ovoglycoprotein enrichment. Other constituents (bands 5–7) were detected on the SDS–PAGE of the "B fraction" (Fig. 5). These constituents have not yet been identified.

## 4. Conclusions

In this work, six fractions from "mucin-free" EW were produced. Four of them were well-characterized high-

recovery yield egg white proteins namely lysozyme, ovotransferrin, ovalbumin and flavoprotein fractions. Their purity levels were 95, 89, 91 and 100%, respectively. In addition, the ovotransferrin fraction contained ovalbumin gene X. To our knowledge, this was the first time that ovalbumin gene X had been detected in albumen. Moreover, by modifying the pH extraction conditions, a fraction containing more than 40% of ovalbumin gene X can be obtained. The two other fractions are enriched in proteins subjected to only few studies because of the difficulty to separate them from major egg white proteins (ovomucoid, ovoglycoprotein) or because of their recent identification in egg white (ovalbumin gene Y). Ovomucoid is usually purified by successive solvent precipitations. Ion-exchange chromatography is an alternative for purifying ovomucoid, which could be the most convenient for further biological activity studies.

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## References

[1] G. Sava, EXS 75 (1996) 433.
[2] V.A. Proctor, F.E. Cunningham, CRC Crit. Rev. Food. Sci. Nutr. 26 (1988) 359.
[3] J.N. Losso, S. Nakai, E.A. Charter, in: A.S. Naidu (Ed.), Natural Food Antimicrobial Systems, CRC Press, Inc., New York, 2000, p. 185.
[4] H.R. Ibrahim, Proceedings of the Xth European Symposium on the Quality of Eggs and Egg Products, Ploufragan, France, 2003, p. 350.
[5] E. Antonini, US patent 4 029 771, 1977.
[6] P. Valentini, G. Antonini, C. Van Hunolstein, P. Visca, N. Orsi, E. Antonini, Int. J. Tissue React. 5 (1983) 97.
[7] Y. Mine, J. Kovacs-Nolan, J. Poult. Sci. 41 (2004) 1.
[8] H. Fujita, H. Usui, K. Kurahashi, M. Yoshikawa, Peptides 16 (5) (1995) 785.
[9] H. Fujita, R. Sasaki, M. Yoshikawa, Biosci. Biotechnol. Biochem. 59 (12) (1995) 2344.
[10] Y. Tsuge, M. Shimoyamada, K. Watanabe, J. Agri. Food Chem. 45 (1997) 2393.
[11] H. Tanizaki, H. Tanaka, H. Iwata, A. Kato, Biosci. Biotechnol. Biochem. 61 (1997) 1883.
[12] K. Watanabe, Y. Tsuge, M. Shimoyamada, J. Agric. Food Chem. 46 (1998) 4501.
[13] K. Watanabe, Y. Tsuge, M. Shimoyamada, N. Ogama, T. Ebina, J. Agric. Food Chem. 46 (1998) 3033.
[14] G. Alderton, H.L. Fevold, J. Biol. Chem. 164 (1964) 1.
[15] E. Li-Chan, S. Nakai, J. Sim, D.B. Bragg, K.V. Lo, J. Food Sci. 51 (1986) 1032.
[16] P. Cautrecasas, M. Wilcheck, Biochem. Biophys. Res. Commun. 33 (1968) 235.
[17] P.R. Levison, S.E. Badger, D.W. Toome, M.L. Koscielny, L. Lane, E.T. Butts, J. Chromatogr. 590 (1992) 49.
[18] P.R. Levison, S.E. Badger, D.W. Toome, M. Streater, J.A. Cox, J. Chromatogr. A 658 (1994) 419.
[19] P.R. Levison, R.M.H. Jones, D.W. Toome, S.E. Badger, M. Streater, N.D. Pathirana, J. Chromatogr. A 734 (1996) 137.
[20] P.R. Levison, in: G. Subramanian (Ed.), Process Scale Liquid Chromatography, VCH, Weinheim, New York, Basel, Cambridge, Tokyo, 1995, p. 131.
[21] P.R. Levison, in: M.S. Verrall (Ed.), Downstream Processing of Natural Products: A Practical Handbook, J. Wiley & Sons Ltd., West Sussex, England, 1996, p. 179.
[22] P.R. Levison, J. Chromatogr. B 790 (2003) 17.
[23] T. Croguennec, F. Nau, S. Pezennec, G. Brulé, J. Agric. Food Chem. 48 (2000) 4883.
[24] M.B. Rhodes, P.R. Azari, R.E. Feeney, J. Biol. Chem. 230 (1958) 399.
[25] C. Guérin, G. Brulé, Sci. Aliments 12 (1992) 705.
[26] S. Mandeles, J. Chromatogr. 3 (1960) 256.
[27] K. Jacobs, L. Shen, H. Benemariya, H. Deelstra, Z Le-bensm Unters F A 196 (1993) 236.
[28] A.C. Awade, S. Moreau, D. Molle, G. Brulé, J.L. Maubois, J. Chromatogr A 677 (1994) 279.
[29] M.C. Vachier, M. Piot, A.C. Awade, J. Chromatogr. B 664 (1995) 201.
[30] T. Croguennec, F. Nau, S. Pezennec, M. Piot, G. Brulé, Eur. Food Res. Technol. 212 (2001) 296.
[31] J. Brooks, H.P. Hale, Biochim. Biophys. Acta 32 (1959) 237.
[32] A. Kato, R. Nakamura, Y. Sato, Agric. Biol. Chem. 34 (1970) 1009.
[33] C. Guérin-Dubiard, G. Brulé, Ind. Aliment. et Agric. 10 (1994) 701.
[34] S. Moreau, F. Nau, C. Guérin-Dubiard, G. Brulé, Int. J. Food Res. Technol. 205 (1997) 329.
[35] J. Hiidenhovi, M. Rinta-Koski, A. Hietanen, S. Mantere-Alhonen, R. Huopalahti, E.L. Ryhänen, Proceedings of the British Nutrition Foundation and the Food Chemistry Group of the Royal Society of Chemistry, Kent, UK, 1999, p. 197.
[36] J. Hiidenhovi, S.H. Aro, V. Kankare, J. Agric. Food Chem. 47 (1999) 1004.
[37] J. Hiidenhovi, J. Mäkinen, R. Huopalahti, E.L. Ryhänen, J. Agric. Food Chem 50 (2002) 2840.
[38] M.B. Rhodes, N. Bennett, R.E. Feeney, J. Biol. Chem. 234 (8) (1959) 2054.
[39] Y. Hidaka, Y. Matsuoka, Nippon Nogeikagaku Kaishi 51 (1977) 165.
[40] V.E. Piskarev, A.M. Shuster, A.G. Rabinkov, Biochem. J. 265 (1990) 301.
[41] P.-F. Rao, S.-T. Liu, Z. Wei, J.-C. Li, R.-M. Chen, G.-R. Chen, Y.-Q. Zhen, J. Food Biochem. 20 (1997) 473.
[42] U.K. Laemmli, Nature 227 (259) (1970) 680.
[43] E. Li-Chan, S. Nakai, Crit. Rev. Poult. Biol 2 (1989) 21.
[44] F. Nau, A. Mallard, J. Pages, G. Brulé, J. Liq. Chrom. Relat. Technol. 22 (8) (1999) 1129.
[45] C. Desert, C. Guérin-Dubiard, F. Nau, G. Jan, F. Val, J. Mallard, J. Agric. Food Chem. 49 (2001) 4553.
[46] R. Heilig, F. Perrin, F. Gannon, J.L. Mandel, P. Chambon, Cell 20 (1980) 625.
[47] F. Nau, M. Pasco, C. Desert, D. Molle, T. Croguennec, C. Guérin-Dubiard, J. Agric. Food Chem. 53 (2005) 2158.