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A new size-exclusion chromatography method for fast rapeseed albumin and globulin quantification

Claire DEFAIX[a,b], Arnaud AYMES[a], Sara ALBE SLABI[a,b], Mélody BASSELIN[a], Christelle MATHE[a], Olivier GALET[b], Romain KAPEL[a] *

[a] Laboratoire Réactions Génie des Procédés, Université de Lorraine, 1 Rue Grandville, 54000 Nancy, 54000 Nancy, France

[b] Avril SCA, 11/13, rue de Monceau, 75008 Paris, France

claire.defaix@univ-lorraine.fr

arnaud.aymes@univ-lorraine.fr

sara.albe-slabi@univ-lorraine.fr

melody.basselin@univ-lorraine.fr

christelle.mathe@univ-lorraine.fr

olivier.galet@groupeavril.com

* romain.kapel@univ-lorraine.fr +33 3 72 74 39 85

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Abstract

The method described in the article aims at the quantification of both main storage proteins, globulins and albumins, in aqueous extract from rapeseed, as an alternative to the current reference methods, Kjeldahl and SDS-PAGE electrophoresis. The new method lies on the analytical separation of extracted compounds by Size-Exclusion High Performance Liquid Chromatography (SE-HPLC) (Biosep-SEC-s2000, 5 µm). The elution of rapeseed extracts with water/acetonitrile/trifluoroacetic acid (45/55/0.1 % v/v) during 30 minutes yields two distinct peaks for the main proteins of rapeseed. Based on the protein extinction coefficients, a calibrationless methodology was developed for their quantification on the basis of the UV signal. The SE-HPLC method was successfully compared to references: Kjeldahl and SDS-PAGE densitometry for the determination of the proportion of each protein. Then, it was successfully applied on two other oleoproteagineous plants, linseed and sunflower.

Keywords: rapeseed proteins, albumins, globulins, SE-HPLC, quantification

1. Introduction

According to FAO statistics, in 2016, rapeseed was the second most produced oilseed after soybeans in the world with a respective production of 69 million tons and 335 million tons. Whereas, for sunflower seeds and linseeds, 47 and 3 million tons were produced during the same year (FAOStats, 2018). Because of their richness in fibers and proteins, meals obtained after oil extraction are currently used as a commodity . These proteins are also interesting for human nutrition due to their nutritional properties (World Health Organisation, 2007).

In oilseed meals, 2 S and 11-12 S proteins constitute the main protein fractions. In rapeseed, 2 S proteins are called albumins or napins, with a molecular weight (MW) of 10 to 15 kDa. They represent between 30 and 40 % of the total proteins in rapeseed. Among their interesting

properties are foaming and emulsifying activities. 12 S protein are called globulins, or cruciferins, and their MW is between 300 and 350 kDa. They represent 50 to 55 % of the total proteins and are suitable to form gels (Crouch & Sussex , 1981) (Aider & Chockry, 2011). In flaxseed and sunflower, similar structure exist with slight variations (Chung, Lei, & Li-Chan, 2005). (Gonzalez-Pérez & Vereijken, 2007).

Currently, protein quantification in aqueous extracts is often done from nitrogen quantification by Kjeldahl method (Sáez-Plaza, Michałowski, Navas, Asuero, & Wybraniec, 2013). The protein amount is deduced from the nitrogen content using a conversion factor (often 6.25 for rapeseed proteins). However, this value has been criticized over the years as it depends on the protein under study (Mariotti, Tomé , & Patureau Mirand, 2008). Furthermore, other biomolecules contain nitrogen. This is taken into account by using another Kjeldahl analysis after selective protein precipitation by trichloroacetic acid (TCA). Eventually, this method gives an overall protein concentration and does not allow specific albumin or globulin quantification. Spectrophotometric methods like BCA or Lowry's method can alternatively be used. These methods are fast with automated microplate systems. But polyphenols co-extracted bias the quantification (Stepanchenko, Novikova, & Mosh, 2011).

SDS-PAGE separates the polypeptides of a complex mixture according to their size (Chiou & Wu, 1999). By densitometry, it is possible to compare the intensity of protein bands (colored by Coomassie blue) and to get an estimated protein content. But this method is long and the results are likely biased . Alternatively, chromatographic methods have been developed to this day to identify and quantify proteins from plants such as rapeseed (Boukroufa, et al., 2017). Even though these methods discriminate between types of proteins, none allows a clear quantification of the protein content.

The hypothesis of this work is that SE-HPLC can be used to accurately quantify the protein content and the proportion of globulins / albumins in rapeseed extract in order to do a follow-up of protein production processes. The methodology described hereafter is calibrationless: amino acids sequences were used to determine two parameters, the extinction coefficient and the nitrogen-to-protein conversion factor used in the Kjeldahl analysis.

The first step consisted of a proof of concept of the ability of the method to separate both proteins. Secondly, two validation steps were carried out using homemade protein isolates. They were used to compare the proposed method to: (i) the Kjeldahl regarding the quantification of the protein content; (ii) and to SDS-PAGE densitometry for the determination of the proportion of each protein in a sample. Thirdly, the SE-HPLC method was compared to both previously cited reference methods for the analysis of aqueous extracts of proteins. Fourthly, a stability study was conducted on the aqueous extracts . Finally, the methodology was applied to two other oleoproteaginous plants: sunflower and flaxseed.

2. Materials and methods

2.1. Materials

2.1.1. Meals

The rapeseed meal used was provided by Olead (Pessac, France): after pressing, a step of oil extraction was carried out with hexane followed by a heating step at 86 °C for 60 minutes to remove the solvent. Its composition was 37.5 % protein, 35.8 % of total dietary fibers, 7.7 % of carbohydrates, 9.4 % of phytates, 7.3 % of minerals, 3.8% water, 2.0 % of polyphenols and 1 % residual oil on a dry matter basis.

Linseed and sunflower meals were also provided by Olead (Pessac, France). Both were subjected to a desolvantation with hexane after the pressing step.

2.1.2. Chemical reactants

Sodium chloride and sodium hydroxide pellets were provided by VWR (Radnor, Pennsylvania, USA). Chlorhydric acid at 37 % was provided by Carlo Erba (Val-de-Reuil, France).

Production of rapeseed protein isolatesAlbumins and globulins and total proteins were extracted and purified for method calibration and validation. The extraction step was carried out in aqueous medium at room temperature with a solid-liquid ratio of 1:9, 0.1 M NaCl and 0.5 $g.L^{-1}$ ascorbic acid (Sigma-Aldrich, Saint-Louis, USA) to prevent oxidation. pH was adjusted to 7 with a solution of NaOH 2 M under agitation. The extraction duration was 30 minutes and the pH was maintained at 7. The solid-liquid separation was realized with a Heraeus Megafuge 16 R centrifugation system (Thermo Scientific, Waltham, Massachusetts, USA) at 10,000 g for 30 minutes. The supernatant was recovered and filtered and the solid was discarded. This first step was common to the production of all three proteins. Purification was achieved by an ultrafiltration procedure with an Äkta Flux 6 (GE Healthcare, Chicago, Illinois, USA) using 4800 cm^2 hollow fibers cartridges (GE Healthcare, Chicago, Illinois, USA) at 2 L/min feed flow and a transmembrane pressure of 1.5 bars. Albumins and globulins were separated using a 500 kDa cut-off membrane. During diafiltration, water or salted water was added to the retentate at the same flow rate as the permeate leaving the system to keep the volume in the retentate constant. Each volume of water equal to the volume of the retentate is called a diavolume (DV). After 7 diavolumeswith salted water (0.2 M NaCl), the permeate rich in albumins was recovered, concentrated 5 times and diafiltrated with 8 DV of water using a 3 kDa membrane. The retentate, rich in globulins, was further purified with the 500 kDa membrane using 3 DV of salted water followed by 2 DV of deionized water. Total proteins were directly purified by diafiltration with the 3 kDa membrane with 10 DV of deionized water. Final retentates were freeze dried with a LTD type GZL-0.5 system (Shanghai Pharmaceutical Machinery Co., Shanghai, China).

To determine purity of all three powders, solutions at a concentration of 5 $g.L^{-1}$ of powder were prepared and homogeneous samples were analyzed according to the official AOAC 979.09 Kjeldahl method (6.25 nitrogen-to-protein conversion factor). Purity was expressed as the ratio of the thus measured protein content divided by the theoretical concentration of the solution.

$$Purity = \frac{Protein\ content\ by\ Kejdahl\ method}{Powder\ content} \quad (1)$$

After freeze-drying, the powders were characterized as follow: 6 % of water, 102 % of proteins and 4 % of phytic acid for albumins; 2 % water, 72 % proteins and 2 % phytic acid regarding globulins; . The total proteins isolate was composed of 5 % water, 94.5 % proteins and 3 % phytic acid. The dry matter was determined according to the method ISO 638:2008. The protein content of the powder corresponds to the purity described previously. The nitrogen-to-protein conversion factor was 6.25 as it is the mostly used value even though it probably overestimates the protein content. And the phytic acid content was determined following the method developed by García-Estepa *et al.* (Garcia-Estepa, Guerra-Hernandez, & Garcia-Villanova, 1999).

2.2. Determination of protein content by Kjeldahl method

Samples were analyzed according to the official AOAC 979.09 Kjeldahl method. The samples were prepared with 4 mL of sulfuric acid (Sigma-Aldrich, Saint-Louis, USA) and a spatula of catalyst Cu-Se (AppliChem, Gatersleben, Germany). The mineralization step was carried out by heating the flasks at 450 °C for 90 minutes in a Büchi SpeedDigester K-439 (Rungis, France): The distillation step took place in a Kjelflex K-360 (Büchi, Rungis, France) with sodium hydroxide 32 % (VWR, Radnor, Pennsylvania, USA) and boric acid (Sigma-Aldrich, Saint-Louis, USA) prepared in solution at 3 %. The titration was carried out with 0.01 M HCl (Honeywell, Morris Plains, New Jersey, USA) in a Titrino Plus 877 from Metrohm (Herisau, Switzerland).

To determine non protein nitrogen, extracts were diluted twice with a 40 % (w/v) trichloroacetic acid TCA (Sigma-Aldrich, Saint-Louis, USA), stirred 30 minutes and centrifuged at 15000 g. The supernatant was analyzed by the Kjeldahl method.

2.3. SDS-PAGE assay and quantification by densitometry

SDS-PAGE reagents were from Bio-Rad (Hercules, California, USA). Two standard protein ladders were used: the first between 10 and 250 kDa and the second between 1.4 and 26.6 kDa. Samples were prepared according to Laemmli method (Laemmli, UK 1970) in reducing conditions and migrated on a 5 % acrylamide staking gel and a 17 % separation gel. The coloration was achieved by Coomassie blue G 250 (Sigma-Aldrich, Saint-Louis, USA). For each SDS-PAGE assay, a standard molecular weight kit was used (Bio-Rad Precision Plus Protein Standard) allowing the determination of molecular weights between 10 and 250 kDa. For band densitometry analysis, a free and open source software developed by the National Institutes of Health (Bethesda, Maryland, USA) called ImageJ was used. From pictures of SDS-PAGE gels, the intensity of the coloration for each band of the gel was determined. A calibration was carried out on globulins and albumins isolate solutions from 0.2 to 4 $g.L^{-1}$ before determination on the aqueous extracts.

The calibration plots for each protein showed a good correlation, with respective R^2 of 0.98 and 0.96 (data not shown) for globulins and albumins. However, the linear portion of the calibration is narrow: protein content should be between 0.2 and 0.8 $g.L^{-1}$ for a reliable determination.

2.4. Quantification of proteins by SE-HPLC

2.5.1 Elution method

SE-HPLC acetonitrile was provided by Biosolve BV (Valkenswaard, the Netherlands) and ultrapure water was generated by a system Purelab Ultra by Elga (High Wycombe, United Kingdom). The chromatographic system was a UFLC machine from Shimadzu (Kyoto, Japan) and the column,

Biosep-SEC-s2000 300 x 7.8 mm, 5 µm, was provided by Phenomenex (Torrance, California, USA). Its separation range (300 kDa – 1 kDa) was chosen because of the molecular sizes of albumins and globulins (14 kDa and 300 kDa, respectively).

The mobile phase was 45 % acetonitrile, 55 % ultrapure water and 0.1 % of trifluoroacetic acid (TFA) from ThermoFisher (Waltham, Massachusetts, USA). Elution was done at 0.6 mL / min flow during 30 minutes. The calibration of the chromatographic column was realized using a gel filtration markers kit for protein molecular weights between 12 kDa and 200 kDa from Sigma-Aldrich (Saint-Louis, USA).

2.5.2 Quantification method

Liquid samples from aqueous extraction or solutions prepared from isolates were filtered on 0.22 µm membranes from Pall (Port Washington, New York, USA). The injection volume was 5 µL and the detection was set to 214 nm. For calibration, a 20 $g.L^{-1}$ (w/v) solution was diluted in a 0.1 M phosphate buffer pH 7 (Sigma-Aldrich, Saint-Louis, USA) to obtain concentrations of 20, 15, 12.5, 10, 7.5, 5 and 1 $g.L^{-1}$. Each stock solution was prepared in triplicate and analyzed using the SE-HPLC method. The protein content of each stock solution was also quantified according to the Kjeldahl method.

Data were extracted and processed for integration using an Excel (Microsoft, Redmond, Washington, USA) spreadsheet in order to retrieve the mass of protein injected. First, peaks were integrated:

$$Area = \int_{V0}^{Vf} A.dV \quad (4)$$

In this equation, V_0 and V_f correspond to the elution volume at the beginning and at the end the protein peak and A is the intensity of the signal.

177 And according to Beer-Lambert law: $A = \varepsilon . l . C$ with ε, the extinction coefficient, and l the length
178 of the detection cell.

179 $$Area = \int_{V0}^{Vf} \varepsilon . l . C . dV = \varepsilon . l . \int_{V0}^{Vf} C . dV = \varepsilon . l . m_{protein\ injected} \quad (5)$$

180 Because the detection of the proposed method is UV, a key parameter was ε, the extinction
181 coefficient. According to Kuipers and Gruppen in a paper from 2007 (Kuipers & Gruppen, 2007),
182 this value was calculated at 214 nm. Subsequently the mass of protein in each sample can be
183 determined.

184 $$m_{protein\ injected} = \frac{Area}{\varepsilon . l .} \quad (6)$$

185 ## 2.5. Validation of the method

186 ### 2.5.1. Aqueous extraction of proteins

187 In order to validate the proposed method, aqueous extractions were carried out under several
188 conditions of pH (from 2 to 9) and salt content (from 0 to 0.3 M). To do so, 10 g of meal was used
189 at a ratio of 10 % solid-liquid. pH was adjusted with NaOH or HCl 1 M. Each extraction was carried
190 out during 30 minutes. A centrifugation step allowed the recovery of the extract.

191 ### 2.5.2. Stability of protein extracts

192 The SE-HPLC analyses of the extracts previously described were compared after 24 hours of
193 storage under three different conditions: at room temperature, 4 °C or -20 °C. The difference of
194 area under protein peaks between time 0 and after 24 hours of storage are compared to assess
195 extracts stability.

196 ### 2.5.3. Statistical analysis to compare the methods

197 In order to compare the SE-HPLC to the references methods, a statistical analysis was carried out
198 in two steps with Excel (Microsoft, Redmond, Washington, USA). First, a Fisher-test was done in

order to compare the variances of the three repetitions of both methods for each validation point. If p-value is superior to 5 %, the variances are deemed equal. The equality or inequality of variances is an input necessary to carry out the next test. Then, a Student T-test was realized in order to compare the expectations taking into account the equality or inequality of variances. If the p-value is superior to 5 %, the expectations of both populations (from the two different methods) were deemed equal and thus, the methods were comparable for the given point of validation.

2.5.4. Validation of the method according to the ICH validation guide

Another step of validation was based on the determination of values advised by the International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use (Validation of Analytical Procedures: Text and Methodology Q2(R1) , 2005). Hence, linearity was established using 7 concentrations for albumins and for globulins. The correlation coefficient, the y-intercept and the slope of the regression line were determined. The range corresponds to the minimum and maximum concentrations analyzed in the present study, taking into account the purity of the isolates. Accuracy was determined by the comparison with the reference method for protein content determination, the Kjeldahl method, on the 5 conditions of extractions previously described. The precision was described by the repeatability, or intraday variation, which is calculated as the variation observed when the same sample is analyzed three times the same day, for 3 concentration levels (20, 12.5 and 5 $g.L^{-1}$). The intermediate precision, or inter-day variation was determined as the variation observed for 3 concentration levels (20, 12.5 and 5 $g.L^{-1}$) measured on three different days. The detection limit and the quantitation limit were calculated by the comparison of the signal and of the noise of the analysis. The detection limit was determined as the minimal concentration that produced a signal 3 times higher than the noise. The quantitation limit was the minimal concentration with a signal 10 times higher than the noise.

3. Results and discussion

3.1. Separation of proteins by SE-HPLC

The aim of the method was to quantify the two main rapeseed proteins (albumins and globulins) by SE-HPLC. This required to choose a column and an elution condition allowing a separation of these two proteins. Globulins and albumins molar weight is around 300 kDa and 14 kDa respectively. As a consequence, Biosep-SEC-s2000 column was selected because its separation range was given between 1 and 300 kDa. The eluent was a water/acetonitrile mixture (55/45 v/v) containing TFA (0.1% v/v) to avoid protein/protein association or aggregation that would yield multiple peaks for a single protein.

Fig. 1 A shows the chromatogram of the isolate at 214 nm. Two main peaks were observed at respective retention times of 9 and 11 minutes. According to the column calibration (data not shown), these retention times corresponded to molar weights around 50 kDa and 10 kDa respectively. Rapeseed globulins (MW = 300 kDa) are composed of 6 subunits of 50 kDa linked together by electrostatic interactions (Schwenke, Raab , Plietz , & Damaschun, 1983) . Albumins (MW = 14 kDa) are composed of 4 and 9 kDa subunits linked by disulfide bridges. Hence, the first peak observed would correspond to globulin subunits dissociated by the elution conditions. Indeed, at the pH of the mobile phase (around 2.5) globulins subunits are positively charged because globulins isoelectric point was reported around ~~7 (Krause & Schwenke, 2001)~~ 5 (Cheung, Wanasundara, & Nickerson, 2014). This probably results in a subunits dissociation and explains that globulins were eluted in a 50 kDa peak. The second peak at around 10 kDa would correspond to albumins. The elution conditions did not contain any reducing agents, so albumins subunits should remain bonded together. The apparent molar weight given by the calibration was slightly below 13 kDa, probably because of the compactness of albumins due to its reported two intra molecular disulfide bonds (Perera, McIntosh, & Wanasundara, 2016).

247 To check that out, each peak was fractionated and analyzed by SDS-PAGE in reducing conditions
248 (with β mercaptoethanol). As shown in Fig. 1 B. Fractions 2 to 5 (between 8 and 10.5 minutes of
249 retention time) were composed of polypeptides with molecular weights around 30 and 20 kDa.
250 This corresponds to the globulin α and β-chains respectively. These two chains are known to be
251 bound by disulfide bridges. As a consequence, they were not dissociated by the elution conditions
252 and are well eluted under a 50 kDa globulin subunit. From fraction 5 to 10, two bands are visible
253 corresponding to low molecular weight proteins and are consistent with the two subunits of
254 albumins of approximately 4 and 9 kDa (that remained associated under elution condition). This
255 conformed with the alleged composition of peaks on the chromatogram. The slight peak
256 overlapping (5% of the total peak area) is composed of a mixture of albumins and globulins.

257 Previous studies used SE-HPLC for the analysis of proteins. Notably, it was reported that SE-HPLC
258 could be used to follow-up a process of enzymatic hydrolysis of milk proteins by the
259 transglutaminase. However, only a comparison of the area under the peaks was carried out and no
260 quantification was done (Faergemand & Qvist, 1997). More specific work was carried out on
261 protein from plants: a study with a Biosep-SEC-s4000 column showed the separation of gliadins
262 and albumins/globulins from wheat after 22 minutes of elution versus 15 minutes in the proposed
263 method. However, no quantification of the protein content was carried out (Bangur, Batey,
264 McKenzie, & MacRitchie, 1997). In 2005, it was shown the separation of wheat proteins between
265 10 and 22 minutes of elution. This method is the closest to the condition used in the present
266 article with a Biosep-SEC-s4000 column and a mobile phase composed of 50 % of acetonitrile, 50
267 % water and 0.05 % TFA. However, it shown an elution of proteins between 10 and 22 minutes
268 with a poor resolution even though two peaks were visible (Singh, 2005) whereas the present SE-
269 HPLC method elutes proteins between 8 and 15 minutes with a good resolution.

3.2. Method validation with protein isolates

The reference assay for food plant protein quantification is the Kjeldahl method. So, the proposed SE-HPLC method was validated by comparison with this method. The protein quantification by Kjeldahl requires the use of a nitrogen-to-protein factor (Ka), since it just quantifies the nitrogen content. The most common Ka used for vegetal proteins is 6.25 (Mariotti, Tomé , & Patureau Mirand, 2008). However, the nitrogen content in proteins depends upon their amino acid sequence. It is possible to calculate a Ka by formula (7) (Mossé , Huet , & Baudet, 1985):

$$Ka = \frac{\sum E_i}{\sum D_i} \quad (7)$$

In this equation, i corresponds to each anhydrous amino acid, E_i is its molecular weight and D_i its nitrogen content. For rapeseed, few studies proposed values for Ka but one estimated it as an average 5.53 (Tkachuk, 1969). The calculation by Mariotti gave an average value of 5.35 (Mariotti, Tomé , & Patureau Mirand, 2008). Yet, these values are likely to change with the proportion of globulins and albumins. As a consequence, it was chosen to validate the method with globulins and albumins isolates using Ka values calculated from their amino acids sequences.

Since no standards of globulins and albumins are commercially available, proteins isolates were produced according to (US Patent No. 8,541,046 B2, 2013). Fig. 2 shows the elution chromatograms at 214 nm of the globulin and albumin isolates prepared. For each isolate, a single peak is observed corresponding to the elution duration observed above. SDS-PAGE analysis confirmed the composition of each isolate. For globulins, a slight contamination by albumins was observed and estimated as 4 % on the SE-HPLC chromatogram. For albumins, the two main bands characteristics of their two subunits were visible by SDS-PAGE with no apparent contamination by globulins. As a consequence, these isolates were considered suitable to carry out the validation of the proposed method.

293 The validation methodology used is shown on Fig. 3. Isolate solutions of globulins and albumins
294 ranging from 1 to 20 g.L^{-1} were prepared and analyzed by SE-HPLC. On SE-HPLC chromatograms at
295 214 nm, the absorbance of albumins and globulins peaks were converted into concentrations
296 using the Beer-Lambert law. The obtained peak was integrated to give an amount of proteins in
297 the sample. To do so, the extinction coefficients of albumins and globulins were calculated with
298 amino acids sequences, as described by Kuipers and Gruppen (2007) on the basis of the UniProt
299 database (P80208 for albumins and Q7XB53 for globulins). For globulins, this protein sequence
300 corresponded to a molecular weight of 55687 Da, a theoretical mass extinction coefficient ε of
301 17.22 and a theoretical nitrogen-to-protein conversion factor, Ka, of 5.46. For albumins, it was
302 14035 Da, the theoretical mass ε was 16.44 and the theoretical Ka was 5.24.

303 The isolate solutions were also analyzed by Kjeldahl method to get the amount of nitrogen. The
304 nitrogen content and the protein concentrations determined by SE-HPLC were plotted against the
305 isolate concentrations. From this plot, an experimental nitrogen-to-protein conversion factor (Ka)
306 was determined as in equation 8.

307 $$Ka = \frac{Protein\ content\ in\ the\ sample\ (SE{-}HPLC)}{Nitrogen\ content\ (Kjeldahl)} \quad (8)$$

308 Then the obtained experimental Ka values were compared to theoretical values calculated from
309 the amino acids sequences.

310 Fig. 3 shows the plots of the protein content by SE-HPLC and the nitrogen content by Kjeldahl as
311 functions of the isolate concentration in the solutions for each protein. For both methods, a good
312 linearity is observed with values of R^2 superior to 0.99. The wide range of linearity, between 1 and
313 20 g.L^{-1}, is particularly convenient as it covers the concentrations of proteins usually found in
314 aqueous extracts. The values of experimental Ka for globulins and albumins deduced from this
315 graph were 5.13 and 4.94 respectively, Table 1.

316 This made relative differences of 6 and 5.7 % from the theoretical values (calculated from protein
317 sequences). This slight discrepancy can be explained by (i) the presence of several isoforms of
318 each protein in a same cultivar of a species leading to an approximation in the values of ε and Ka
319 (ii) an approximation in the calculation of the theoretical ε as the folding of the protein might
320 change UV absorption; (iii) a contribution to the absorption by remaining polyphenols in the
321 isolates; or (iv) a flawed optical path in the HPLC system. As a consequence, these results show
322 that the use of the SE-HPLC would give very similar results to the reference method, Kjeldahl.

323 ### 3.3. Application of the SE HPLC method for total proteins quantification in
324 rapeseed meal extracts

325 The main interest is to be able to quantify rapeseed proteins in aqueous extracts from meal
326 solid/liquid extraction. The main parameters that may affect the extraction of one or both
327 proteins are the pH and the ionic strength. As a consequence, aqueous extracts were prepared in
328 several conditions of pH and ionic strength: pH 2 0 M, pH 5 0 M, pH 7 0 M, pH 7 0.1 M and pH 9
329 0.3 M (Table 1). These conditions were chosen in order to get various protein concentrations and
330 globulins/albumins compositions. The extracts were analyzed by SE-HPLC and Kjeldahl. Kjeldahl
331 analysis required an additional step of precipitation with TCA in order to determine the true
332 protein content in samples. Since Ka values of globulins and albumins are very close (respectively
333 5.46 and 5.24), a Ka value of 5.35 was chosen whatever the globulin / albumin proportion.

334 Fig. 4 represents the stacking of chromatograms at 214 nm for three extraction conditions: pH 2 0
335 M NaCl, pH 7 0 M NaCl and pH 9 0.3 M NaCl. On the right side is a zoom of the protein peaks. As
336 expected, it shows that the composition of the extract depends very much on the extraction
337 condition. For example, the first peak (of globulins) is bigger at pH 7 and 9 than at pH 2.

338 Other peaks were visible after 15 minutes. Because the separation was based on size-exclusion,
339 these peaks corresponded to low molecular weight molecules. Since phenolic compounds are

soluble in water, they are co-extracted with proteins. Moreover, common chromatographic methods to detect and quantify phenolics usually use a UV detection at 325 nm. In the present study, the chromatograms at 325 nm (data not shown) show the presence of several signals at these retention times. They are very likely to correspond to free phenolic compounds. Nevertheless, the presence of these peaks after 15 minutes, did not impact the quantification of rapeseed proteins. In any case, globulin and albumin signals were not biased with other solutes absorbing at 214 nm. Hence, the SE-HPLC method was suitable for proteins quantifications from liquid complex extracts.

The results of Kjeldahl and SE-HPLC quantifications are featured in Table 1. A statistical investigation was carried out to compare both sets of data. For all sets of data, the Student T-test p-value was higher than 0.05, resulting in the equality of means. It is to note that the p-value for the T-test is very low, with 0.046, regarding the pH 5 condition: there is a small probability of H0 being false for this extract only. For all the other conditions, the statistical tests indicate an equivalency between both methods to determine the soluble protein content in an aqueous extract. Nevertheless, the results obtained with both methods show very close values. As a consequence, the proposed method can be used as a substitute of the Kjeldahl method for quantifying soluble proteins. The SE-HPLC presents many advantages: (i) it is very convenient because it is fully automated; (ii), it is less dangerous for the user (the Kjeldahl method requires the use of strong acids and bases); (iii) it generates less effluents; (iv) it is quick as one run lasts 30 minutes.

3.4. Application of the SE HPLC method for albumins/globulins composition determination

The Kjeldahl method does not discriminate between globulins and albumins. That is not the case of the proposed SE-HPLC method. To determine the concentration of one of the proteins in a

364 mixture, the only referenced method is SDS-PAGE densitometry. Hence, the SE-HPLC method was
365 compared to SDS-PAGE densitometry for the quantification of globulins and albumins in aqueous
366 extracts. Table 1 shows the values obtained by both methods for the albumin content in the
367 aqueous extracts. The average values between both methods present up to 19 % difference (for
368 the condition pH 7 and 0.1 M NaCl). As seen before, various compositions of globulins are found
369 between the extraction conditions. These differences can be quantified with both methods.

370 For three of the conditions (pH 2, pH 7 0.1 M NaCl and pH 9 0.3 M NaCl), there was a significant
371 difference between the methods ($p<0.05$). The other two conditions (pH 7 0 M and pH 5 0 M)
372 were found statistically equal. However, for the SDS-PAGE densitometry, very large standard
373 deviations were observed which makes the comparison more difficult.

374 As a consequence, compared to SDS-PAGE densitometry, the SE-HPLC method would be easier to
375 carry out as no particular preparation is required to dose the proteins. Whereas, to prepare SDS
376 samples, it is necessary to dilute the sample quite accurately as the linear parts of the calibrations
377 are between 0.2 and 0.8 $g.L^{-1}$. Moreover, SDS-PAGE is a method which is longer to apply because:
378 gels must be prepared before the migration step; there are several steps of staining and
379 destaining; and finally, the computational densitometry treatment of the scan or picture of the
380 gel. With a four-gel SDS-PAGE system, up to 36 samples can be loaded in a single run with one
381 marker per gel. The total time including all operations was estimated at 18 hours which would be
382 30 minutes per sample. Hence, depending on the number of samples to be analyzed, the average
383 time of analysis per sample would be between 30 minutes (for 36 samples) and 18 hours (for one
384 sample). And, contrary to the SE-HPLC analysis, many time-consuming operating steps are
385 required. Indeed, the SE-HPLC method requires only the filtration of the samples. Furthermore,
386 the narrow linearity range will imply to try different sample dilutions thus, even increasing the
387 overall duration. Moreover, the processing of the data with the SDS-PAGE densitometry also

388 involves several steps that were found to induce a bias as shown by the large standard deviations.
389 The Kjeldahl method would require a total time of 4 hours to analyze 5 samples (total nitrogen
390 and non-protein nitrogen analyses), including one blank and one standard analysis. It corresponds
391 to 48 minutes per sample without determination of the globulin and albumin contents. Moreover,
392 it implies a full-time operator exposed to concentrated and dangerous substances. On the
393 contrary, the SE-HPLC method requires only 30 minutes per analysis once the method is set up
394 and after a 30 minutes' equilibration of the system. There are few steps requiring the intervention
395 of an operator, less dangerous substances and a computer-assisted processing of the data.

396 ### 3.5. Stability study of extracts

397 Proteins are known to be sensitive to their medium: the presence of other molecules such as
398 polyphenols for example, that are interacting with proteins and can be oxidized under alkaline
399 conditions; pH and ionic strength are directly linked to protein precipitation (Purkayastha, et al.,
400 2014). All these factors may cause a variation in what is observed by SE-HPLC. Moreover, as shown
401 in Fig. 1, polyphenols are detected at 325 nm. These molecules are very sensitive to oxidation, and
402 hence, their presence in the aqueous medium after extraction, justifies the need to investigate the
403 stability of the samples.

404 Thus, analyses by SE-HPLC were carried out on extracts produced in a variety of conditions of pH
405 and ionic strength. Fig. 5 shows the results of the stability study under several conditions of
406 storage, room temperature, 4 °C and -20 °C, after 24 h or 48 h. The results were compared to the
407 initial analysis of each extract.

408 As expected, a larger variation was visible on extracts stored at room temperature, all the more
409 after 48 hours. However, pH seemed to affect the stability of certain extracts: the variation was
410 the largest for the samples produced at pH 5 or 7 with 0 or 0.1 M of NaCl. On the contrary, the set

of conditions with the most stable extract is pH 9 and 0.3 M NaCl which presented a variation of only 1 % at most whatever the storage condition.

As a consequence, this stability study showed the importance of storage conditions particularly for extracts produced at a neutral pH. These samples must be stored under the coldest conditions as possible to preserve them and constrain alterations.

3.6. Validation of the method according to the ICH validation guide

The area under the peak (in µAU.L) of both globulin and albumin isolates was plotted against the concentration in the sample (data not shown) between 1 and 20 $g.L^{-1}$ of isolate powder. Seven points of concentration were considered with two to three replicates. The concentration in the sample was determined according to the Kjeldahl method using a nitrogen-to-protein coefficient of 5.35. Regarding globulins, it was found a correlation coefficient of 0.9927, the y-intercept was set at 0 and the slope of the regression was 81.5. As for albumins, it was found a regression coefficient of 0.9959with a y-intercept of 0 and a slope of 75.0. Hence, for both proteins, very good correlations were found. The data regarding the ICH validation is shown in supplementary data, Table S.2.

Taking into account the purity of the isolates (with a Ka value of 5.35), the range of concentrations studied was determined. For albumins, it was between 0.4 and 16.2 $g.L^{-1}$ and for globulins, it was 0.6 to 12.0 $g.L^{-1}$. As a consequence, the method was successfully developed to cover the usual range of concentrations found in rapeseed protein extracts.

For albumins, the repeatability, or intra-day variation was found with a value of 2.8 %. The intermediate precision, or inter-day variation, was 3.4 %. For globulins, repeatability was 0.3 % and intermediate precision was 0.9 %. These values are below 5 % and in consequence, the precision of the method was deemed validated regarding the ICH procedure.

For albumins, the detection limit was found at 0.010 $g.L^{-1}$ and the quantitation limit was 0.033 $g.L^{-1}$. Similar values were found for globulins with, respectively, 0.008 $g.L^{-1}$ and 0.026 $g.L^{-1}$.

3.7. Application of the method on other oleoproteaginous resources: examples of linseed and sunflower

Sunflower and linseed are two other major oleoproteaginous produced all over the world. Hence, the same issues regarding the need of a reliable and quick analysis of the proteins are emerging. Indeed, sunflower proteins are also composed of two major types: globulins (11 S), also called helianthinin, and albumins (2 S) with respective proportions in the meal of about 60 % and 30 %. Their molecular weight is similar to the same proteins in rapeseed (Gonzalez-Pérez & Vereijken, 2007). Linseed, of flaxseed, also have their own 11-12 S globulins, also called linin, and 2 S albumins or conlinin. However, these proteins have been the subject of fewer studies to this day that is why limited data is available regarding this resource (Chung, Lei, & Li-Chan, 2005). As a consequence, the approach used for rapeseed proteins is applied to both linseed and sunflower.

Supplementary figure, Fig.S. 1, shows the chromatograms of linseed and sunflower with their associated SDS-PAGE.

Regarding linseed proteins, two main peaks were observed on the chromatograms. The first peak has a retention time between 8 and 10.5 minutes: it is coherent with a high molecular weight protein, globulins. The second peak, between 10.5 and 14 minutes, would correspond to low molecular weight protein, albumins. These observations were completed by SDS-PAGE analysis after a fractionation every 30 seconds between 7.5 and 15 minutes. In the first fraction, two bands were visible: one thin around 30 kDa and the other one wider between 20 and 25 kDa. These would be the result of the dissociation of disulfide bonds from subunits of globulins. In the second fraction, several bands were present between 6.5 and 15 kDa, consistent with albumins.

In respect to sunflower, two peaks were also present on the chromatograms, the first between 8 and 10.5 minutes and the second between 10.5 and 15 minutes, probably corresponding to respectively globulins and albumins. The SDS-PAGE showed that the first peak, fraction 1, was composed of a high-molecular weight protein, dissociated in 3 bands corresponding to the 3 main subunits of globulins: α and α' chains with 30 to 33 kDa and β chain with 21 kDa. Fraction 2 showed the presence of several bands between 16 to 18 kDa corresponding to sunflower albumins.

As before, thanks to sequences it was possible to determine Ka values and extinction coefficients ε for each protein (data not shown). The sequences for linseed were taken from (Nwachukwu & Aluko, 2018) and the calculation gave an average Ka of 5.56. The sequences for sunflower were found in the Uniprot database P19084 (chain) for albumins and P19084 for globulins and resulted in an average Ka of 5.58.

Regarding linseed, the total proteins content in the extract determined by the SE-HPLC method was 1.72 $g.L^{-1}$ versus 1.64 $g.L^{-1}$ with the Kjeldahl method, which represents a relative difference of 4.8 %. As for sunflower, the respective values observed by SE-HPLC and Kjeldahl were 5.70 $g.L^{-1}$ and 6.41 $g.L^{-1}$ meaning a relative difference of 11 %.

As a result, these preliminary experiments prove the validity of the SE-HPLC method on these resources as well. Because rapeseed (*Brassica napus*), sunflower (*Helianthus*) and linseed (*Linum usitatissimum*) are different biologically and because the SE-HPLC method works on all three, it is very likely that the proposed method will also work with other oleoproteaginous plant proteins.

Conclusion

This study proved the possibility to use SE-HPLC to characterize protein-rich aqueous extracts from rapeseed: indeed, both the soluble protein content and the proportion of each major protein type

was determined by this method. It was shown to give statistically equivalent results compared to the Kjeldahl method, for protein isolates but also for proteins in aqueous mediums of extraction. The comparison with SDS-PAGE densitometry was also concluding for the analysis of protein isolates even though very small concentrations were required. However, it was likely biased by the presence of polyphenols in aqueous extracts resulting in interferences in SDS-PAGE gels.

Furthermore, the proposed method allows not only the quantification of the protein content in a sample but also the determination of the proportion of each major proteins. Moreover, this method presents several major advantages: it is quick, with a 30 minutes analysis per sample; no sample preparation is required after filtration; the amount of effluent generated is moderate compared to Kjeldahl; it is accurate and reliable. Finally, it was shown to be usable also on other oleoproteaginous resources.

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Figure captions

Fig. 1: Total proteins isolate analysis, A. Chromatograms of the SE-HPLC method at 214 and 325 nm ; B. Fractionation of the main peaks observed in SE-HPLC and SDS-PAGE analysis of the obtained fractions.

Fig. 2: Globulins and albumins isolates analysis by SE-HPLC at 214 nm and SDS-PAGE.

Fig. 3: Methodology for the validation of the SE-HPLC method. Light blue boxes represent analytical steps and grey boxes show the calculated values. Below are the values obtained by both methods represented as functions of the isolate concentration. The Ka is the ratio of both slopes.

Fig. 4: Stacked chromatograms of extracts at 214 nm and for three extraction conditions. The blue line corresponds to pH 2 0 M NaCl, the orange to pH 7 0 M NaCl and the green to pH 9 0.3 M NaCl. On the right side of the figure is a zoom of the proteins peaks.

Fig.5: Stability study of several rapeseed protein extracts under different storage conditions (room temperature, 4 °C and -20 °C) after 24 h (A) or 48 h (B). Values on top of the bars represent the absolute relative difference of area with the initial measure.

Tables

Table 1: Comparison of the SE-HPLC method with the Kjeldahl method and with the SDS-PAGE densitometry.

True protein content according to extraction condition	SE-HPLC method (N=3)	Kjeldahl (N=3)	Statistical comparison
			P-value T-test (two tails)
pH 2 0 M NaCl	10.5 ± 1.16	11.8 ± 0.81	0.197
pH 5 0 M NaCl	10.7 ± 0.76	12.3 ± 0.57	0.046 *
pH 7 0 M NaCl	11.0 ± 0.64	10.7 ± 1.04	0.691
pH 7 0.1 M NaCl	16.8 ± 0.79	16.3 ± 0.99	0.464
pH 9 0.3 M NaCl	21.4 ± 2.30	20.1 ± 0.45	0.441
Proportion of albumins	SE-HPLC method (N=3)	SDS-PAGE densitometry (N=3)	Statistical comparison
			P-value T-test (bilateral)
pH 2 0 M NaCl	90.7 ± 0.45	95.8 ± 1.06	0.002 *
pH 5 0 M NaCl	54.5 ± 2.27	55.2 ± 2.29	0.712
pH 7 0 M NaCl	30.7 ± 1.44	37.9 ± 4.33	0.052
pH 7 0.1 M NaCl	50.2 ± 1.20	57.0 ± 1.99	0.007 *
pH 9 0.3 M NaCl	50.3 ± 0.44	61.6 ± 3.02	0.023 *

*The highest part of the Table contains the data used for the characterization of the homemade protein isolates of globulins and albumins from rapeseed meal. The second part consists of the data used to compared the SE-HPLC method with references, Kjeldahl and SDS-PAGE densitometry. N corresponds to the number of repetition of the experiments. Experimental values are reprensented as mean ± the standard deviation. In the statistical analysis, values followed by * are below the signification threshold of 5 %.*

595 # Figures

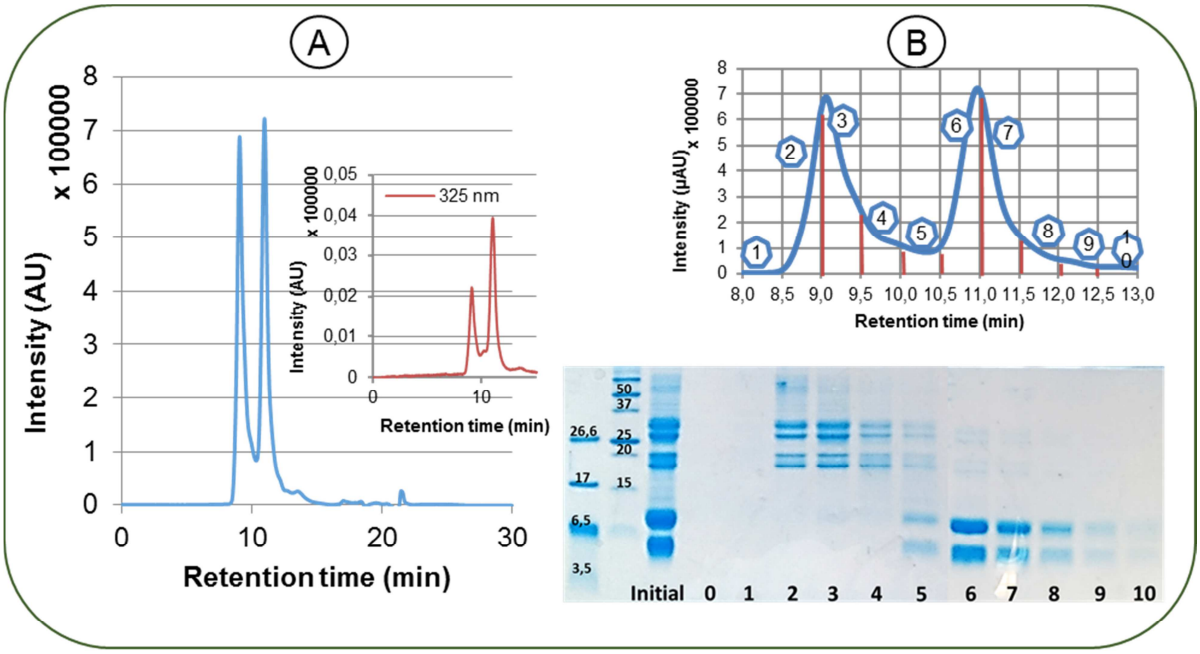


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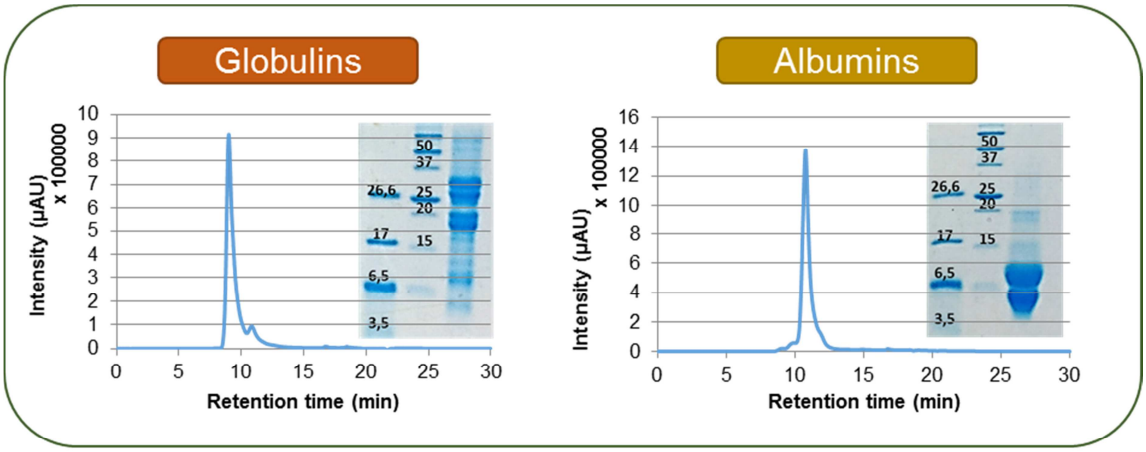


Fig. 2: Globulins and albumins isolates analysis by Size-Exclusion at 214 nm and SDS-PAGE.

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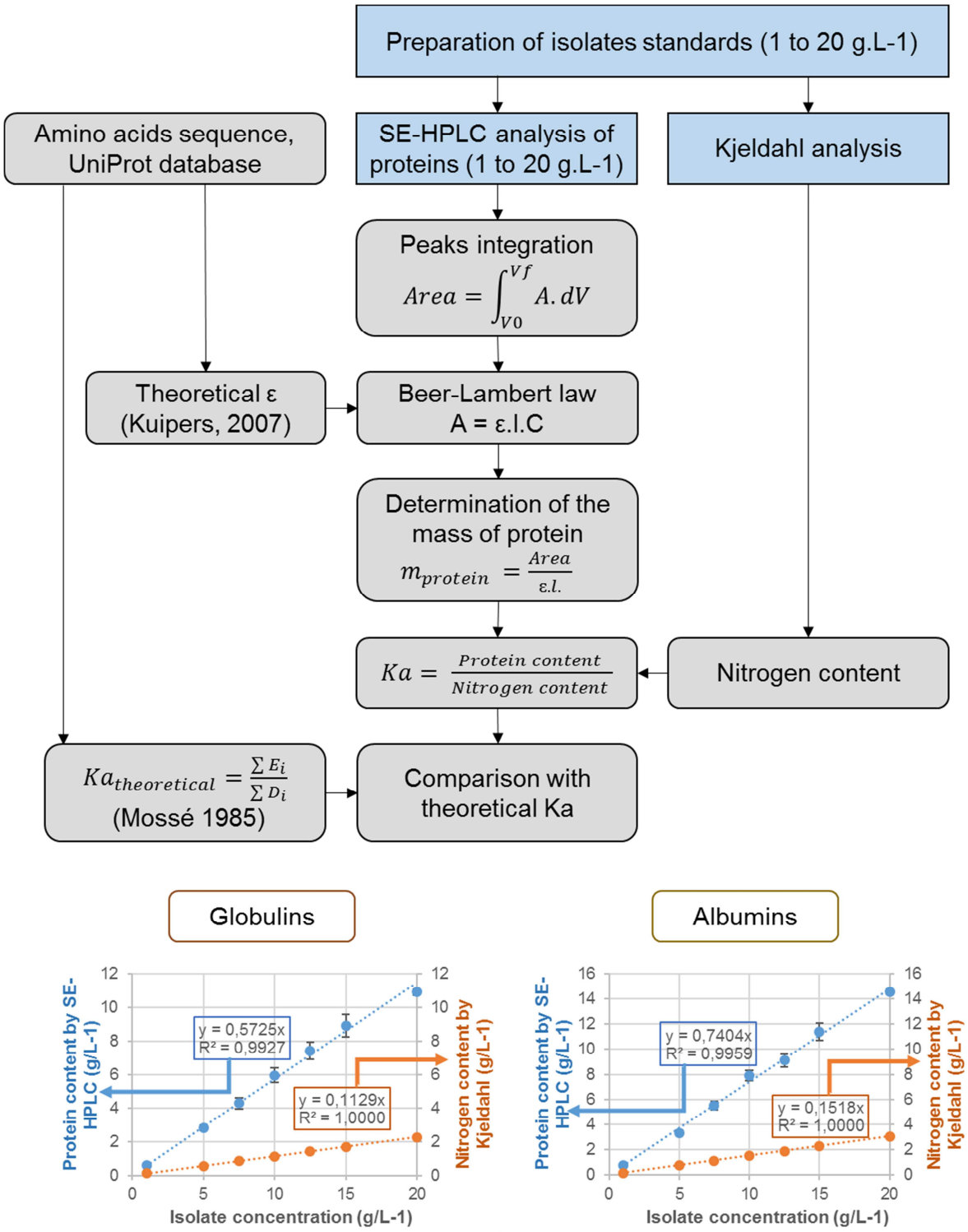


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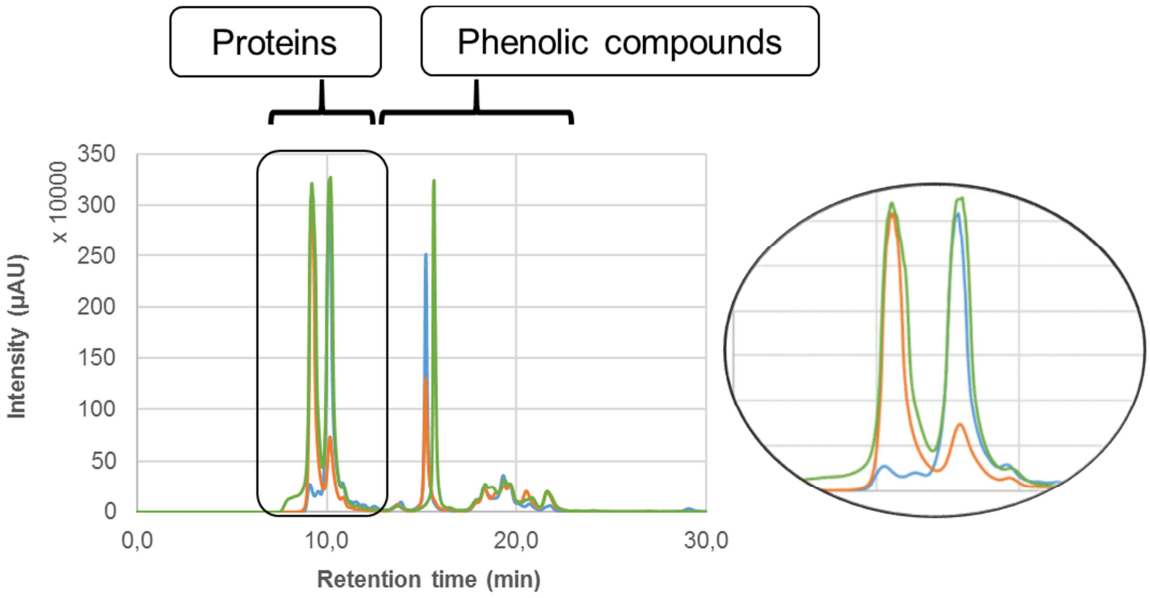


Fig. 4: Stacked chromatograms of extracts at 214 nm and for three extraction conditions. The blue line corresponds to pH 2 0 M NaCl, the orange to pH 7 0 M NaCl and the green to pH 9 0.3 M NaCl. On the right side of the figure is a zoom of the proteins peaks.

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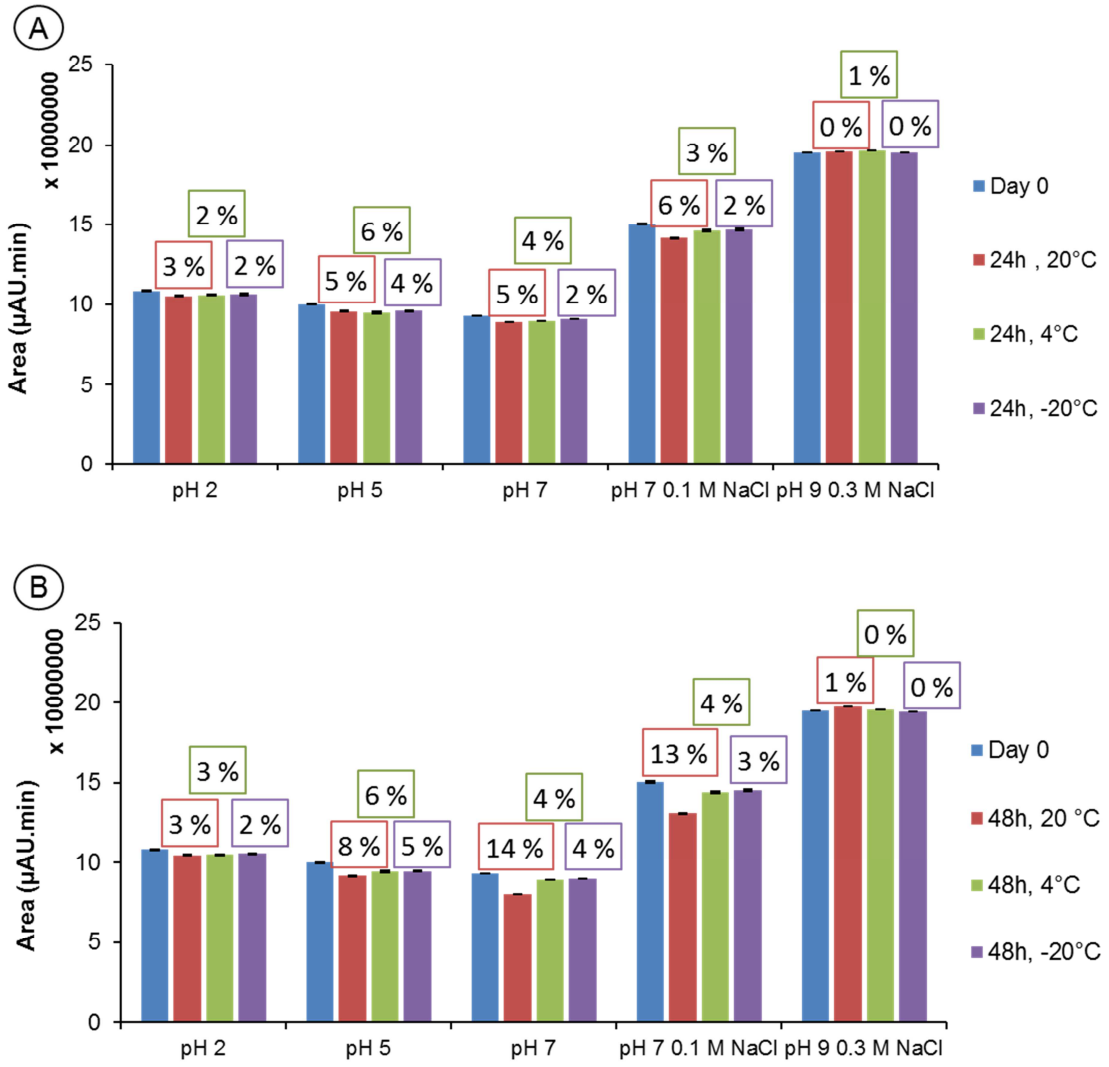


Fig.5: Stability study of several rapeseed protein extracts under different storage conditions (room temperature, 4 °C and -20 °C) after 24 h (A) or 48 h (B). Values on top of the bars represent the absolute relative difference of area with the initial measure.

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Supplementary material

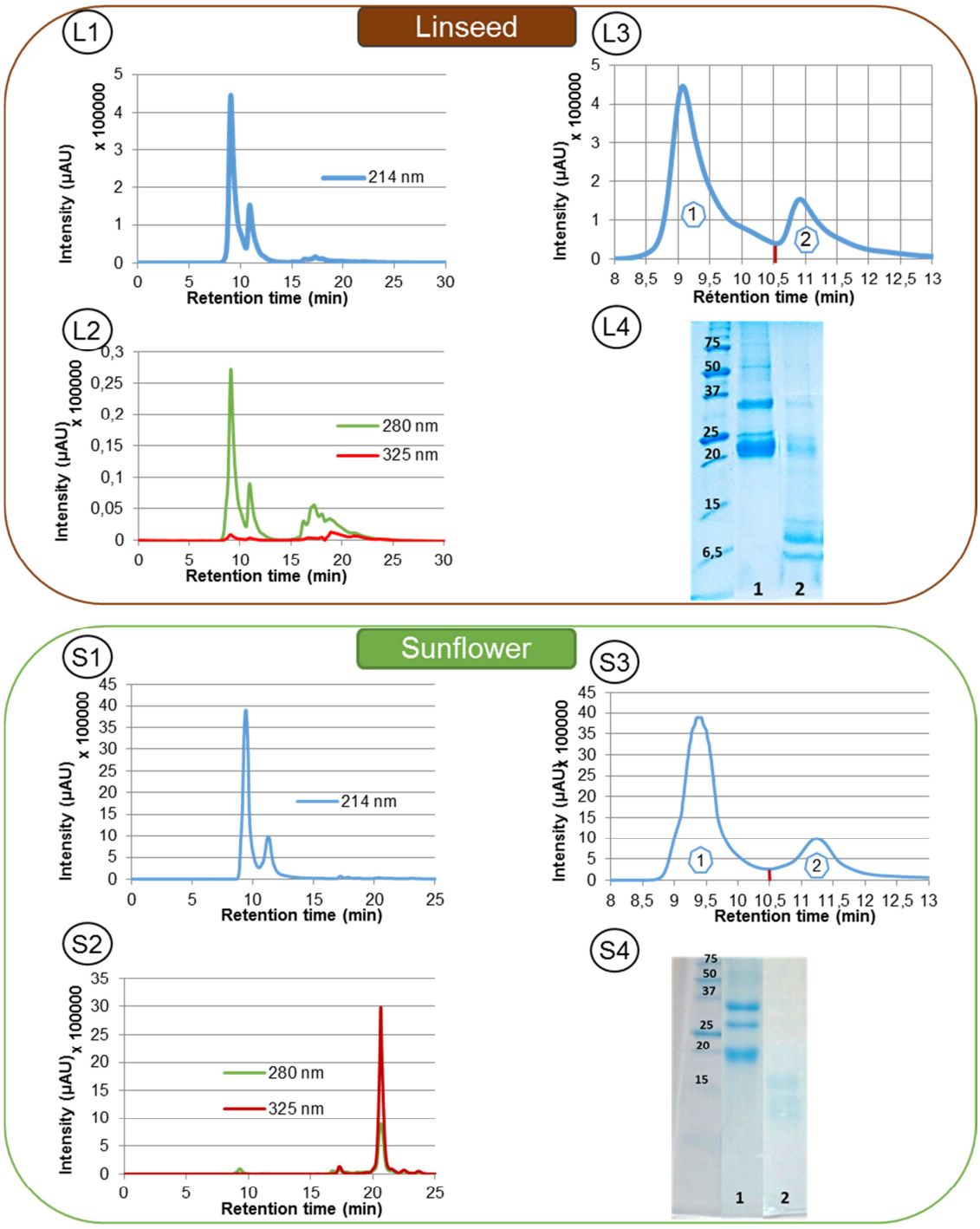
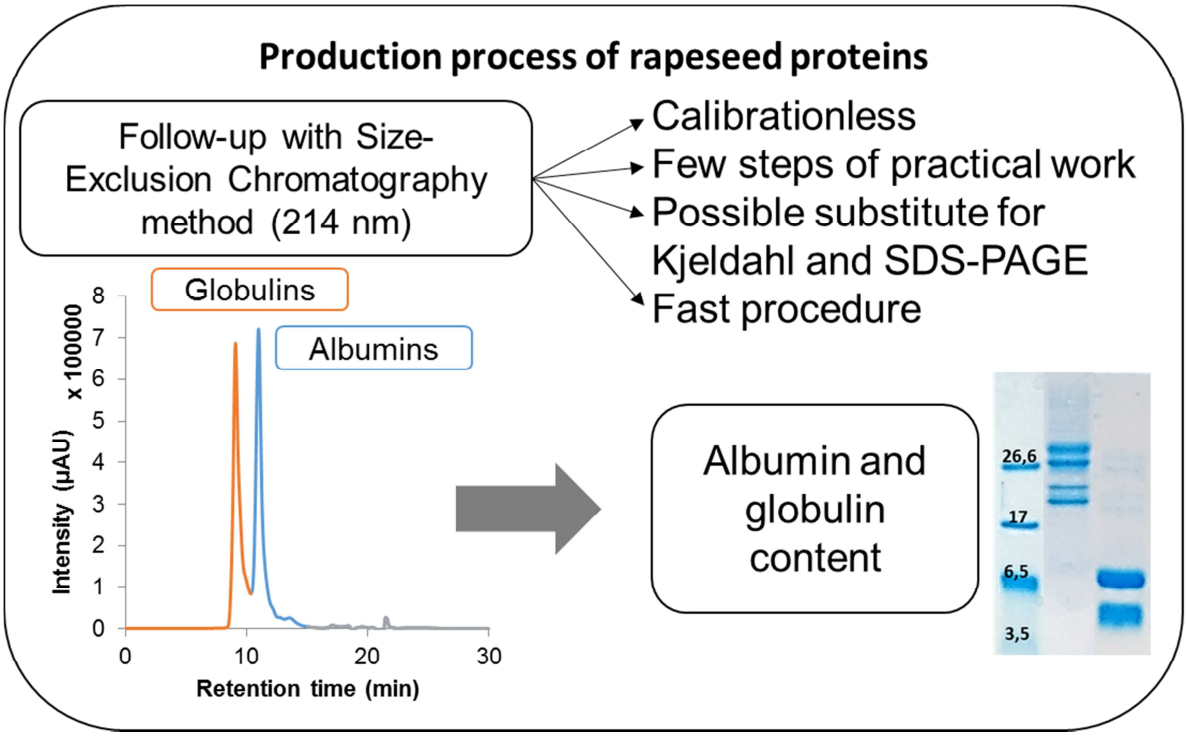


Fig. S. 1: L1 and S1 are the chromatograms at 214 nm ; L2 and S2 are the stacked chromatograms at 280 nm and 325 nm ; L3 and S3 are zooms from 214 nm chromatograms and show the different fractions shown on SDS-PAGE gels in reducing conditions, L4 and S4.

603 Table S.2: Validation of the method according to the International Conference on Harmonisation (ICH) guideline.

	Globulins	Albumins
Regression coefficient	0.9927	0.9959
y-intercept	0	0
Slope	81.5	75.0
Concentration range	0.6 to 12.0 g/L^{-1}	0.4 to 16.2 g/L^{-1}
Repeatability (intra-day variation)	0.3 %	2.8 %
Intermediate precision (inter-day variation)	0.9 %	3.4 %
Detection limit	0.008 g/L^{-1}	0.010 g/L^{-1}
Quantitation limit	0.026 g/L^{-1}	0.033 g/L^{-1}

604 ## Graphical abstract



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