Short Communication

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Determination of Protein in Lupin Using Liquid Chromatography Coupled with Organic Carbon Detector and Organic Nitrogen Detector (LC-OCD-OND): Validation of a New Method

Maria V. Chandra-Hioe[1*], Alice Antony[2] and Jayashree Arcot[1]

[1]ARC Training Centre for Advanced Technologies in Food Manufacture, School of Chemical Engineering, UNSW Sydney, Chemical Sciences Building (F10) High Street (gate 2), Sydney NSW 2052, Australia

[2]UNESCO Centre for Membrane Science and Technology, School of Chemical Engineering, UNSW Sydney, Chemical Sciences Building (F10) High Street (gate 2), Sydney 2052, Australia

*Correspondence to:

Maria V. Chandra-Hioe
ARC Training Centre for Advanced Technologies in Food Manufacture, School of Chemical Engineering, UNSW Sydney
Chemical Sciences Building (F10) High Street (gate 2), Sydney NSW 2052, Australia
E-mail: m.v.chandra-hioe@unsw.edu.au

Abstract

The Kjeldahl or Dumas methods used to estimate the crude protein in foods measure total organic nitrogen of foods. The limitation of these methods is that the estimated nitrogen is not solely derived from proteins. This is the first study to determine the protein content of Australian sweet lupin using size-exclusion chromatography interfaced with organic carbon detector and organic nitrogen detector (LC-OCD-OND), a method that is widely used in water research. Protein was initially extracted into 10 mM phosphate buffer saline, filtered and diluted prior to analysis. Measured protein in the lupin flour and the lupin protein isolate were 1.6 g and 6.1 g per 100 g, respectively, in agreement with results obtained from Bicinchoninic assay. The coefficients of variation for both intra and inter-variability studies were 5%. The work presented here introduces a fast and simple protein extraction from solid state prior to LC-OCD-OND analysis that specifically measures only soluble protein in lupins.

Keywords

Protein, Food, Size exclusion, Chromatography, Organic carbon detector, Organic nitrogen detector

Abbreviations

LC-OCD-OND: Liquid Chromatography-Organic Carbon Detector-Organic Nitrogen Detector; HgO: Mercury (II) Oxide; CO_2: Carbon Dioxide; H_2O: Water; NO_2: Nitrogen Oxide; SO_2: Sulphur Dioxide; PBS: Phosphate Buffer saline; DOM: Dissolved Organic Matter; kDa: Kilo Dalton; CV: Coefficient Variation

Introduction

In food composition studies, protein contents in food are generally obtained by measuring the total nitrogen or total organic nitrogen, and the value is multiplied by the Kjeldahl factor to estimate the total protein content in food. A factor of 6.25 is commonly used based on the assumption that protein contains 16 percent of nitrogen and a 5.70 conversion factor has been proposed for plant-based proteins due to their higher nitrogen contents [1]. In the traditional Kjeldahl method, nitrogen containing compounds in food are converted to ammonium sulphate after digestion with sulphuric acid at its boiling point. Ammonium sulphate undergoes reaction with excess alkaline solution resulting in ammonia vapour, from which the percentage of nitrogen can be determined [2], and this reaction is catalysed by metal oxides, such as HgO. The limitations of the Kjeldahl method

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are that the use of both concentrated acidic solutions at a high temperature and hazardous catalyst(s) cause increased health and safety risks. Another method involves the combustion of food that generates CO_2, H_2O, NO_2 and SO_2. In the Dumas method, NO_2 is formed and converted to nitrogen, which is then measured by a thermal conductivity detector. The drawback of these methods is that nitrogen estimated in food is not necessarily derived from protein [2].

Liquid chromatography coupled with organic carbon detector and organic nitrogen detector (LC-OCD-OND) is commonly employed to study the dissolved organic matter (DOM) in water matrices [3]. This technique is based on size exclusion, in which various DOM components are separated into fractions according to their molecular weights [4]. One of these fractions is a biopolymer that consists of polysaccharides, proteins and amino sugars. This fraction has a molecular weight greater than 20,000 g mol^{-1} and starts to elute between 26-32 minutes. The biopolymer fraction itself consisting of polysaccharides, proteins and amino sugars is measured by the organic carbon detector, whereas the corresponding peak from the organic nitrogen detector is representative of proteins [5]. The organic nitrogen detector has an advantage that it is capable of distinguishing the organic bound nitrogen present in biopolymers and inorganic bound nitrogen found in ammonium, nitrite and urea; whilst primary nitrate remains unaltered [3]. Assuming that all the nitrogen is bound in proteinaceous matter and assuming a typical carbon to nitrogen mass ratio (C:N) of 3 for proteins, the nitrogen content can be calculated from the two values, nitrogen carbon ratio and quantified organic nitrogen. The amount of bound nitrogen in the biopolymers can be determined, which represents protein contents. Other peaks indicate compounds with molecular weight ranges between 350-1000 Da. Peak representing molecular weight of 350 Da indicates the presence of sugars and amino acids. This study aimed to determine the protein contents in lupin flour and its protein isolate using LC-OCD-OND. Australian sweet lupin is a rich source of plant-based protein and currently there is an interest to use this commodity as food ingredients for human consumption.

Materials and Methods

Bovostar bovine serum albumin (66 kDa) used for characterisation of a protein peak was acquired from Bovogen Biologicals (East Keilor, Australia). Tryptophan used for identifying a peak of low molecular weight (204 Da) and other analytical grade chemicals, such as phosphate buffered saline (10 mM PBS) and potassium nitrate, were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Milli-Q water with conductance ≤ 0.1 µS/cm was sourced from a Millipore system (Bedford, MA, USA). *Suwanee* humic acid and fulvic acid reference standards were obtained from the International Humic Substance Society (St. Paul, MN, USA). Commercial lupin flour was provided by Coorow Seeds (Coorow, Western Australia), and chickpea flour and soy flour were obtained from retail shops in Sydney Metropolitan Area (NSW, Australia).

Lupin protein isolate

The lupin flour (20 g, n = 14) was initially defatted using hexane and then processed in aqueous fractionation to obtain lupin protein isolate [6] with minor modifications. Milli-Q water was aliquoted to defatted flour and the pH was adjusted to 9.0 with 1 M sodium hydroxide. Samples were placed on a ten-roller mixer (Thermo Scientific, Sydney, Australia) for 2 hours and centrifuged (Thermo Scientific, Sydney, Australia) at 10,000 g for 15 minutes at ambient temperature. The supernatants containing the protein-rich fraction were adjusted to pH 4.5 with 1 M hydrochloric acid, placed on the ten-roller mixer and centrifuged. Afterwards, the supernatants were freeze-dried (Scanvac Coolsafe Labogene, Lynge, Denmark) overnight, ground into fine powder and stored in the freezer for further analysis.

Sample preparation

Samples of de-fatted lupin flour and lupin protein isolate (0.01 g, in duplicate) were initially dissolved in 5 mL of 10 mM PBS buffer and sonicated (Hwashin Technology Co., Seoul, Korea) for 10 minutes. After that, samples were placed on the ten-roller mixer at room temperature for 1 hour and centrifuged. The supernatants were immediately analysed for total organic carbon, which will be described in detail below, and then they were stored in the fridge for crude protein determination using LC-OCD-OND and Bicinchoninic assay [7]. Pre-weighed bovine serum albumin (m = 0.0355 g) was initially dissolved in 5 mL of 10 mM PBS and treated as previously described. L-tryptophan (m = 0.0202 g) was added into 1 L of Milli-Q water and mixed using a magnetic stirrer to obtain a homogenous solution. This sample was used for peak detection of the low molecular weight nitrogen. Similarly, samples of lupin flour, chickpea flour and soy flour were prepared for the LC-OCD-OND analysis. In addition, flour samples of lupin, chickpea and soy (in solid form) were directly analysed for their nitrogen content by Dumas combustion method (LECO TC600, Sydney, Australia).

Determination of total organic carbon

At first, the supernatants were diluted with Milli-Q water and filtered using 0.45 µm RC syringe filter (Sartorius, Sydney, Australia), before the concentrations of their total organic carbon and dissolved organic carbon (Shimadzu TOC analyser, Kyoto, Japan) were determined. Samples were further diluted as required to obtain a total organic carbon reading of approximately 5 µg/L.

LC-OCD-OND analysis

LC-OCD-OND analysis (DOC Labor, Karlsruhe, Germany) was carried out according to Henderson et al. [8]. A weak cation exchange column (Toyopearl HW-50S, Tosoh Bioscience, Tokyo, Japan) was employed to separate a wide range of molecular weights based on their steric interaction. The mobile phase system was 28 mM phosphate buffer (pH 6.6) and the flow rate was 1.1 mL/min. The molecular masses were calibrated with *Suwanee* humic acid and fulvic acid reference standards and the detector was calibrated according to the manufacturer's recommendation [3]. The

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sensitivity of the OND was tested using potassium nitrate as per manufacturer's recommendation.

To assess the intra-assay variability expressed in percentage of coefficient of variation (CV), lupin samples (n = 8) were simultaneously prepared, as previously described, on the same day and then analysed on the same run. The inter-assay variability was tested by analysing the lupin samples (n = 2) that were freshly prepared every day for 4 consecutive days. The intra-variability and the inter-variability studies were carried out to determine the precision of the method.

Data analysis

ChromCALC software (DOC Labor, Karlsruhe, Germany) was employed for peak integration and quantitation. External standard calibration curve was prepared employing the potassium nitrate solutions to calculate the dissolved organic nitrogen in samples. The data on dissolved organic nitrogen was used to compute the protein content in plant-based samples by multiplying percentage of nitrogen (% N) by 5.7 to obtain percentage of protein.

Results and Discussion

Peak identification

Dissolved carbon and nitrogen matter were chromatographically separated and identified by organic carbon, organic nitrogen and UV detectors. In this study, bovine serum albumin with a known molecular weight (approximately 66 kDa) was used for peak characterisation by OND. The protein peak eluted at 30 minutes of retention time and showed UV-active components (Figure 1). Besides distinct peak identification, the OND chromatograms 1 and 2 display the injection's precision of the same sample (Figure 1). The OND chromatogram 3 (Figure 1) shows lower signal intensity than both chromatograms 1 and 2 as a result of sample dilution.
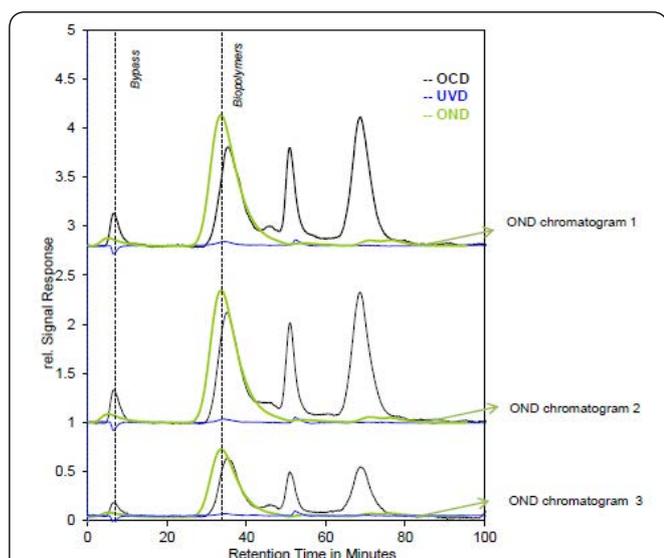

Figure 1: Peaks of bovine serum albumin (OND chromatograms 1 and 2) eluting at 30 minutes. Dilution of the same sample gave rise to less signal intensity as shown in OND chromatogram 3.

Validation parameters

A standard calibration curve covering 13.9-695 μg/L of potassium nitrate was linear ($r^2 = 0.99$) and the lowest standard concentration was set as the limit of detection. The precision expressed as the coefficient of variation for both intra-assay and inter-assay variability studies was 5%.

Measuring protein in lupin samples

Figure 2 shows peaks, which eluted at 30 minutes corresponding to hydrophilic fractions with a high molecular weight. No peaks were detected after 60 minutes, which could point out to amino acids in lupin isolate.

The LC-OCD-OND method was then applied to determine de-fatted lupin flour, which contained 1.6 g protein/100 g and was not significantly different when determined by Bicinchoninic assay (2.1 g/100g; p>0.05). The protein content in the lupin protein isolate was 6.1 g/100 g and was also comparable to Bicinchoninic assay with 5.9 g/100 g. As previously described, the same samples were used for both Bicinchoninic assay and LC-OCD-OND that measured only dissolved nitrogen. The comparable results obtained from these methods suggested, that both LC-OCD-OND and Bicinchoninic assay would have measured soluble protein rather than insoluble protein, indicating that polar side chains, such as basic and acidic amino acids, are present in the soluble protein [9].
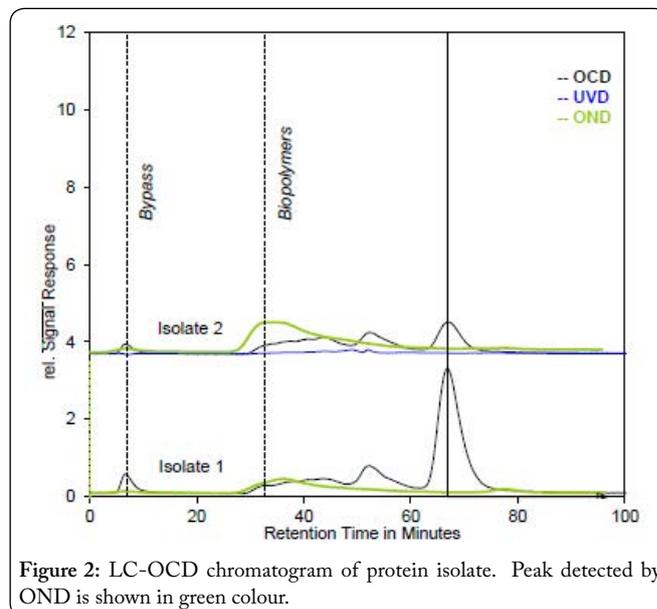

Figure 2: LC-OCD chromatogram of protein isolate. Peak detected by OND is shown in green colour.

Protein measured using LC-OCD-OND were lower than Dumas combustion analysis in all samples (Table 1). The plausible reason for these differences is that LC-OCD-OND specifically measures dissolved organic nitrogen represented by the protein peak, whilst dissolved inorganic nitrogen is eluted as inorganic nitrogen peak. However, in the Dumas method, both organic and inorganic nitrogen present in food samples was subjected to combustion, leading to higher measured nitrogen content that potentially could be derived from non-protein source. As previously reported, protein measured

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using a Dumas method generally show higher values than the Kjeldhal method [10]. Another reason would be that amino acids also occur in other forms, such as amides, glutamine and asparagine [9], which might contribute to higher measured protein values in Dumas method.

Mean protein in lupin flour determined by the BCA assay was 22.1 ± 1 g/100 g and was not significantly different compared to the value analysed by LC-OCD (p > 0.05, Table 1). Similarly, the mean difference in protein contents between samples of chickpea flour and soy flour was not significant (p > 0.05). Samples of chickpea flour contained 6.2 ± 0.1 g and 5.1 ± 0.3 proteins per 100 g, determined using BCA assay and LC-OCD, respectively. The protein contents in soy flour were 13.2 ± 0.8 and 11.7 ± 0.4 g/100 g when samples were analysed using BCA assay and LC-OCD, respectively.

Table 1: The mean protein (g/100 g) measured in selected samples using LC-OCD-OND and Dumas combustion analysis (± corresponds to standard deviation).

Samples	Protein (g/100g; LC-OCD-OND)	Protein (g/100g; Dumas)
Lupin flour	22.2 ± 3	42.8 ± 0.6
Chickpea flour	5.1 ± 0.3	22.4 ± 0.5
Soy flour	11.7 ± 0.4	34.4 ± 0.4

We reported for the first time an optimised method to determine protein contents of foods using LC-OCD-OND that is commonly used for water matrices. In particular, this study highlighted a new and simple approach to prepare solid samples suitable for LC-OCD-OND. The experimental data obtained from LC-OCD-OND and BCA assay was comparable, suggesting that both methods measured soluble protein in foods. Protein contents determined by Dumas method were higher than LC-OCD-OND, possibly due to combustion of non-protein nitrogen. The advantage of chromatographic separation based on molecular weight and the use of organic nitrogen detector was that this technique specifically detected dissolved nitrogen for quantitation of protein in lupin. Importantly, this study lays the groundwork for future research on solid to liquid mass transfer or solid-liquid extraction prior to LC-OCD-OND analysis.

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