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Size-exclusion HPLC as a sensitive and calibrationless method for complex peptide mixtures quantification

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ABSTRACT

This work describes an original methodology to quantify complex peptide mixtures by size-exclusion high-performance liquid chromatography (SE-HPLC). The methodology was first tested on simulated elutions of peptide mixtures. For this set of experiments, a good estimation of the total peptide concentration was observed (error less than 10 %). Then 30 fractions obtained by ultrafiltration of hydrolysates from two different sources were titrated by Kjeldahl or BCA analysis and analysed by SE-HPLC for an experimental validation of the methodology. Very good matchs between methods were obtained. The linear working range depends on the hydrolysate but is generally between 0.2 and 4 g L^{-1} (i.e. between 10 and 200 μg). Moreover, the presence of organic solvents or salts in samples does not impact the accuracy of the methodology contrary to common quantification methods. Hence, the findings of this study show that total concentration of complex peptide mixture can be efficiently determinate by the proposed methodology using simple SE-HPLC analysis.

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1. Introduction

Protein hydrolysates are complex peptide mixtures obtained by enzymatic proteolysis of proteins from various vegetable or animal resources (e.g. soy, wheat, rapeseed [1–3], milk or fish [4,5]). Their main applications are in different fields of human nutrition (clinical, sport or regular nutrition [6,7]) or as ingredient for food product texturations due to their functional properties (such as emulsifying, foaming [8,9]). Besides, hydrolysates may contain biologically active peptide like antioxidative [10], opioid [11], antihypertensive [12] or antimicrobial peptides [13] that enlarge their potential of use in other high added-value industrial areas like nutraceutrics, food security or cell culture medias. Their functional properties or bioactivities are often improved by enriching mixtures in targeted peptides [14–16] or peptide fractions [17] by membrane filtration or chromatography [14–19].

The total quantification of hydrolysates or fractions are achieved either by Kjeldahl method (nitrogen quantification) or by spectrophotometric assays. Kjeldahl analysis consists in a mineralization of organic nitrogen into ammonia, titrated by an acid [20]. This procedure is time-consuming and requires relatively large amount of peptide fraction sample (between 0.2 and 5 g according to the sample concentration, nitrogen/proteins converting factor and apparatus sensitivity [21]). Furthermore, this method can not be used directly when the sample contains acetonitrile (due to the large input of nitrogen) which is classically used in separation processes based on hydrophobic interactions. Spectrophotometric methods are based on the formation of colored complexes coming from reagent reactions and peptide properties (like Lowry assay or bicinchoninic acid (BCA) assay [12,22–24]). These methods are faster than Kjeldahl analysis, and require low sample amounts. However, the accuracy of the assay strongly depends on the calibration of the system. Unfortunately, the calibration (often realized with the initial hydrolysate) may introduce a bias for a peptide fraction quantification because of a modification of peptide composition or environment (salts, solvents, etc.).

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Peptides are well known to possess intrinsic UV absorbance properties classically monitored at 214 nm. This property results in a complex contribution of peptide bonds and amino acid lateral chains (each one being able to have a varied contribution). Recently, it has been shown that the molar extinction coefficient at 214 nm (ε_{214nm}) of a peptide can be precisely calculated on the basis of its amount of peptide bond and its peptide composition [25]. This allows to quantify a pure peptide of known aminoacid composition by using the Beer–Lambert law but not a complex peptide mixture.

Hydrolysates or peptide fractions are systematically analysed by size exclusion high-performance liquid chromatography (SE-HPLC) in order to assess their peptide molecular weight distribution [9,23,26–28]. The aim of this work is, first, to propose an original methodology that uses SE-HPLC for fast and accurate total peptide quantification that does not necessitate calibration for each type of protein hydrolysate. The methodology was tested on simulated chromatograms of peptide mixtures in order to evaluate its applyability on controlled peptide molecular weight distributions and compositions. Then, accuracy, repeatability and linearity of the methodology were investigated using two different protein hydrolysates. In a third part, the quantifications of 30 actual different ultrafiltration fractions obtained from the methodology, Kjeldahl analysis and BCA assay were compared. Eventually, the influence on the SE-HPLC assay of the potential chemical environment (organic solvent and salt) related to hydrolysate preparation techniques was investigated.

2. Methodology

Theoretically, it is possible to calculate the concentration of a peptide mixture from SE-HPLC analysis by applying the the Beer–Lambert law to each point 'x' of the chromatogram (in order to convert absorbances into concentrations) and to integrate the overall concentration signal. To do so, Eq. (1) can be used:

$$C_{\text{sample}} = \frac{1}{V_{\text{inj}}} \int \frac{A_x}{\varepsilon_x l} \text{dV} \quad (1)$$

A_x and ε_x standing for the absorbance and the molar extinction coefficient for each point 'x' of the chromatogram, dV a fraction of the elution volume and V_{inj} the injection volume.

The difficulty lies on assigning a proper molar extinction coefficient to every chromatogram point. For pure peptides, Kuipers and Gruppen [25] have demonstrated that the molar extinction coefficient at 214 nm can be calculated using Eq. (2):

$$\varepsilon_{\text{pure peptide}} = (n_{\text{amino acid}} - 1)\,\varepsilon_{\text{bond}} + \sum_{i=1}^{20} \left(\varepsilon_{aa,\,i} \times n_{\text{amino acid},i}\right) \quad (2)$$

Eq. (2) can be rearranged as in Eq. (3):

$$\varepsilon_{\text{pure peptide}} = (n_{\text{amino acid}} - 1)\,\varepsilon_{\text{bond}} + \overline{\varepsilon}_{aa} \times n_{\text{amino acid}} \quad (3)$$

with $\varepsilon_{\text{bond}}$, the molar extinction coefficient of peptide bond, $n_{\text{amino acid}}$, the total number of the amino acid in the peptide and $\overline{\varepsilon}_{aa}$, the mean molar extinction coefficient by amino acid calculated from the peptide sequence.

SE-HPLC separation is based on solute steric hindrances and is not resolutive enough to separate the peptides from a complex mixture one from anothers. So each point 'x' of the chromatogram represents the UV signal of a peptide mixture with close molar mass.

We assumed that 'x' is a mixture of peptides having a mean molar mass (MM_x) that can be determined by the column calibration which is classically under the form of Eq. (4):

$$\text{MM}_x = 10^{a \times V_x + b} \quad (4)$$

with V_x, the elution volume of the point 'x', a and b the coefficients of the calibration curve.

It comes, that the number of amino acids to be used in Eq. (3) can be calculated by Eq. (5):

$$n_{\text{amino acid}} = \frac{\text{MM}_x}{\overline{\text{MM}}_{aa}} \quad (5)$$

with $\overline{\text{MM}}_{aa}$, the mean amino acid molar mass of the hydrolysate.

Other physico-chemical properties (charge, hydrophilicity...) do not modify SE-HPLC separation much. So we also hypothesized that aminoacid compositions of peptide mixture 'x' and hydrolysate are the same. As a consequence, $\overline{\text{MM}}_{aa}$ and $\overline{\varepsilon}_{aa}$ in 'x' should not differ from the hydrolysate.

Thus, hydrolysate aminogram (or the aminogram of the protein hydrolyzed) can be used to calculate $\overline{\text{MM}}_{aa}$ and $\overline{\varepsilon}_{aa}$ by applying Eqs. (6) and (7), respectively:

$$\overline{\text{MM}}_{aa} = \sum_{i=1}^{20} w_i \times \text{MM}_i \quad (6)$$

MM_i and w_i standing for the molar mass and the mass ratio of amino acid i in the hydrolysate aminogram, respectively.

$$\overline{\varepsilon}_{aa} = \sum_{i=1}^{20} x_i \times \varepsilon_{\text{amino acid, i}} \quad (7)$$

with $\varepsilon_{\text{amino acid,i}}$, the molar extinction coefficient of amino acid i and x_i, the molar ratio of amino acid i in the hydrolysate aminogram.

Taking as a whole, the molar extinction coefficient to apply in Eq. (1) is calculated as follows for each point x:

$$\varepsilon_x = \varepsilon_{\text{bond}} \times \left(\frac{10^{a \times V_x + b}}{\overline{\text{MM}}_{aa}} - 1\right) + \overline{\varepsilon}_{aa} \times \frac{10^{a \times V_x + b}}{\overline{\text{MM}}_{aa}} \quad (8)$$

The overall methodology for the quantification of peptide mixture concentration on the basis of its SE-HPLC chromatogram is summarized in Fig. 1.

3. Material and methods

3.1. Material

Sodium hydroxide pellets and sodium chloride were supplied by Carlo Erba Reactifs (Val de Reuil, France). HPLC grade eluents (water and acetonitrile) were both purchased from Fisher Scientific (Geel, Belgium). Trifluoroacetic acid was obtained from Arcos Organics (Geel, Belgium). For Kjeldahl analysis, sulfuric acid and chloridric acid was obtained from Carlo Erba Reactifs, ammoniac and hydrogen peroxide from Fisher Scientific. The reagents for the BCA test came from Pierce Biotechnology (Rockford, IL, USA). Synthetic peptides used for the column calibration were obtained (GeneCust, Dudelange, Luxembourg).

3.2. Size exclusion chromatography analysis

Samples (hydolysates and ultrafiltration fractions) were analyzed by SE-HPLC using a Superdex peptide 10/300 GL column (10 × 300 mm, GE Healthcare) connected to a Shimadzu model LC20 system (Shimadzu Corporation, Japan). 50 μL of sample were injected onto the column kept at 35 °C. The mobile phase consisted in water and acetonitrile in a 70/30 proportion (v/v) with 0.1 % TFA (v/v). Samples were eluted at a flow rate of 0.5 mL min^{-1}. UV signal was measured at 214 nm using a cell with an optical path of 1 cm. Hydrolysate and UF fraction concentrations were between 0.2 and 2 g L^{-1}. Hydrolysates were also prepared between 0.25 and 20 g L^{-1} to determinate the linear working range of the methodology. Each sample was filtered through 0.22 μm filter prior analysis. Chromatograms were exported in Excel spreadsheets to apply the methodology of quantification.

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