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In-situ protein determination to monitor contamination in a centrifugal partition chromatograph

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ABSTRACT

Centrifugal partition chromatography (CPC) works with biphasic liquid systems including aqueous two-phase systems. Metallic rotors are able to retain an aqueous stationary phase able to purify proteins. But the adhesion of proteins to solid surface may pose a cross-contamination risk during downstream processes. So it is of utmost importance to ensure the cleanliness of the equipment and detect possible protein contamination in a timely manner. Thereby, a direct method that allows the determination of the effective presence of proteins and the extent of contamination in the metallic CPC rotors was developed. This in-situ method is derived from the Amino Density Estimation by Colorimetric Assay (ADECA) which is based on the affinity of a dye, Coomassie Brillant Blue (CBB), with protonated N^+ groups of the proteins. In this paper, the ADECA method was developed dynamically, on a 25 mL stainless-steel rotor with various extents of protein contaminations using bovine serum albumin (BSA) as a fouling model. The eluted CBB dye was quantified and found to respond linearly to BSA contamination up to 70 mg injected. Limits of detection and quantification were recorded as 0.9 mg and 3.1 mg, respectively. While the non-specific interactions between the dye and the rotor cannot currently be neglected, this method allows for in situ determination of proteins contamination and should contribute to the development of CPC as a separation tool in protein purification processes.

1. Introduction

Centrifugal liquid-liquid chromatography, including countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC), is a chromatographic separation technique based on the partition of solutes between two immiscible liquid phases with no solid support [1–3]. One of the two immiscible phases is retained in the column by centrifugal force fields; it is called the stationary phase. The other phase is the mobile phase; it percolates through the stationary one. CCC and CPC have numerous advantages such as a high loading capacity and no loss of solute since it is always possible to recover any material trapped in a liquid phase. These advantages were used over the past years to purify biomolecules, especially proteins, by CCC and CPC modes [4,5]. Aqueous Two Phase Systems (ATPS) were found very efficient for protein purification [6,7]. As their name says, ATPSs are composed by two immiscible aqueous phases. This is obtained either dissolving two polymers in water or dissolving a polymer and a salt or an ionic liquid and a salt. ATPSs combine a high biocompatibility and selectivity for biomolecules [8]. These solvent systems were proved effective in biopurification due to their high water content and low interfacial tension, which make them gentle towards proteins [9,10]. However, CCC was found unable to retain efficiently an aqueous liquid stationary phase likely due to the low ATPS interfacial tension [3]. CPC, with its constant centrifugal field and its rotor of interconnected chambers was able to retain ATPSs allowing for protein purification [1–3].

One of the issues when working with proteins is the risk of contamination of the CPC equipment. Indeed proteins can easily adsorb on the rotor material. Adhesion of proteins to solid surface can occur under various conditions and cause problems for biotechnology manufacturers. For example, in case of food

Abbreviations: CPC, Centrifugal partition Chromatography; BSA, Bovin Serum Albumin; ADECA, Amino Density Estimation by Colorimetric assay; PBS, Phosphate Buffer Salin; CBB, Coomassie Brillant Blue; FDA, Food Drug Administration; pI, Isoelectric point.

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manufacturing, proteins can form a fouling which is an unwanted deposit on the equipment surface [11]. Thereby, an insufficient cleaning may result in the development of bacteria and biofilm formation [12]. Moreover, the adherent proteins may pose a cross contamination risk [13]. For these safety reasons, the cross-contamination risks are strictly controlled through governmental organizations, such as the U.S Food and Drugs Administration (FDA). These Agency documents clearly establish the required expectation for cleaning procedure validation [14]. CPC is no exception. So, one of the most important issues for its development in the protein industry is to ensure the cleanliness of the equipment after a purification in order to avoid cross-contamination and hence to be able to detect protein contamination.

A cleaning method was specifically developed by Chollet [15] for CPC rotors. This method consists in alternate rinsing steps of water, 0.5M sodium hydroxide solution and sulfuric acid solution at 2.10^{-5} M, repeated twice. While it follows the FDA standards and the Good Manufacturing Practice (GMP), this method is only available in French. The validity of the cleaning procedure is controlled either by visual criterion opening the rotor and swiping the disks surface, or by the protein determination in the various rinse solutions via the Bradford method [16]. Unfortunately, the ultimate rinsing solutions are highly diluted, and their analysis requires a highly sensitive detection system. Although the validation of the cleaning protocol by swiping is soundproof, it is only accessible to instrument suppliers, as it requires the mechanical opening of the rotor. The industrial users do not have the facilities to tighten disks and to equilibrate weights after closure of this rotating device.

In the present work we propose an in-situ strategy to determine the state of protein contamination inside the rotor without opening the machine. This in-situ method derived from the Amino Density Estimation by Colorimetric Assay (ADECA) method [17]. This method was established to rapidly quantify grafted proteins on a solid support such as 96-well plates. It is based on the affinity of a dye, the Coomassie Brillant Blue (CBB), with protonated amino groups. The ADECA method consists in three steps: first a fixation step (or staining) ensures that the dye is bound to the surface material by an N^+-dye complex formation. Next a washing step removes any unbound dye. Last, the dye bounded to proteins is eluted by a pH switch which breaks the N^+-dye complex and the quantification of grafted protonated groups is directly related to the amount of released CBB dye. Thereby, this method should accurately indicate if any traces of proteins remain in a rotor after a full cleaning. The CBB dye would stick to such traces and any blue color seen during the acid wash would point remaining proteins. To set up this method in CPC instruments, we applied the ADECA protocol to a commercial 25 mL stainless steel rotor and the optimal conditions were determined. A graduated range of protein contamination was simulated using bovine serum albumin.

2. Experimental

2.1. Materials

The dye Coomassie Brillant Blue CBB-G250 (CBB, >99%, $C_{45}H_{44}N_3NaO_7S_2$, MW = 854 g/mol, a triphenylmethane dye with two benzene-sulfonic acid and three amine groups), potassium carbonate (>99.5%; MW = 138.2 g/mol), potassium bicarbonate (>99.5%; MW = 100.12 g/mol) and phosphate buffer saline (PBS) as well as the protein bovine serum albumin (BSA 96%, MW = 66463, pI = 4.7) were from Sigma Aldrich (Saint-Quentin Fallavier, France). Ethanol absolute was from ThermoFisher (Villebon-sur-Yvette, France).

The pH measurements are performed in the overall hydro-organic solution. The effective hydrogen activity in aqueous/organic solutions can be only estimated using water calibrated pHmeter and will be stated as “apparent pH”.

2.2. Instrumentation

The CPC instrument is a hydrostatic apparatus model, FCPC-A from Kromaton Rousselet-Robatel (Annonay, France) with interchangeable rotors. A stainless steel 316 rotor with a volume of 25 mL was mainly used in this study. For comparative assays, two prototype rotors were assessed: a stainless steel 316 rotor with a volume of 80 mL and a titanium rotor with a volume of 46 mL. The internal surface was calculated thanks to the cell and channel dimensions provided by the manufacturer and was evaluated at 0.38 m^2 for the 25 mL rotor, 0.61 m^2 for the 80 mL rotor and 0.31 m^2 for the 46 mL titanium rotor.

A Spot Prep II integrated system from Armen Instruments (Saint-Avé, France, Gilson USA) was used. This equipment is the assembly of a quaternary pump, an automatic loop injection valve fitted with a 1 mL sample loop, a UV/Vis spectrophotometer dual wavelength set up at 259 nm and 280 nm and a fraction collector.

2.3. Rotor cleaning procedure

After protein impregnation and/or ADECA implementation, the used rotors were fully cleaned according to Common Industrial Protocol, i.e. alkaline solution pH 14, for the equivalent of 3 column volumes.

2.4. Preparation of solutions

Solutions for the staining step were prepared by dissolving 500 mg of CBB in 100 mL of ethanol and 50 mL of glacial acetic acid (CH_3COOH) and stirred. After complete dissolution, deionized water was added up to a final volume of 1 L. The final composition of the staining solution was 0.05% (w/v) CBB, 10% ethanol, 5% CH_3COOH and 85% H_2O (v/v). The apparent pH is 2.4.

The composition of the washing solution was the same as that of the staining solution, i.e. 10% ethanol, 5% CH_3COOH and 85% H_2O (v/v) but with no CBB. For pH studies in the range 2.4–12, acetic acid or potassium carbonate was added until the desired target pH was reached.

The composition of the elution solution was 50% (v/v) EtOH and 50% carbonate buffer pH 12.

2.5. Extent of proteins contamination in various rotors

Five BSA proteins standard solutions were prepared in the range of 0 mg/mL to 200 mg/mL in phosphate buffer saline (PBS) pH = 7.4 or in carbonate buffer pH = 9 and pH = 12. The simulated contaminations were performed on a clean rotor by injecting 1 mL of protein solution in the rotor using the chromatographic system then rinsing by the buffer solution. It was previously checked that no significant adsorption happens in PEEK tubings and in the injection device. Blanks were performed by injecting phosphate buffer without protein.

2.6. Implementation of a dynamic ADECA method in a CPC rotor

The ADECA method developed to quantify the amount of grafted proteins on a surface contains three different steps. To transfer this static method performed in 96-well plates to a dynamic method in CPC rotor, the three ADECA steps were adapted as follows: **A-The staining step** was carried out pumping the staining solution at 10 mL/min during 15 min; i. e a volume of 150 mL staining solution; **B-The washing step** was achieved with a flow rate of 10 mL/min