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Purification of Coomassie Brilliant Blue G-250 by multiple dual mode countercurrent chromatography

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ABSTRACT

Commercial samples of Coomassie Brilliant Blue G-250 (CBB) were not pure enough to give reliable results when used as indicator of amine content in biological material. The polar and apolar impurities produce unacceptable biases in the results. Counter current chromatography (CCC) was used to purify significant amounts of CBB. The liquid system heptane/1-butanol/water 2:3:4 (v/v) was appropriate to separate crude CBB in three groups of components: polar, partitioning in the aqueous lower phase, intermediate, partitioning well between the aqueous and organic phases, and apolar, preferring greatly the organic phase. The dual-mode way of using a CCC chromatograph was found appropriate for the separation injecting the crude CBB in the middle of a two coil CCC instrument. A multi dual-mode purification was performed allowing to eliminate the polar impurities in the aqueous phase at the column tail and the apolar ones in the organic phase at the column head, trapping the purified dye inside the CCC column. 200 mg of purified CBB were obtained from 1 g of crude CBB in 3 h using as little as 150 mL of butanol and 70 mL of heptane with 200 mL of water. The purified CBB gave total satisfaction in testing amine content in polyclonal antibody containing monolith pipettes.

1. Introduction

Biomolecules and amines immobilized on solid supports were developed for micro-volume reactions and/or biocatalysis [1–3]. They found important uses in enzymatic conversion [4], molecular recognition [5] or biosensors engineering [6]. These supports need to be fully characterized. Spectroscopic techniques were used for that purpose but they are limited to accessible surfaces [7–11]. Other techniques including molecular labeling or protein total analysis have been described but these applications remained destructive analytical methods, lacked in terms of specificity or sensitivity or required the use of expansive reagents for the assays [12,13]. Recently, a simple and non-destructive colorimetric analytical method to quantify protonated amino groups in proteins, peptides or any amino containing structure adsorbed on solid supports has recently been proposed [14,15]. It was called the ADECA method for "Amino Density Estimation by Colorimetric Assay". This non-destructive method is based on specific and reversible Coomassie dye-amino group binding to positively charged amino groups. The surface containing protonated amino groups is stained by the dye. Next the excess dye is washed out without disrupting the dye-amino bonded part. Finally, a special sodium carbonate methanol buffer is used to release the attached dye whose colorimetric measure is easily related to the amino group concentration [15]. The ADECA method can be applied to a wide variety of supports of very differing hydrophobicity. Being reversible, the method is non-destructive. It means that the very same surface that was characterized by the ADECA method can be readily used for further coupling application or protein reaction [14,15]. However, the method requires the use of an extremely pure Coomassie Blue as the staining agent to avoid biases measuring the desorbed dye amount. Regular and commercial grade Coomassie Blue will produce biased results due to non-specific bindings [16].

Coomassie Brilliant Blue (CBB) is a triphenyl methane dye extensively used to stain proteins in electrophoretic profiles and in solution quantification. There are however two types of CBB dyes: CBB R-250 where R refers to the reddish tint of the product and CBB G-250 where G is related to the greenish tint of the dye [17]. CBB G differs from CBB R-250 with only two additional methyl groups (Fig. 1). The methylated dye is the preferred dye used in protein quantification assays by the Bradford method [18]. Coomassie is a trademark name still detained by Imperial Chemical Industries (ICI, later acquired by Akzo Nobel Inc.), and the 250 code is related to the dye content. In fact, both varieties of CBB commercially available very often contain up to 50% impurities accompanying the main staining agent [19–21] making them unusable with modified solid supports. It is therefore critical to purify the staining agent from high binding impurities in order to achieve better analytical performances and reproducible assays. A low pressure LC purification

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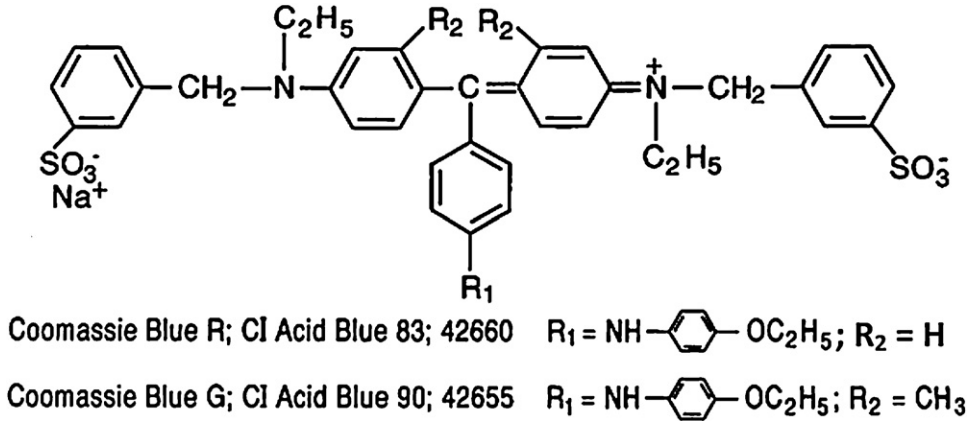

Fig. 1. Coomassie Brilliant Blue R-250 and G-250 molecular structures.

method has already been reported treating 10 g of commercial dye using 3 L of chloroform and 1 L of methanol and producing about 4 g of a purified Coomassie dye (yield 40%) [21].

In this work we propose to use counter-current chromatography (CCC) to purify Coomassie Blue in an efficient way using greener solvents. CCC is a chromatographic technique that involves partitioning of solutes between a mobile phase and stationary phase both liquids, without any solid support [22,23]. The CCC properties that will be essential in such purification are (i) the loading capacity of a CCC column and (ii) the lack of irreversible adsorption with a liquid stationary phase. The whole volume of the liquid stationary phase is accessible for the injected solutes which explains the very high loading capability observed with CCC columns [24]. Also, if a solute is strongly retained in the stationary phase, it can be easily recovered by extruding the all-liquid column content. This operating mode was called Elution Extrusion Counter-Current Chromatography (EECCC) [25]. It is even possible to switch the phase roles during purification, the stationary phase becoming the mobile phase and vice versa. This dual-mode way to work with a CCC column also allows for full recovery of all injected materials [26]. In this work, we propose to increase the Coomassie Blue purification productivity by using the dual-mode method in a sequential way as recently described as the multi dual mode method [27,28].

2. Materials and methods

2.1. Chemicals

Solvents used for mixtures screening and thin layer chromatography (TLC) experiments were of analytical grade. Heptane and methanol were purchased from Fisher (Loughbourough, Leicestershire, UK). 1-Butanol and acetic acid were from Riedel de Haën (Seelze, Germany). Ethyl acetate was from SDS-Carlo Erba (Val de Reuil, France). Four different Coomassie Blue dyes were obtained from Fisher Thermo Scientific, Coomassie Blue G-250 product number PI-20279, Sigma–Aldrich, Coomassie Blue G-250 product B5133 (L'Isle d'Abeau Chesne, France) and Acros, Coomassie Blue G-250, product C/P541/46 (Geel, Belgium), two different batches of CBB Acros C/P541/46 were used over time, Lot #1 and #2. The CBB B5133 of Sigma was sold as a Coomassie purified dye with more than 90% dyes (compared with ~50% for the other products). The structures given in Fig. 1 correspond to m.w. 826 and CAS number 6104-59-2 for the R-250 form and m.w. 854 and CAS number 6104-58-1 for the G-250 form. Sigma–Aldrich compound B5133 served as standard in TLC controls. Fig. 2A shows the TLC plates obtained with the commercial products confirming the enhanced purity of Sigma B5133. Water was distilled and purified by an ELGA Purelab UHQ-MK3 system (Veolia Waters System, Bucks, UK).

2.2. Countercurrent chromatography experiments

The CCC experiments were conducted on a Spectrum™ Dynamic Extraction (Slough, United-Kingdom). This J-type instrument has a rotor radius of 85 mm, and two symmetrical and balanced sets of coiled bobbins. Each set contains a preparative coiled bobbin with an approximate internal volume of 70 mL (66.5 mL for preparative coil P1 and 69 mL for preparative coil P2, coil tubing diameter is 1.6 mm) and an analytical coil with an internal volume of 10.5 mL for analytical coil A1 and 10.0 mL for analytical coil A2 with tubing diameter of 0.8 mm. The β_r-value which is the ratio of the bobbin radius to the rotor radius ranges from 0.64 to 0.81 for coils A1 and A2 and from 0.52 to 0.86 for the preparative bobbins. The instrument can produce a centrifugal field of $240 \times g$ (about 2350 m s^{-2}) at 1600 rpm allowing for high flow rates. The external non-chromatographic volumes due to the flying and connecting leads add a total volume of about 4.4 mL. The dual mode and multiple dual mode experiments were performed working with the two preparative coils connected in series (140 mL volume).

Two isocratic Shimadzu pumps LC6A (Kyoto, Japan) were use for each liquid phase. Switching between the descending mode (lower phase pumped from bobbins head to bobbins tail) and the ascending mode (upper phase from tail to head) was automated using an ASTED XL Gilson (Villiers le Bel, France) equipped with two Rheodyne 6-ports valves 7010 (Cotati, USA). The valves were remotely controlled through the ASTED keyboard and driven for multiple dual mode experiments with a minimum time-cycle of 0.1 min. Collected fractions were dried on a Rotavap Büchi instrument (Flawil, Switzerland). An UV detector Shimadzu SPD-6A (Kyoto, Japan) was used to monitor the solutes on line. The signal was processed by an A/D converter (ULYS, Datalys, Saint-Martin d'Heres, France) and visualized on a PC computer running the software AZUR 4.0 (Datalys). The simulation calculi used for process optimization were realized on a PC computer with a Microsoft Excel (2003 version) spreadsheet.

2.3. Coomassie Blue control

All Coomassie Blue fractions were controlled by TLC as described in [21]. The eluting phase was a mixture of 1-butanol/acetic acid/water in 75/10/5 (v/v) proportion. The TLC plates were silica gel 60 F254 on aluminum 4 cm × 10 cm plates purchased from Merck (Darmstadt, Germany) (Fig. 2). Detection is very easy by

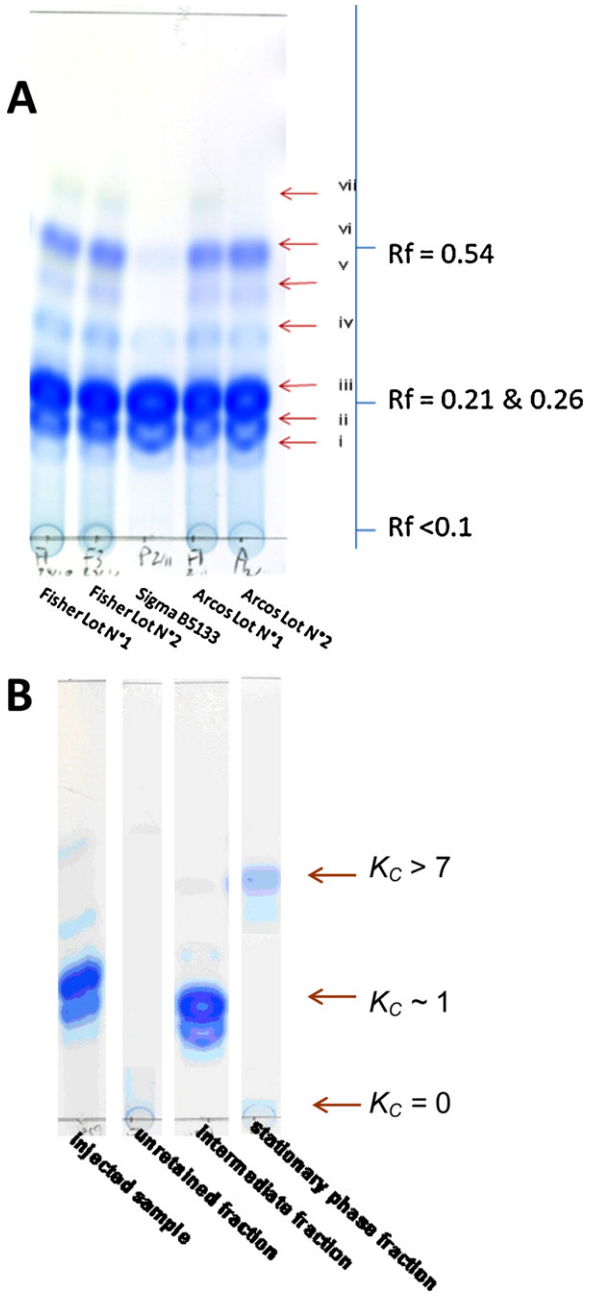

Fig. 2. (A) TLC analysis of the five commercial Coomassie dyes. Eluting phase: 1-butanol/acetic acid/water 75:10:5 (v/v), aluminum plate of Merck Silica gel 60-F254. Identified spots lay from (i) to (vii) with respective retention factors (Rf): 0.14, 0.21, 0.26, 0.38, 0.46, 0.54, 0.64. (B) TLC analysis of the isocratic CCC eluted fractions of 5 mg Coomassie Acros Lot #2 separated by isocratic CCC. Column spectrum 140 mL; 1600 rpm; liquid system: heptane/butanol/water 2:3:4 (v/v); mobile phase: aqueous lower phase in the head-to-tail descending direction, 5 mL/min, V_M = 34 mL; stationary phase: organic upper phase, Sf = 76%; V_S = 106 mL. Collection times after injection: unretained fractions: 7–8 min; intermediate fractions: 24–34 min; stationary phase: after 1 h elution and extruding the stationary phase.

simple visual examination. UV254 nm light was used to confirm the eluted spots location, especially for the polar little colored fraction.

2.4. Polyclonal antibody organic monolith

The driving force for the CBB purification was the accurate measurement of the polyclonal antibody concentration on a new organic monolith designed for a specific microsystem. A solid phase extraction (SPE) test of the purified dye was performed using the actual newly prepared organic monolith to assess the specific or non-specific binding properties of the purified dye fractions obtained. The hydrophobic support material is a highly porous organic monolithic with a high specific area. This organic monolith was synthesized in glass pipettes following a protocol adapted from [29]. Prior to synthesis, the glass pipettes were treated with an anionic commercial surfactant TDF 4 (Franklab, France) and thoroughly washed with deionized water and dried. The pre-synthetic mixture contained glycidyl methacrylate, ethylene di-methacrylate, 1,4-butanediol, 1-propanol and water in the volume ratio 9:3:10:6:1.5. The mixture was carefully de-oxygenated by gentle sonication under reduced pressure. Photo-polymerization was initiated by adding trace amounts (less than 1%) of 2,2′-azoisobutyronitrile. The pretreated glass pipette was filled with the mixture and placed in an UV-oven Crosslinker Biolink (VWR International, France) under 365 nm UV light with 6 J/cm^2 energy for 30 min. The poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) polymer was photo-catalytically formed. After washing with acetonitrile for 100 min to remove the porogen propanol and 1,4-butanediol solvents, a strongly hydrophobic stationary phase with a highly porous monolithic structure is obtained in the pipette [29]. Prior to monolith polymerization, the inner surface of the glass pipette was treated with 3-(methacryloxypropyl)-trimethoxysilane coupling agent to promote polymer linkage to the glass surface. The reaction was conducted filling the glass pipette with a 5% (v/v) solution of the organic silane reagent in a 95:5% (v/v) methanol/ethanol mixture and letting react for at least 1 h. Finally the coupling solution was washed out with methanol and the pipettes were dried before use. All polymerization and coupling reagents were purchased from Sigma–Aldrich (L'Isle d'Abeau, France).

These organic monolith containing pipettes were loaded with polyclonal antibodies that are rich in positively charged amino groups. The ADECA method is very adapted for determining their concentration, hence the need of purified Coomassie dye. The loading procedure is as follows: 1 mL solution containing 750 μg polyclonal antibodies was obtained from Covalab (Villeurbanne, France). It was percolated by positive pressure and left onto the monolith inside the pipette overnight to react with or load on the organic monolith surface. After at least 12 h at room temperature, the whole solution was pushed out of the pipette with 10 mL of 0.01 M phosphate-buffer + 0.15 M NaCl solution (pH 7.4).

The non-specific binding property of the Coomassie Blue samples on the monolith stationary phase was evaluated by comparing elution profiles of CBB on grafted with antibody pipettes with profiles obtained with non-grafted pipettes. Following the ADECA method, the methanolic eluates were collected after SPE experiments and monitored by UV–vis spectrometry and TLC analysis.

3. Results and discussion

3.1. Optimizing the liquid system for CCC purification

In any CCC separation, the first step is to select the biphasic liquid system that will give the desired separation. This liquid system selection corresponds to the simultaneous and indissociable selection of the mobile phase and the stationary phase. Indeed, both liquid phases must be in thermodynamic equilibrium so any change in one phase induces changes in the other phase.

A concentrated 10 g/L Coomassie Blue solution in 50% ethanol was prepared. One drop of this concentrated solution was added to each liquid system in test tubes. The color repartition in the two liquid phases was visually examined. The heptane (or hexane)–ethyl acetate–methanol–water system (HEMWat) is one of the most frequently used system in CCC separations [22–24].

However no proportions belonging to the HEMWat system were found appropriate for the CBB purification. Only the lower aqueous phase of the biphasic systems obtained was stained blue. Liquid systems more polar than the HEMWat system involve 1-butanol. The heptane/butanol/water 2:3:4 system produced two liquid phases in which the CBB solution seemed to partition. An isocratic run was performed using the upper organic phase as the stationary phase and the lower aqueous phase as the mobile phase in the head-to-tail direction. It showed a good separation of the commercial Coomassie Blue obtained from Acros with an unretained almost colorless fraction eluting with the mobile phase volume (partition coefficient close to zero) and a deep blue fraction eluting near the column volume (partition coefficient close to one). Extruding the organic stationary phase after using two column volumes of mobile phase, a third light blue fraction was recovered in it, meaning that components with large partition coefficients were separated by this apolar liquid phase. Fig. 2B shows the TLC plates obtained analyzing the CCC fraction along with the corresponding Rf TLC retention parameters and K_C estimated distribution ratios or partition coefficients.

3.2. Scaling-up the purification with multi-dual-mode CCC

The multi-dual mode (MDM) way to use a CCC column was introduced by Delannay et al. [27]. Actually, the concept was developed with different names by other authors: the True Moving Bed method can be viewed as a MDM process with a CCC hydrostatic column [30]; the Intermittent CCC (ICCC) method is essentially the same as the MDM one [31]. A full theoretical model for MDM recently appeared [28].

In the MDM separation process performed in this work, repetitive dual mode steps were performed injecting a new volume of crude sample at each step in the middle of the CCC column. Taken individually, a single cycle of the MDM process has two steps changing both nature of the mobile phase and flowing direction. In our theoretical model, we called the first step, the classical elution mode coded CM. Logically, the second step is the dual mode step coded DM [28]. It was demonstrated that the MDM method was virtually increasing the column length since the injected compounds were moving back and forth with longer chromatographic pathways. Fig. 3 illustrates the concept simulating middle column injection by use of two coils serially connected: a mixture of five compounds is initially injected in the middle of an equilibrated CCC column. The mobile phase is the lower aqueous phase flowing in the head-to-tail direction. Defining the solute distribution ratio or partition coefficient as the ratio of the solute concentration in the organic phase over its concentration in the aqueous phase, Compound 1 is very polar, mostly located in the aqueous phase with a very low partition coefficient, so it is not retained and elutes soon at the column tail (Fig. 3A). Next, both the phase nature and flow direction are switched (Fig. 3B). Compounds 4 and 5 are apolar with high partition coefficients so they were moving very slowly with the initial aqueous lower phase. After the phase role switch, Compounds 4 and 5 are moving very fast with the upper organic phase toward the column head. The dual mode step is maintained long enough for Compounds 4 and 5 to elute. Next the phase nature and flowing direction are changed again completing the first cycle and simultaneously, another volume of the five model compounds is injected in the middle of the CCC column so that the concentration of Compounds 2 and 3 is increased, Compounds 1 being recovered in the aqueous phase collected at the column tail (Fig. 3A). Model Compounds 4 and 5 are collected at the column head (Fig. 3B).

3.2.1. Single dual-mode separation

Coomassie Brilliant Blue can be seen as a mixture with three compound families: (i) the polar part with very small partition coefficients lower than 0.1 in the specially selected heptane/1-butanol/water 2:3:4 liquid system, (ii) the valuable and desired part with intermediate partition coefficients between 0.8 and 1.3 and (iii) the apolar part with large partition coefficients higher than 7. The described MDM method is adapted working on the liquid system used and flow rates and volumes used in each step so that both the un-retained polar part and a large portion of the desired part of the dye is collected in the head-to-tail or descending step with the aqueous polar mobile phase. All the apolar part of the dye is collected in the tail-to-head or ascending step with the organic apolar upper phase.

Using our previously published theoretical treatment [28], it is possible to estimate the mobile phase volume needed in the initial classical mode head-to-tail step so that the most polar fraction of the Coomassie Blue sample is eluted out the column. Introducing the relative distance, x_i, that a solute traveled in the column and setting $x = 0$ at the injection point (in the middle of the two coil experimental set-up, we define positive x value going toward the column tail and negative x value going toward the column head). When a x_i value reaches 1, the solute is seen eluting at the column tail. If x_i reaches −1, the solute elutes at the column head. The general equation for x_i is:

$$x_i = \frac{V}{V_{Ri}} \tag{1}$$

in which V is the aqueous phase volume used in the head-to-tail descending mode and V_{Ri} is the solute retention volume expressed by:

$$V_{Ri} = V_M + K_{Ci}V_S \tag{2}$$

K_{Ci} is the distribution ratio or partition coefficient of the solute that will be taken as:

$$K_{Ci} = \frac{[\text{con.}i] \text{ in organic upper phase}}{[\text{conc. } i] \text{ in aqueous lower phase}} \tag{3}$$

The V_M and V_S volumes are respectively the mobile and stationary phase volume in one bobbin. In a first approximation, we will describe the two bobbins as identical with the same phase volumes in them and the same volume $V_C = V_M + {}_iV_S$. In the tail-to-head ascending step, the K_{Ci} parameter does not change (Eq. (3)), the V_M volume becomes the stationary phase volume and the V_S volume becomes the mobile phase volume maintaining their values. So applying Eq. (2) with these changes, the solute retention volume, V'_{Ri}, becomes:

$$V'_{Ri} = V_S + \left(\frac{1}{K_{Ci}}\right) V_M = \frac{V_{Ri}}{K_{Ci}} \tag{4}$$

A volume V' of organic phase in the tail-to-head ascending direction will move all solutes by a distance x'_i opposite at x_i so that a negative sign is introduced:

$$x'_i = -\frac{V'}{V'_{Ri}} \tag{5}$$

Fig. 4 shows the single dual mode purification of 1 mL of 10 g/L Coomassie Blue (10 mg injected). The stationary phase retention factor, $Sf = V_S/V_C$ was adjusted to 46% for the two coils. The V_M volume is 38 mL (V_S volume 32 mL) per coil. The selected flow rate was 2 mL/min. 77 mL (V_{CM}) of aqueous phase were used in the classical mode step to elute the polar part of the dye along with the intermediate polarity desired portion of the dye in 38.5 min (left part of Fig. 4). The apolar components of the CBB sample have K_C values higher than 7. Using the K_C value of 8, as the smaller value for the apolar components of the sample, Eq. (2) gives an average classical mode retention time of $38 + 8 \times 32$ or 294 mL. Eq. (1) tells that these compounds moved down by $x = 77/294 = 0.26$ or less. This is one fourth of the bottom coil length during the first CM step. Now,

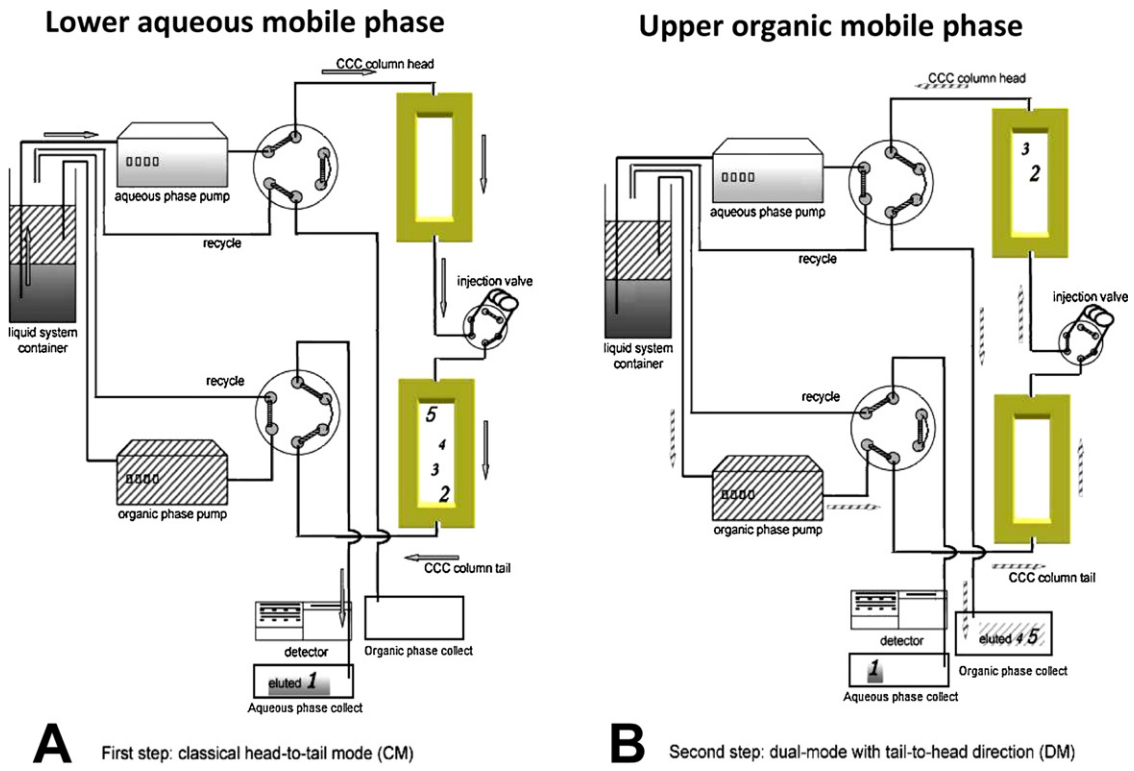

Fig. 3. Diagram of the multiple dual mode set-up in the head-to-tail or descending position (A) and tail-to-head or ascending position (B). Solute 1 elutes immediately in the descending step, and solutes 4 and 5 with a high affinity for the upper phase elute in the second ascending step, while the remaining solutes 2 and 3 see increased separation going back and forth in the following dual mode steps.

Step II, switching phase role and flowing direction, will move these apolar compounds up the coils (Fig. 3B). Eq. (4) gives the new average retention time of the apolar fraction as $V'_R = 294/8 = 37$ mL (or less for compounds with higher K_C). The band has a distance $x = -1.26$ to travel up to the first coil head (negative sign for upward traveling). So the needed organic phase volume as given by Eq. (5) is $V' = 1.25 \times 37 = 46$ mL of upper organic phase or 23 min elution time. The vertical orange bar marks the dual mode switching time and the apolar part of the sample is collected in the organic phase as shown on the right part of Fig. 4. The TLC control showed no spot in the Rf zone 0–0.26 assessing the purification of the sample.

3.2.2. Trapping multi-dual mode purification

The MDM method will work to separate two compounds only: one eluting in one phase at the column tail, the other being recovered in the other phase at the column head. The unique partitioning of the Coomassie sample obtained in our biphasic liquid system will be used. The sample in the heptane/1-butanol/water 2:3:4 (v/v) liquid system has three well distinguishable fractions: a polar water soluble and almost unretained fraction ($K_C \sim 0$), the desired fraction partitioning well between the two phases with an average K_C value of 1, and the apolar fraction favoring greatly the upper organic phase with an average K_C value of 8. The MDM experimental protocol will be set so that the unwanted polar fraction is eliminated eluting in the aqueous phase at the CCC column tail and the other unwanted apolar fraction is eliminated in the organic phase at the CCC column head. The desired dye fraction will stay purified but trapped inside the CCC column. Solubility is the limiting factor in this way of using a CCC column. Since the purified dye concentration increases in the CCC column, non linear behavior and/or precipitation could occur ruining the separation. In the selected biphasic liquid system, heptane–butanol–water 2/3/4 (v/v), CBB partitions almost equally between the two organic and aqueous phases. However, if this is true at low concentration, a non linear adsorption isotherm would make higher amounts of CBB going preferentially in one phase or in the other. It was found that the butanol rich aqueous phase of the selected liquid system was able to dissolve up to a concentration of ~75 g/L at room temperature (23 ± 2 °C). The solution becomes so dark that it becomes impossible to see through, missing possible micro crystals. When filtering a 75 g/L CCC solution, no crystals were observed; but here also, the filter becomes dark blue colored possibly hindering the view of crystals. 75 g/L will be arbitrarily taken as the maximum CBB concentration.

Knowing the distribution ratio range of the components of our sample, the following MDM experiment was optimized. A concentrated 71 g/L dark black solution of Coomassie Brilliant Blue (Acros lot #2) was prepared in the aqueous phase of the heptane/1-butanol/water 2:3:4 (v/v) liquid system. Fig. 3 CCC set-up was used and loaded with the upper phase of the heptane/1-butanol/water 2:3:4 (v/v) liquid system. Then it was equilibrated in the descending head-to-tail direction with the lower aqueous phase at 5 mL/min and 1000 rpm rotor rotation. The stationary upper phase retention ratio, *Sf*, was calculated as 46% on the two coils. Next the flow rate was decreased to 2 mL/min and the rotor rotation was increased to 1100 rpm to insure a tight hold of the liquid phase inside the two coils during the successive switches of flowing direction and phase nature. 1 mL (71 mg) of the concentrated solution was injected in between the two coils and eluted in the head-to-tail direction (aqueous mobile phase) for 5 min or 10 mL. Next, the MDM process was started switching for the organic upper phase and the tail-to-head flowing direction for 10 min or 20 mL after which the head-to-tail direction and aqueous mobile phase was resumed but injecting another mL (71 mg) of the dye. The process was continued with regular switch every 10 min for a total of 7 injections (500 mg). After the seventh DM step, another DM cycle was done

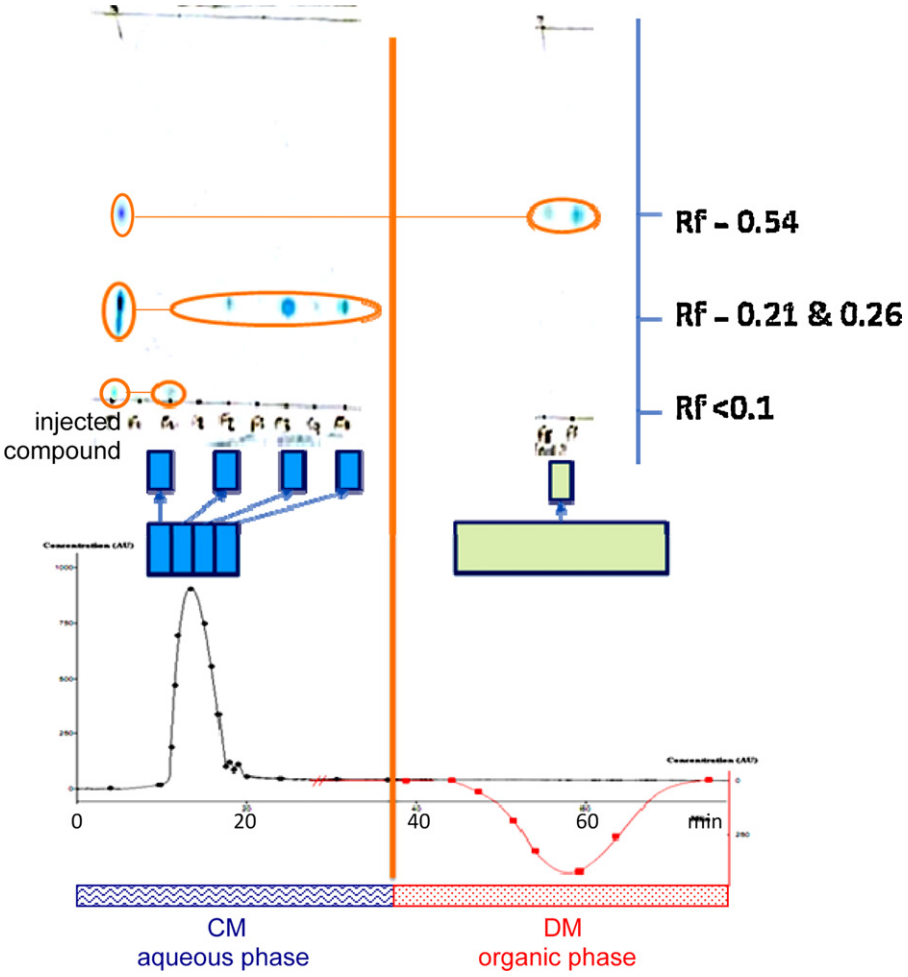

Fig. 4. Separation of polar and non-polar compounds in Coomassie Blue G-250 (Acros #1) by dual-mode elution and control by TLC. CCC conditions: heptane/1-butanol/water 2:3:4 (v/v) system. 1000 rpm; both H → T descending and T → H ascending flow rates: 2 mL/min; *Sf* = 46%; injection volume: 1 mL (10 mg); classical descending CM step for 38.5 min or 77 mL aqueous phase (wavy blue band); dual mode DM step until complete elution of the hydrophobic fraction at 78.5 min after 80 mL organic phase (dotted red band). TLC conditions: silica gel on aluminum Plates 60 F254, 1-butanol/acetic acid/water 75:10:5 (v/v) eluting phase. The TLC controlled fractions are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

without injecting more CBB. A 35 min head-to-tail aqueous mobile phase step (70 mL) finished the purification allowing recovering the dark colored concentrated and purified CBB fraction that the MDM method trapped in the column.

Fig. 5 shows the actual UV trace obtained during a typical trapping MDM purification. Table 1 lists the calculated position of all bands during the experiments using the proposed model. A line of the table corresponds to a particular band introduced at a particular time. The line allows to follow the motion of the band. Especially, following a dye band, it is calculated that it moved back and forth around its injection point without leaving the column. A column of Table 1 gives the CCC column content at a particular time. For example, at time 65 min, the blue color indicates that it is an aqueous phase head-to-tail step and that seven bands are present in the column. The dye trapping is seen by the accumulation of three dye bands located at 29% (0.29) inside the lower coil and another dye band located at 14% (0.14) of the lower coil. There are also two bands of apolar impurity, one close to the injection valve (0.07 or 7% in the lower coil) and the other one at 41% (negative −0.41) in the upper coil. The last band is a single band of polar impurity located at 54% (0.54) in the lower coil.

The following assumptions were made for computation: (i) the polar, dye and apolar bands have exactly the distribution ratio values of respectively $K_C = 0$, 1 and 8. Actually the bands gather different compounds of similar distribution ratios explaining why rounded bands are seen on the UV chromatogram (Fig. 5). The listed x_i values must be taken as the location of the center of the bands. (ii) The CCC set-up is made with two identical CCC columns of 70 mL. This is not exactly the case: if the total set-up volume was 140 mL, exact measurements returned coil volumes of 66.5 mL and 69 mL for the upper and lower coils, respectively with 1 mL of injection loop and 3.5 mL of connecting tubing.

Evaporating the 70 mL collected after the MDM experiment allowed to recover an average of 186 mg of purified CBB (yield 37%) whose TLC plate showed several close spots but a total absence of polar and apolar impurities (Fig. 2B, intermediate fraction). Each MDM experiment is done in 190 min using 220 mL of aqueous phase and 160 mL of organic phase. The average time throughput is 1 mg/min. Calculating all conditions to obtain 1 g of purified CBB, we found that 17 h are needed using 0.34 L heptane, 0.81 L butanol and 1.05 L water. Not counting water, the “greenest” possible solvent, this productivity compares well with previous results using less green solvents: 0.25 L of methanol and 0.75 L of chloroform per gram of purified CBB [21].

3.3. Assessing the quality of the purified dye

To assess the analytical quality of the purified dye, its binding properties were investigated using pipettes containing a hydrophobic monolithic coated (or bonded) with a polyclonal

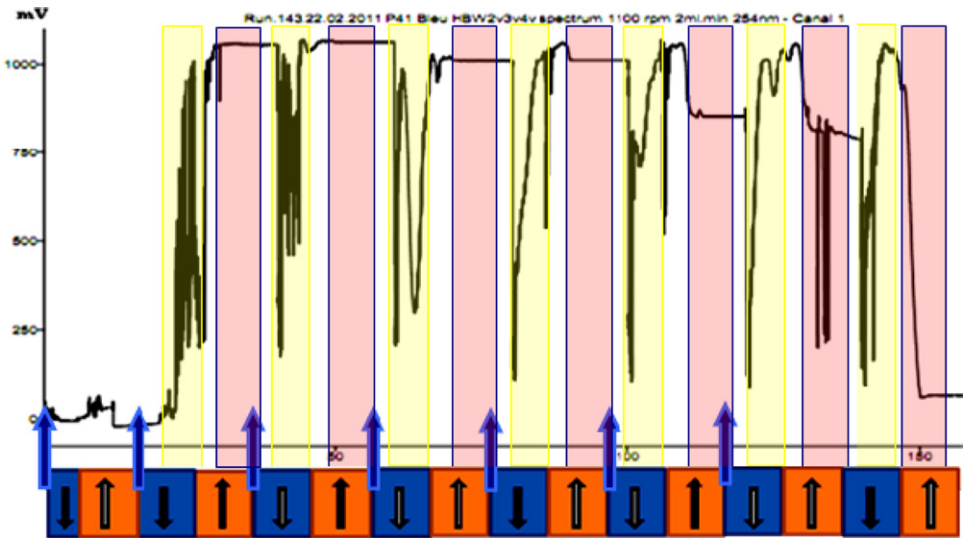

Fig. 5. 254 nm UV trace obtained during a "trapping" multi-dual-mode experiment for the purification of 500 mg of CBB G-250 (Acros #1). Two coils serially connected (see Fig. 3 for experimental set-up). Total volume 140 mL. Rotor rotation: 1100 rpm; flow rate (both phases): 2 mL/min; *Sf* = 46%. Blue steps refer to head-to-tail descending elution step with the aqueous phase and orange to tail-to-head ascending step with the organic upper phase. Repetitive 1 mL injections are indicated by arrows. The light yellow bands show the polar fraction elution (almost colorless) collected in the aqueous phase. The pink bands shows the apolar blue fractions collected in the organic phase. The purified CBB fraction was recovered in a long 70 mL head-to-tail aqueous phase elution after 155 min not shown due to complete detector saturation. See Table 1 for full modeling of band locations inside the CCC columns. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

antibody needed in another study. The adsorption-elution procedure called ADECA method was used. First, the purified CBB was assayed using pipette without polyclonal antibody. This allows to determine non specific adsorption by the organic monolith. Five different pipettes were loaded with a 0.1 g/L CBB aqueous solution at pH 2 by acetic acid. The acidic medium turns all amines into positively charged ammonium cations that interact by coulombic attractions with the CBB sulfonate groups. Then, the pipettes are rinsed with pure pH 2 buffer to flush out all excess dye. The fixed CBB is released by flushing the pipette with a known volume of methanolic solution of sodium carbonate (pH ~11), collecting the eluant and measuring its optical density [15]. Fig. 6 shows that the CCC purified dye gave results comparing well with those of the purified CBB compound B5133

Table 1
Computed localisation x_i of each chromatographic bands inside Fig. 3 CCC set-up during Fig. 5 MDM experiment.

Time (min)		5	15	25	35	45	55	65	75	85	95	105	115	125	135	145	155	190
Volume (mL)		10	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	70
		aq.	org.	aq.	org.	aq.	org.	aq.	org.	aq.	org.	aq.	org.	aq.	org.	aq.	org.	aq.
injection	fraction	down	up	down	up	down	up	down	up	down	up	down	up	down	up	down	up	down
	polar	0.26	0.26	0.79	0.79	eluted												
1	dye	0.14	-0.14	0.14	-0.14	0.14	-0.14	0.14	-0.14	0.14	-0.14	0.14	-0.14	0.14	-0.14	0.14	-0.14	eluted
	apolar	0.03	-0.51	-0.44	-0.99													
	polar			0.53	0.53	eluted												
2	dye			0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	1.00
	apolar			0.07	-0.48	-0.41	-0.95											
	polar					0.53	0.53	eluted										
3	dye					0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	1.00
	apolar					0.07	-0.48	-0.41	-0.95									
	polar							0.53	0.53	eluted								
4	dye							0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	1.00
	apolar							0.07	-0.48	-0.41	-0.95							
	polar									0.53	0.53	eluted						
5	dye									0.29	0.00	0.29	0.00	0.29	0.00	0.29	0.00	1.00
	apolar									0.07	-0.48	-0.41	-0.95					
	polar											0.53	0.53	eluted				
6	dye											0.29	0.00	0.29	0.00	0.29	0.00	1.00
	apolar											0.07	-0.48	-0.41	-0.95			
	polar													0.53	0.53	eluted		
7	dye													0.29	0.00	0.29	0.00	1.00
	apolar													0.07	-0.48	-0.41	-0.95	

A positive x_i value indicates that the band is located in the lower coil (Fig. 3); a negative value x_i indicates that the band is located in the coil above the injection valve. $x_i = 1.00$ means that the band is eluting at the CCC column tail in the aqueous phase; $x_i = -1.00$ means that the band is eluting at the CCC column head in the organic upper phase. Distribution ratios for polar, dye and apolar fractions are respectively: $K_C = 0$, 1 and 8. $V_M = 38$ mL; $V_S = 32$ mL for both coils. Eqs. (1) and (5) were used for aqueous or organic phase motion, respectively. See Fig. 5 caption for all experimental conditions. Horizontal reading allows following the column motions of a particular band; vertical columns give the CCC columns content at a particular time. The column colors correspond to Fig. 5 notation.

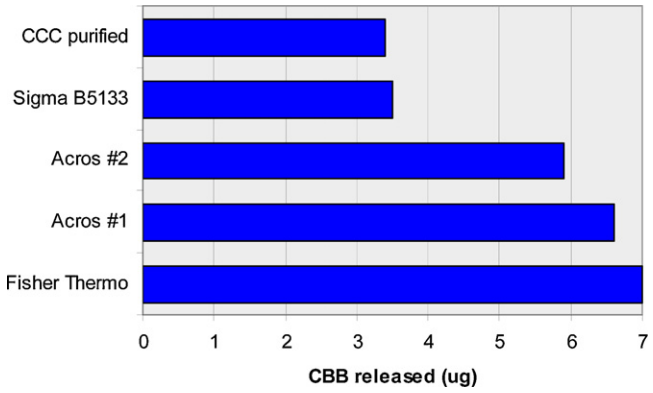

Fig. 6. Amount of Coomassie Brilliant Blue G-250 (CBB) released by an organic monolith. Higher amounts indicate non amine specific interactions introducing a bias in measurements.

of Sigma. 43% less dye was adsorbed onto the organic monolith using the CCC purified CBB compared to the Acros lot #2 from where it came. It clearly shows that much less non amino-specific interactions occur between the surface and the purified dye.

Similar measurements were made with pipettes containing the proteinized material. After staining and washing, the blue colored methanolic solution was collected and its optical density was measured giving the amount of CBB released. Once corrected by the amount due to the monolith itself (Fig. 6), it gives the amino group concentration hence the antibody concentration when no interfering colored compounds were involved [15]. Here again, the results obtained with the CCC purified dye compared well with those produced by the purified commercial dye. The purified CBB allows the same decrease of analytical bias, and achieve a better exactitude of the ADECA method. Furthermore a decrease of the noise level was observed with the purified CCC dye with sharp color changes during the elution steps. This also led to better sensitivity and reproducibility of the method.

4. Conclusion

CCC is the only chromatographic technique offering wide different possibilities to separate compounds playing with the mobile phase or the stationary phase, both liquids. The presented MDM purification of the CBB dye was not optimized for enhanced yield and throughput. It is well possible that a higher raw CBB concentration could be used. Also, the described "trapping" MDM method was indeed concentrating the purified fraction inside the column. Our goal describing this particular purification is to point out the versatility of the CCC technique. The presented way of purifying a sample "trapping" it inside a CCC column cannot be the best purification way since it cannot be performed continuously. In this case, it is necessary to stop the process to recover the purified fraction when the limiting maximum concentration is approached. During our experiments, it was possible to see the dye through the semi-transparent Teflon® tubing connecting the injection valve between the two coils. A small part of it was lost filling the injection loop since this 1 mL loop was part of system containing some trapped purified CBB. These losses significantly decreased the production yield. This problem could easily be solved changing the injection procedure; it would greatly enhance the recovered amount of purified CBB fractions. The important point is that CCC mounted in MDM configuration can produce significant amount of purified material using environmental friendly solvents. The non-optimized yield of 500 mg of purified CBB per working day (~8 h) using only 170 mL of heptane, 405 mL of 1-butanol and 0.5 L of water was obtained with a 140 mL bench scale CCC instrument and classical LC equipment. It must be pointed out that the whole presented purification was possible because the solvent choice allowed for a large separation of the three groups of compounds to separate. The solvent selection is one of the most critical parameters in CCC separations.

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