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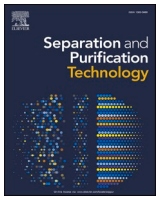

# Evaluation of ethanol-based aqueous two-phase systems performance for application in centrifugal partition chromatography (CPC)

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ABSTRACT

Aqueous two-phase systems (ATPS) have recently been proposed for Centrifugal Partition Chromatography (CPC). This type of biphasic system has several advantages over conventional aqueous-organic liquid–liquid systems, namely, high biocompatibility owing to its large water content. The most commonly used ATPS are polymer-based systems. However, owing to their poor phase separation hydrodynamics compared to aqueous-organic systems, the use of short-length water-miscible alcohols has been evaluated as an alternative to polymers. In this study, an ATPS composed of ethanol and dipotassium hydrogen phosphate ($K_2HPO_4$) is proposed to overcome the common hydrodynamic drawbacks of polymer-based ATPS. The Ethanol/$K_2HPO_4$ system exhibited a very short settling time (ts = 30.85 ± 0.80 s), high interfacial tension (IFT = 6.38 mN/m), significant density differences between the phases (Δρ = 353.40 kg/m$^3$), and low viscosity of the coexisting phases at 25 °C (maximum μ = 3.79 ± 0.11 mPa.s), making it an ideal choice for implementation in CPC apparatus. A comprehensive study of this biphasic system was conducted using CPC equipment by carefully adjusting the operating parameters, specifically, the mobile phase flow rate ($F$), rotational speed ($\omega$), and mode of operation, that is, ascending (upper phase is mobile) or descending (lower phase is mobile). In this particular CPC equipment, the use of polymers as phase-forming compounds in ATPS resulted in higher CPC pump pressures compared to ethanol or phosphate salt aqueous solutions, thus confirming the superior performance of the ethanol/salt ATPS. The results obtained using this ATPS demonstrated that high $F$ and $\omega$ values lead to lower stationary phase retention ($S_F$) and higher backpressures, respectively. $S_F$ remained relatively constant and above 50% for $F \leq 6$ mL/min but significantly decreased for $F > 6$ mL/min. This trend confirms that hydrostatically stable conditions can be achieved by employing the Ethanol/$K_2HPO_4$ system in the CPC mode by controlling $F$ (*i.e.,* the hydrodynamics of the mobile phase). The partition of the model solutes gallic acid and L-tyrosine was also evaluated using the ethanol/$K_2HPO_4$ system. The critical operating CPC parameters were compared, confirming the significant potential of the ethanol/salt ATPS for successful commercial application of this chromatographic technique in the separation of small value-added biomolecules.

## 1. Introduction

Centrifugal Partition Chromatography (CPC) is one of the most common types of liquid–liquid chromatography techniques and is a technological variant of Counter-Current Chromatography (CCC) [1,2]. In contrast to CCC, which works with coiled tubing, CPC creates a uniform centrifugal field by rotating its chambers around a single axis, allowing a continuous separation process. This technique is also called hydrostatic CCC, in which the flow rate ($F$) and rotational speed ($\omega$) of the rotor are limited by the maximum working pressure of the rotary joints (backpressure or pressure drop) [2,3]. As a liquid–liquid chromatographic technique, CPC uses the immiscible phases of a liquid biphasic system as mobile and stationary phases. The constant centrifugal field generated led to retention of the stationary phase in the column, with the mobile phase (carrying the sample) passing through it. CPC stands out for its simplicity, preparative capacity, versatility in the selection of stationary and mobile phases with no irreversible solute adsorption, and scalability [4,5].

The separation process of liquid–liquid chromatography-based technologies is driven by the partitioning of the components towards the immiscible phases according to their affinities for each phase [4],

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**Nomenclature**

*Greek Letters*

| | |
|---|---|
| μ | viscosity (mPa·s) |
| Δμ | viscosity difference (mPa·s) |
| ρ | density (kg/m$^3$) |
| Δρ | density difference (kg/m$^3$) |
| ω | rotational speed (rpm) |
| $\omega_{max}$ | maximum rotational speed (rpm) |

*Symbols*

| | |
|---|---|
| F | flow rate (mL/min) |
| $S_F$ | stationary phase retention (%) |
| ts | phases' settling time (s) |
| $t_R$ | solute's retention time (min) |
| $t_{Rc}$ | calculated retention time (min) |
| $t_{Re}$ | experimental retention time (min) |
| $V_C$ | column volume (mL) |
| $V_D$ | column dead volume (mL) |
| $V_M$ | mobile phase volume (mL) |
| $V_R$ | solute retention volume (mL) |
| $V_{RD}$ | solute retention volume in descending mode of operation (mL) |
| $V_{RA}$ | solute retention volume in ascending mode of operation (mL) |
| $K_D$ | partition coefficient |
| IFT | Interfacial tension (mN/m) |

*Abbreviations*

| | |
|---|---|
| ATPS | Aqueous Two-phase System |
| CPC | Centrifugal Partition Chromatography |
| CCC | Counter-Current Chromatography |
| LLE | Liquid-Liquid Extraction |

*Subscripts*

| | |
|---|---|
| $K_2HPO_4$ | di-potassium hydrogen phosphate |
| $K_3PO_4$ | tri-potassium phosphate |
| PEG-1000 | Poly(ethylene) glycol with an average molecular weight of 1000 g/mol |
| PPG-400 | Poly(propylene) glycol with an average molecular weight of 400 g/mol |
| EtAc | Ethyl acetate |

and the separation efficiency is strongly influenced by the hydrodynamic interactions of the stationary and mobile phases inside the chambers. In turn, the flow regime and its hydrodynamic effects are influenced by i) the physical properties of the two-phase system, such as density (ρ), viscosity (μ), and interfacial tension (IFT); ii) the characteristics of the chambers, such as shape, volume, and material; and iii) operating parameters, such as *F*, *ω*, and the mode of operation (*i.e.*, the less dense phase is mobile and passes through the more dense stationary phase in an upward direction (ascending mode) or the more dense phase is mobile and passes through the less dense stationary phase in a downward direction (descending mode)) [6,7]. This hydrodynamic behavior can be observed through effects such as mass transfer, phase back-mixing, stationary phase retention ($S_F$), and backpressure. $S_F$ is defined as the amount of liquid stationary phase retained in the column over the column volume [8,9]. The backpressure in hydrostatic countercurrent machines, such as the CPC, is higher than that in hydrodynamic countercurrent machines (CCC) and is limited by the utilization of rotary joints, which possess a maximum pressure limit that restricts the selection of operating parameters for each biphasic system [10]. Furthermore, the operating parameters should be selected to obtain high stability of the stationary phase in the column (*i.e.*, a high $S_F$) because the $S_F$ influences the solute retention and column efficiency, with a higher $S_F$ resulting in better peak resolution [8,11]. To achieve a high resolution and satisfactory CPC performance, the selection of operating parameters based on hydrodynamic behavior must be understood for a given biphasic system. To date, classical aqueous-organic systems for the purification of natural products have been mainly used in CPC, with many studies reporting the selection of operating parameters for these systems. However, for the purification of more complex biomolecules, the use of classic organic solvents (*e.g.*, ethyl acetate, hexane, and heptane) can cause conformational changes in their structures and loss of biological functionality [12].

Aqueous Two-Phase Systems (ATPS) have recently been proposed as biocompatible and sustainable platforms for the separation and purification of biological products [13]. Owing to the water-rich nature of their phases, the use of ATPS in biotechnological processes has been widely investigated for a plethora of biomolecules, which are frequently described as simple, amenable, biocompatible, and easily scalable separation platforms [3,13]. Interestingly, there are some promising studies showing the use of ATPS in liquid–liquid chromatography equipment as efficient and selective biphasic systems for the separation of valuable biomolecules, such as immunoglobulin Y [14], PEGylated cytochrome *c* conjugates [3], and mixtures of lysosomes and myoglobin [15]. However, the commonly used ATPS in CPC are composed of polymers, resulting in systems with high viscosity and very low interfacial tension, which makes it difficult to retain the stationary phase in the chambers and the backpressure within the working limits of some chromatographic equipment [16]. To overcome the limitations of polymer-based ATPS, short-length water-miscible alcohols have been proposed as alternatives to polymers owing to alcohols-based ATPS advantages such as short separation times, low viscosity of coexisting phases, low cost of phase-forming compounds, and ease of solvent recycling and polishing of extracted solutes [17]. However, it should be noted that many proteins are not compatible with the alcohol-rich phase of these biphasic systems, being denatured by alcohols, which is a major drawback of this system type [18,19]. Nevertheless, these systems have been previously used in the separation and purification of some enzymes, such as lipase [19] and DNA polymerase [20]. In this sense is quite important to evaluate the stability of target biomolecule before application of an extraction system.

Alcohol/salt ATPS composed of ethanol and dipotassium hydrogen phosphate ($K_2HPO_4$) can effectively and selectively separate various biomolecules, such as 2,3-butanediol [21], crocins [22], genistein, and apigenin [23]. Thus, ethanol-based ATPS can overcome the major drawbacks of common polymer-based ATPS, providing better hydrodynamics, while still maintaining the stability and biological activity of several small added-value biomolecules. Despite these interesting features, to the best of our knowledge, the integration of ethanol-based ATPS with CPC has never been addressed. Thus, the present study investigates the effect of the CPC operating modes and parameters, *e.g.*, $S_F$, *F*, and *ω,* on the system's hydrodynamics and overall performance using an Ethanol/$K_2HPO_4$ ATPS, relating the obtained results with the physicochemical properties of the ATPS phases (*i.e.*, conductivity, density, viscosity, and IFT) and the system's settling time ($t_s$). The partition of the model solutes (gallic acid and L-tyrosine) was used to assess the efficiency of this type of ATPS for the separation of biomolecules.

## 2. Materials and methods

### 2.1. Material

The chemical compounds used in this study were polypropylene

glycol with an average molecular weight of 400 g/L (PPG-400, $C_3H_6O_2$, Sigma-Aldrich®), dipotassium phosphate ($K_2HPO_4$, ≥98%, Merck KGaA®), tripotassium phosphate ($K_3PO_4$, ≥95%, Exodus Scientific®), and absolute P.A. ethanol ($C_2H_6O$, ≥99.8%, Exodus Scientific®). Double-distilled ultrapure water was used in all preparations, which was passed through a reverse osmosis system and then treated by ion-exchange filtration using a Millipore Milli-Q system (18 MΩcm). The solutes used in the partition experiments were gallic acid ($C_7H_6O_5$, 97.5–102.5%, Sigma-Aldrich®) and L-tyrosine ($C_9H_{11}NO_3$, ≥98%, Sigma-Aldrich®).

### 2.2. Physicochemical characterization of the ATPS

To determine its physicochemical properties, ATPS with a total mass of 15 g was prepared gravimetrically ($\pm 10^{-4}$ g) and mixed with the required phase-forming agents in the following mass ratios (wt%):19.5 $K_2HPO_4$ + 19.5 Ethanol + 61.0 $H_2O$. The mixture was stirred in a vortex mixer for 4 h and maintained in a thermostatic bath until the components were completely solubilized and equilibrated at 25 °C. After equilibration between the phases, the top and bottom phases were separated using a glass Pasteur pipette. The pH and conductivity values were measured at 25 °C using a portable pH meter and Metrohm ®/Model 914 conductometer. The water content of the phases (% v/v) was measured by volumetric Karl Fischer titration at 25 °C using a Karl Fischer 852 Titrando (Metrohm®). Density and viscosity measurements were performed at atmospheric pressure and at 25 °C using an automated SVM 3000 Anton Paar rotational Stabinger viscosimeter densimeter. The $t_s$ values (*i.e.*, the time required for complete separation of the two phases with no emulsion layer at the interface) were determined according to the methodology described by Jorge et al. [24].

The IFT values of the ATPS were recorded using the pendant drop method and Dataphysics Instruments GmbH, OCA20 (Germany) at room temperature. Using a Hamilton DS 500/GT syringe, connected to a stainless-steel needle with a diameter of 0.52 mm, and a very slow continuous dispense (0.06 $\mu L/s^1$), a drop of the salt-rich phase was dispensed within the alcohol-rich phase until a clear image of the drop's

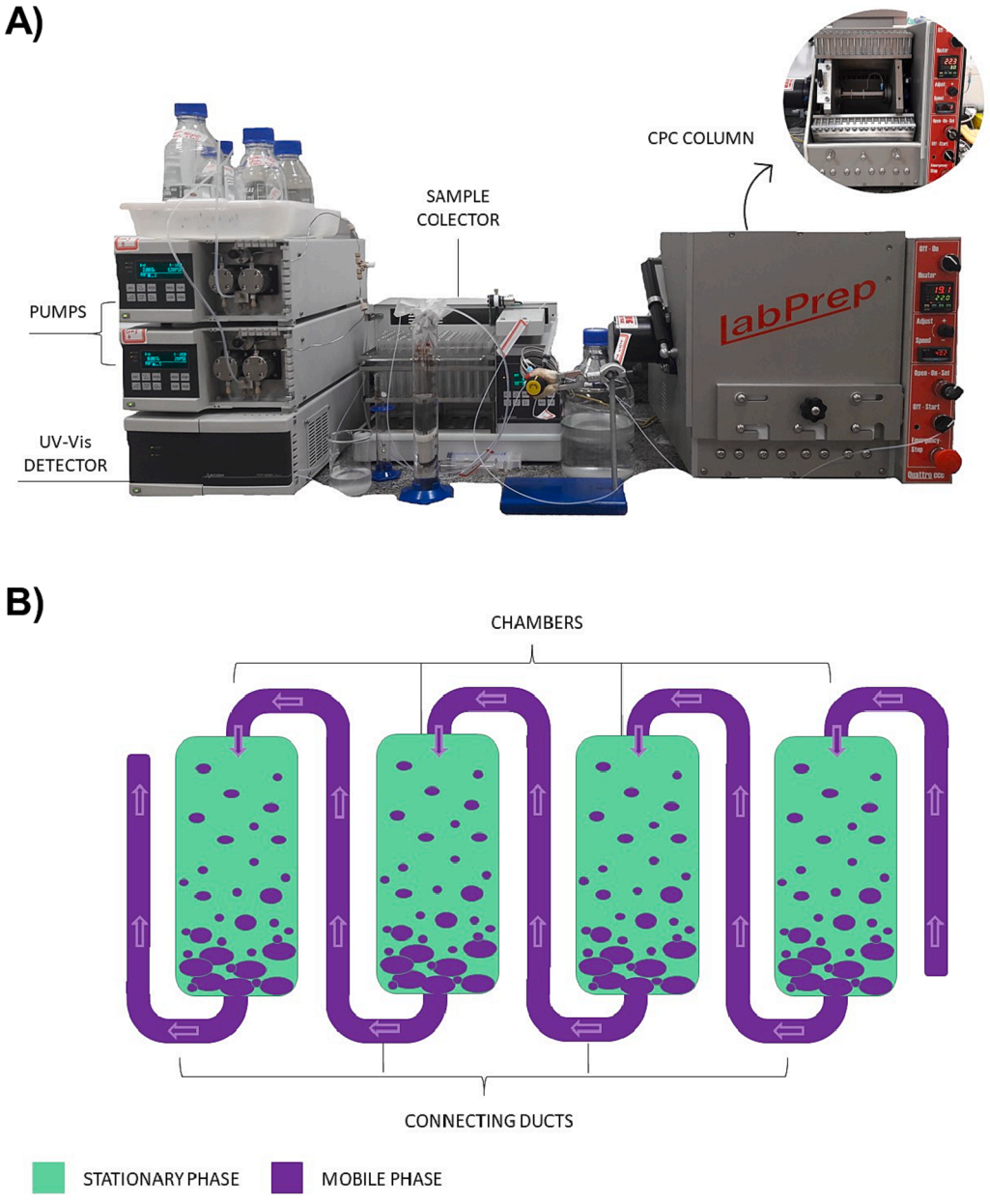


**Fig. 1.** Quattro LabPrep™ CPC equipment (A) and schematic representation of a CPC equipment (B).

profile could be observed. With the drop's image, the shape of the pendant drop was analyzed by fitting to the Young-Laplace equation with the software module SCA20, using the gravitational acceleration (g = 9.8018 m/s$^2$) and latitude (lat = 40) values in accordance with the assay location. The densities of the phases required for the calculation of the IFT from the drop image data were obtained as described in this section.

### 2.3. CPC apparatus

CPC assays were performed using a hydrostatic equipment model Quattro LabPrep$^{TM}$ CPC (AES-QuikPrep Ltd., Newquay, United Kingdom), with a column volume ($V_C$) of 200 mL and dead volume ($V_D$) equivalent to 11% of the column volume (*i.e.*, $V_D$ = 22 mL). The equipment had two columns with $V_C$ of 200 mL and 470 mL and a maximum speed ($\omega_{max}$) of 800 rpm. Each column consisted of a pair of opposing rotors made of three stainless steel plates with layers of polytetrafluoroethylene between them to provide a high-pressure seal. The central plate has multiple identical chambers interconnected by ducts. A picture of the equipment and schematic representation of the rotor are shown in Fig. 1.

For all CPC tests, a $V_C$ of 200 mL was used, aiming for lower solvent consumption and shorter duration of the experiments. Prior to each test, the column was equilibrated with the stationary and mobile phases. Stationary phase filling was performed using the two available flow pumps at a rate of 80 mL/min (40 mL/min for each pump), with $\omega = 100 \pm 3$ rpm, in the ascending or descending operation mode to be used in the test. The stationary phase flow was performed with the sample injection valve in the "inject" position, which allowed the stationary phase to pass through the loop and expel the previously contained phase.

To ensure the complete filling of the column with stationary phase, the flow was continued until the baseline of the CPC chromatogram remained constant, resulting in a stationary phase volume injection of between 1.8 and 2.2 × the $V_C$ (equivalent to injecting 360–440 mL). After the stationary phase filling was completed, mobile phase filling was performed with specific $\omega$ and $F$ values for each test, and the sample injection valve was placed in the "load" position, where $V_M$ did not pass through the loop. A manual injection valve (Upchurch Injector, V450) was used for sample injection.

For phase pumping, two preparative high-efficiency liquid chromatography pumps model ECP2050 (ECOM, Prague, Czech Republic) with a maximum flow rate of 50 mL/min and a maximum pressure of 5861 kPa were used. The phases were detected and collected using a UV–Vis detector (TOY 14 DAD) and collector (ECF2096), both from ECOM (Prague, Czech Republic).

For CPC tests, ATPS were prepared with a total variable volume ranging from 2 to 4 L, according to the demand of each assay. The mixture was vigorously stirred and equilibrated overnight at 25 °C, using a separatory funnel. The separated phases were degassed in an ultrasonic bath for 10 min before use.

### 2.4. Evaluation of pump pressure as a function of aqueous solutions of ATPS phase-forming compounds

To understand the influence of different types of ATPS phase-forming compounds on the CPC pump pressure, an initial screening was performed using aqueous solutions of PPG-400 (10, 20, 40, and 60 wt%), $K_3PO_4$ (10, 20, and 30 wt%), and ethanol (10, 20, 30, and 40 wt%). The effect of the injection flow rate on the pump pressure was evaluated by varying the operating parameters between 5 and 40 mL/min while $\omega$ was fixed at 0 rpm. To assess the effect of $\omega$ on the pump pressure, injection flows of 5, 15, and 30 mL/min were used, with $\omega$ set at 0, 200, 400, and 600 rpm. Because there were only significant differences between the ascending and descending modes when two immiscible liquid phases were present (which is not the case because this study only used aqueous monophasic solutions of the phase-forming compounds), the descending mode was randomly selected for this initial screening. After the column was completely filled with these solutions, CPC was adjusted to the desired injection flow rates and $\omega$ values. Once the flow of the aqueous solution (the same solution contained in the column) was initiated, the pressure indicated by the pumps was recorded every 2 min for a total analysis duration of 100 min.

### 2.5. Backpressure determination

The effects of varying $F$ (from 2 to 10 mL/min, with a fixed ω = 400 rpm), $\omega$ (from 200 to 800 rpm, at $F = 2$ mL/min), and operating mode on the backpressure were evaluated for the Ethanol/$K_2HPO_4$ ATPS. The column was first filled with the stationary phase (bottom phase in the ascending mode or top phase in the descending mode). The stationary phase was pumped at a flow rate of 80 mL/min and $\omega = 100 \pm 3$ rpm in the respective operating mode of each test. The stationary phase was filled with the sample injection valve at the injection position, which allowed the stationary phase to pass through the loop and push out the phase that was previously contained in the column. The mobile phase was then pumped with the respective $F$ and $\omega$ values of each assay over 100 min, and the backpressures displayed by the pumps were recorded.

### 2.6. Measurement of $S_F$

The influence of increasing $F$ and $\omega$ on $S_F$ in the Ethanol/$K_2HPO_4$ ATPS was evaluated for the two operating modes. The column was filled with the stationary phase, as previously described. The mobile phase was pumped at $F$ ranging from 1 to 10 mL/min (for an intermediate $\omega$ of 400 rpm), whereas $\omega$ ranged from 200, 400, 600, and 800 rpm (for $F = 1$, 2, 3, and 6 mL/min). The $F$ and $\omega$ ranges were selected according to the maximum pressure limit obtained in backpressure studies. All tests were performed in ascending and descending operating modes.

The $S_F$ was measured according to the volumetric method described by Schwienheer et al. [12]. In brief, the stationary phase displaced from the column owing to pumping of the mobile phase was collected in a measuring cylinder until the phases reached a steady state (confirmed by the appearance of the mobile phase front and a straight baseline on the detector). Because the volume of the stationary phase decreases even after the steady state is reached (bleeding), the volume of the stationary phase was collected for 150 min for all the tests.

$$S_F\ (\%) = \frac{V_C + V_D - V_M}{V_C} \quad (1)$$

where $V_C$ and $V_D$ are the column and dead volumes, respectively. $V_M$ is the volume of the mobile phase inside the column, and is equivalent to the displaced volume of the stationary phase. $V_D$ was considered constant based on the information provided by AECS-QuikPrep, which stated that 11% of the chambers' total volume constitutes the dead volume, *i.e.*, considering the total chamber volume ($V_C$) is 200 mL, a constant $V_D$ of 22 mL was used in the calculations.

### 2.7. Partitioning of model-solutes using CPC

To determine the gallic acid and L-tyrosine partition coefficients ($K_D$), an ATPS with a total mass of 10 g was used. A solution with concentration of 0.1 g/L was used to form the salt-rich phase of the biphasic mixture (*i.e.*, instead of 61 wt% of $H_2O$, these ATPS were formed with 61 wt% of an aqueous solution of 0.1 g/L of the model-solutes).

After weighing the components, the systems were allowed to equilibrate for 30 min and then centrifuged for 15 min at 5000xg to ensure complete phase separation. The phases were carefully separated and analyzed for UV–Vis absorbance using an Infinite M200PRO microplate reader. All readings were performed at wavelengths corresponding to the maximum absorbance ($\lambda_{max}$), which was determined using prior scanning analysis for each solute. The maximum absorbance peaks of

gallic acid and L-tyrosine were defined as 266 [25] and 223 [26] nm, respectively, and all spectra of the samples were retrieved to register their absorbance values and confirm that the peaks were correct. The $K_D$ values were calculated from the absorbances of each ATPS phase using Eq. (2) [27]:

$$K_D = \frac{A_T}{A_B} \quad (2)$$

where $A_T$ and $A_B$ are the absorbance values of the solutes in each phase of the biphasic system, respectively. Note that the subscripts "$T$" and "$B$" correspond to the "top" and "bottom" phases, respectively.

Subsequently, the partition of the model solutes using CPC was analyzed. A gallic acid partitioning study was conducted in four different assays to study the effect of varying $F$ (*i.e.*, $F = 1$, 2, and 4 mL/min) using $\omega = 600$ rpm and the descending mode of operation. The injected samples consisted of 20 mL of the bottom phase of Ethanol/$K_2HPO_4$ ATPS, formed with a 61.0 wt% aqueous gallic acid solution at a concentration of 0.50 g/L instead of 61 wt% of pure water (the total gallic acid concentration considering the total mass of the ATPS was approximately 0.31 g/L). For the L-tyrosine partitioning assay, the ascending mode of operation was used with $F = 2$ mL/min and $\omega = 600$ rpm. The injected sample consisted of 20 mL of the ATPS top phase, containing 61.0 wt% aqueous L-tyrosine solution at a concentration of 0.35 g/L.

Understanding the partitioning of solutes (and impurities) in a given biphasic system is crucial for estimating the separation performance of CPC. Once the $K_D$ of the target molecule is known and the stationary and mobile phase volumes in the column are determined, position of the chromatographic peak and the solute retention volume in descending ($V_{RD}$) and ascending ($V_{RA}$) modes can be calculated using respectively Eqs. (3) and (4) [4,8]:

$$V_{RD}(mL) = V_M + K_D{}^*(V_C - V_M) \quad (3)$$

$$V_{RA}(mL) = V_M + \frac{1}{K_D}{}^*(V_C - V_M) \quad (4)$$

$V_C$ is the sum of the stationary and mobile phase volumes, since the column is composed of these two phases only. Using $V_R$, in the descending mode – $V_{RD}$, or ascending mode - $V_{RA}$, it was possible to estimate the retention time ($t_R$) of the solutes using Eq. (5):

$$t_R\ (\text{min}) = \frac{V_R}{F} \quad (5)$$

where $t_R$ is the retention time of the solute, $V_R$ (mL) is the solute retention volume, and $F$ (mL/min) is the flow rate of the mobile phase.

## 3. Results and discussion

### 3.1. Physicochemical properties of Ethanol/$K_2HPO_4$ ATPS

Phase separation and solute partitioning in ATPS are governed by the physicochemical properties of the system, which vary according to the nature of the phase-forming components, characteristics of the target molecules, and overall thermodynamic variables of the system (*i.e.*, pH, temperature, and pressure) [13]. Furthermore, the hydrodynamics and performance of CPC are highly dependent on the physicochemical properties of the biphasic system used in the separation process, and characterization of each immiscible phase of the tested system is of utmost importance [2,16]. Thus, to understand the suitability of ethanol-based ATPS hydrodynamics for CPC and the subsequent extraction of biomolecules using this technique and system, the physicochemical properties of each coexisting ATPS phase (*i.e.*, conductivity, density, viscosity, IFT, and pH) were determined, as well as their volume, weight, water content, and $t_s$. The obtained results are displayed in Table 1, along with the physicochemical properties of a "commonly used" ATPS in CPC composed of polyethylene glycol with an average molecular weight of 1000 g/mol (PEG-1000) and $K_2HPO_4$, and of a conventional LLE system regularly used in CPC systems composed of ethyl acetate (EtAc) and water, retrieved from Schwienheer et al. [16] for comparison.

As indicated in Table 1, the recorded pH values were found to be identical and almost neutral in both phases (pH ≈ 7), which can help stabilize the targeted small biomolecules and is another advantage of using an Ethanol/$K_2HPO_4$ ATPS in the separation and/or purification of these compounds [18,28]. The considerably higher conductivity (approximately 20-fold) and density of the bottom phase provided the main indication of the presence of significant amounts of salt, confirming that the bottom phase is the salt-rich phase of the ATPS [24,29]. The studied ATPS presented a very good formation hydrodynamics, as demonstrated by the very quick complete phase splitting ($t_s = 30.85 \pm 0.80$ s), with these results being associated with the low viscosities and high differences in the density values of the phases [24].

The continuous bleeding of the stationary phase is one of the biggest disadvantages of the "commonly used" ATPS in CPC (*i.e.*, ATPS composed of polymer and salts, such as PEG-1000/$K_2HPO_4$/$H_2O$ (Table 1)), and it is expected that a high-density difference, low viscosity of the phases, and high interfacial tension will decrease bleeding by improving phase coalescence [16]. When the values of $\rho$, $\mu$ and IFT of the studied ethanol-based system are compared with those of polymer/salt ATPS, it is possible to conclude that alcohol/salt system have significantly higher $\Delta\rho$ (Table 1) (density differences of other polymer/salt ATPS range between 79.9 and 218.7 kg/m$^3$) [16,30]; a considerably lower viscosity for the top phase, since the use of polymers in the

**Table 1**
Physicochemical properties of the Ethanol/$K_2HPO_4$/$H_2O$ ATPS at 25 °C.

| ATPS' composition | Ethanol/$K_2HPO_4$/$H_2O$* | | PEG-1000/$K_2HPO_4$/$H_2O$** | | EtAc/$H_2O$*** | |
|---|---|---|---|---|---|---|
| ATPS' phases | Ethanol-rich top phase | Salt-rich bottom phase | Polymer-rich top phase | Salt-rich bottom phase | EtAc-rich top phase | $H_2O$-rich bottom phase |
| Volume (mL) | 7.20 ± 0.3 | 6.20 ± 0.2 | – | – | – | – |
| Weight (g) | 6.79 ± 0.26 | 7.89 ± 0.12 | – | – | – | – |
| pH | 10.05 ± 0.02 | 9.60 ± 0.01 | – | – | – | – |
| Water content(% v/v) | 66.11 ± 0.75 | 67.03 ± 0.60 | – | – | – | – |
| Conductivity (mS/cm) | 6.94 ± 0.30 | 133.50 ± 1.80 | – | – | – | – |
| $\rho$ (kg/m$^3$) | 959.10 ± 5.50 | 1312.50 ± 7.60 | 1084.1 | 1157.8 | 892.8 | 996.3 |
| Density difference, $\Delta\rho$ (kg/m$^3$) | 353.40 | | 73.7 | | 103.5 | |
| $\mu$ (mPa.s) | 2.53 ± 0.01 | 3.79 ± 0.11 | 5.4 | 1.7 | 0.47 | 1.07 |
| IFT (mN/m) | 6.38 ± 0.74 | | 0.06 | | 6.45 | |
| $t_s$ (s) | 30.85 ± 0.80 | | – | | – | |

*ATPS studied in this work and composed of 19.5 wt% Ethanol /19.5 wt% $K_2HPO_4$/61.0 wt% $H_2O$.
**"Commonly used" ATPS composed of 12.5 wt% PEG-1000/6.8 wt% $K_2HPO_4$/75.0 wt% $H_2O$; data from [16].
***Conventional LLE system composed of 50 wt% EtAc/ 50 wt% $H_2O$; data from [16].

polymer/salt systems leads to higher viscosities in the polymer-rich top phase than in the ethanol-rich top phase of the studied alcohol/salt ATPS (Table 1) (viscosities of other types of polymer/salt systems vary between 1.41 and 2.8 mPa·s for the salt-rich phase and from 5.23 to 23.6 mPa·s for the polymer-rich phase) [16,30]; and a higher IFT value than the "commonly used" polymer/salt systems (Table 1) (also being higher than the IFT of other polymer/salt ATPS reported, with the values for those systems varying between 0.3 and 2.44 mN/m) [16]. Compared to the conventional aqueous-organic system composed of EtAc and water (Table 1), the $\Delta\rho$ value for the tested alcohol/salt ATPS was 3-fold than that recorded for the EtAc/$H_2O$ biphasic system, and considerably higher than those of several other systems composed of conventional organic solvents and water reported elsewhere [2]. The IFT values were very similar for both systems; however, the viscosities of the phases of the conventional systems were lower than those of the ethanol-based ATPS (Table 1).

Overall, the obtained results indicate that alcohol-based ATPS possesses ideal properties for the best performance of a CPC-based technique, owing to physicochemical properties closer to those required for a good hydrodynamic performance in CPC than the "commonly used" ATPS and similar properties to the conventional aqueous-organic systems used in CPC [16]. To fully understand the impact of ethanol-based ATPS properties and their suitability for CPC, the effect of the operating parameters on the process hydrodynamics and extraction of biomolecules is discussed in the following sections of this work.

### *3.2. Effect of aqueous solutions of ATPS' phase-forming components in CPC pump pressure*

To confirm the good hydrodynamic suitability of ethanol-based ATPS for CPC applications, the effects of varying the injection flow rate and $\omega$ on the pump pressure of the equipment were analyzed for aqueous solutions containing polymers, namely propylene glycol with an average molecular weight of 400 g/mol (PPG-400), ethanol, and tripotassium phosphate ($K_3PO_4$). Fig. 2 shows the CPC pump pressure registered for aqueous solutions with different concentrations of each compound and for injection flows varying between 5 and 40 mL/min at $\omega = 0$ rpm.

From Fig. 2, it can be observed that the use of PPG-400 in this type of CPC equipment is less desirable, as the pressure of the system is significantly higher (between 0 and 4965 kPa) for this compound than for ethanol or $K_3PO_4$ (between 0 and 1000 kPa for both). It should be noted that these pressures were obtained only by injecting each compound or aqueous solution separately and not as part of a biphasic mixture, making it possible to analyze the impact of each individual compound (PPG-400, ethanol, or $K_3PO_4$) on the backpressure of the system. This indicates that the use of polymers as phase-forming compounds in ATPS leads to considerably superior pump pressures compared to ethanol or phosphate salts. This is associated with the increase in viscosity for systems containing polymers because higher viscosities lead to higher friction forces within the equipment's chambers and reduce the ability of the liquid to pass through, increasing the backpressure of the system [10,16]. These friction forces are proportional to the viscosity of the liquid, and Fig. S1 (Supporting Information) confirms the superior viscosity of PPG-400 solutions ($\mu$ varies between 2 and 22 mPa·s) when compared to ethanol and phosphate salt aqueous solutions ($\mu$ varies between 1 and 5 mPa·s) [16]. To obtain further insights concerning the ATPS phase-forming compounds at the CPC pump pressure, aqueous solutions containing 20 wt% of each phase-forming component were injected into the equipment at a constant flow rate of 15 mL/min and in descending mode, varying $\omega$ between 0 and 600 rpm. The results are shown in Fig. 3.

As previously stated, Fig. 3 also shows that the pump pressure of a 20 wt% PPG-400 aqueous solution was higher (approximately 483 kPa) than that of aqueous solutions containing the same concentration of ethanol and phosphate salt (between 310 and 448 kPa). These

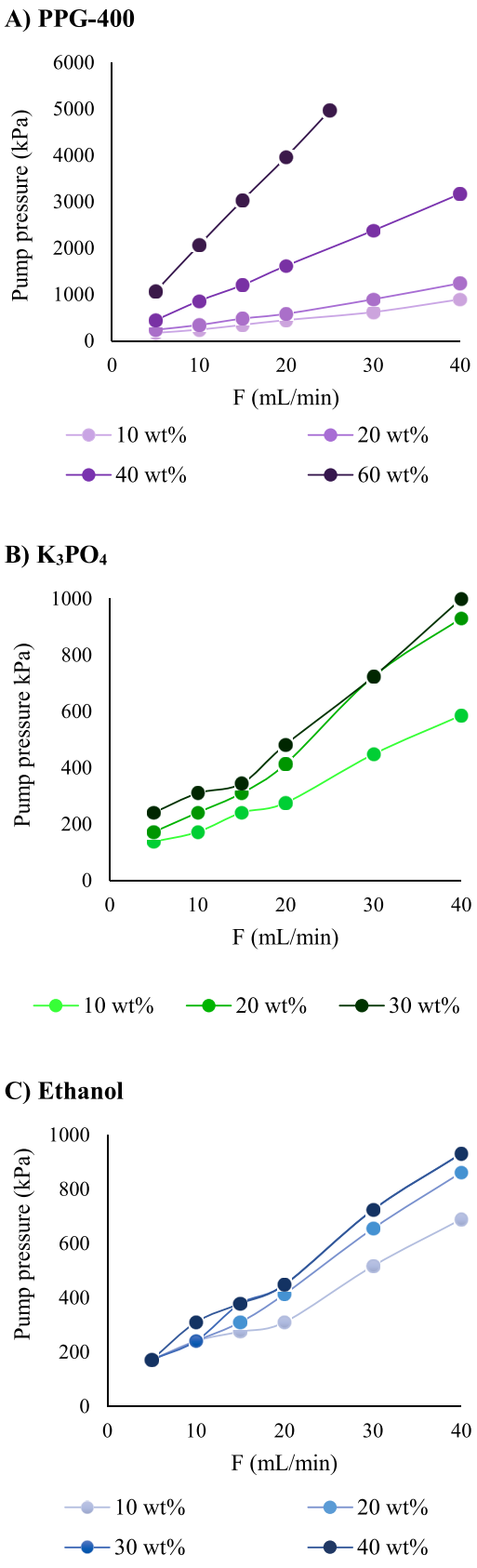


**Fig. 2.** CPC pump pressure (kPa) of different aqueous solutions (wt%) of **A)** PPG-400 (purple), **B)** $K_3PO_4$ (green) and **C)** ethanol (blue) for injection flow rates varying between 5 and 40 mL/min without rotation ($\omega = 0$ rpm).

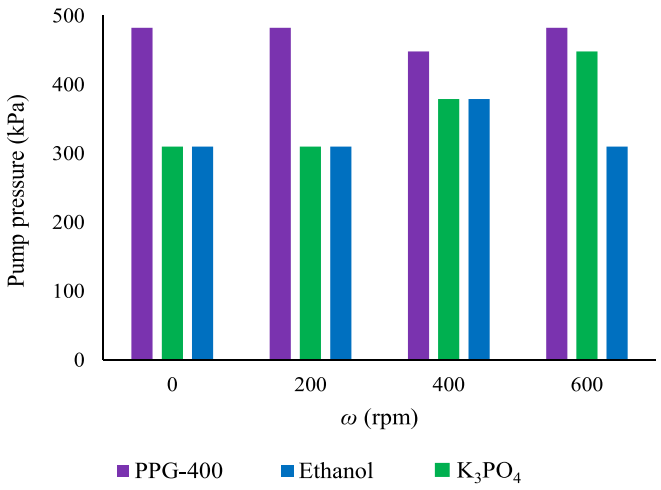


**Fig. 3.** Pump pressure (kPa) of aqueous solutions containing 20 wt% of PPG-400 (purple), $K_3PO_4$ (green) and ethanol (blue) for an injection flow rate of 15 mL/min, in descending mode and $\omega$ between 0 and 600 rpm.

differences are more prominent at $\omega = 0$ and 200 rpm, as the pump pressure values for the PPG-400 aqueous solution are almost twice as high as those of aqueous solutions of ethanol and phosphate salt, and are (once again) explained by the considerably higher viscosity of the polymer aqueous solutions and their higher friction forces in comparison to the alcohol and salt/water solutions studied.

Considering all these data and because these pump pressure values are all obtained using the same equipment and operating conditions, it is possible to affirm that the use of biphasic mixtures based on polymers in this type of equipment will present worse hydrodynamic performance than ethanol and phosphate salt-based ones. It has been reported elsewhere that ATPS composed of different phosphate salts ($K_3PO_4$, $K_2HPO_4/KH_2PO_4$, and $K_2HPO_4$) and ethanol have similar properties and hydrodynamic behavior [31]. The results from Fig. S1 confirm the potential of the ethanol/$K_2HPO_4$ ATPS in CPC-based separation processes, and that the comparison of results from this work with others must always consider the use of different equipment and their impact on the process.

### 3.3. Effect of operating parameters on CPC backpressure using Ethanol/$K_2HPO_4$ ATPS

Backpressure is considered one of the main factors limiting successful CPC performance. According to Van Buel et al. [32], the overall backpressure in a CPC column corresponds to the sum of individual hydrodynamic and hydrostatic contributions. The hydrodynamic contribution is due to the friction of the liquid with the walls of the channels and ducts in the column. Therefore, this contribution is influenced by the column geometry and properties of the liquid, particularly the viscosity. The hydrostatic contribution is caused by the centrifugal force (owing to $\omega$), density difference between the mobile and stationary phases in the ducts and channels, and $S_F$ [32,33]. Fig. 4 presents the effect of increasing $\omega$ on the backpressure of the CPC process using the studied ATPS at a constant $F = 2$ mL/min in both the ascending and descending operating modes.

As shown in Fig. 4, an increase in $\omega$ (which maintains a stable stationary phase in the column) directly results in an increase in the backpressure for both operating modes. In hydrostatic machines, chambers filled with the stationary phase are gradually balanced by the passage of the mobile phase, causing a linear increase in backpressure; this phenomenon is also observed in this type of biphasic system [4]. For these operating conditions, a linear increase in backpressure occurs until approximately 50 min, when the backpressure reaches its maximum according to the applied $\omega$. From this point onward, the top and bottom phases reached a complete steady state, and the pressure stabilized [33].

The CPC units have a higher backpressure than the CCC units, and the maximum backpressure is limited by the use of rotary joints. The pressure drop of CPC is composed of a hydrodynamic part (dependent on $F$ and $\mu$) and a hydrostatic part (dependent on $S_F$ and $\Delta\rho$), which limits the rotational speed that can be applied by the rotary joints of that type of process [10]. To reduce the backpressure, the use of a less viscous phase as the mobile phase is often advised, as it decreases the friction force between the phases (which is proportional to the viscosity of the continuous phase) and reduces the pressure drop of the equipment [10,16]. However, for the Ethanol/$K_2HPO_4$ ATPS, the use of the ascending mode (*i.e.*, use of the less viscous ethanol-rich phase as the mobile phase) did not result in considerably lower pressure drops compared to the descending mode. Because $F$ and $\Delta\rho$ are constant in both operating modes, the observed tendencies are related to the similar viscosities observed for both ATPS phases, which results in identical frictional forces between the phases of each operating mode, inducing

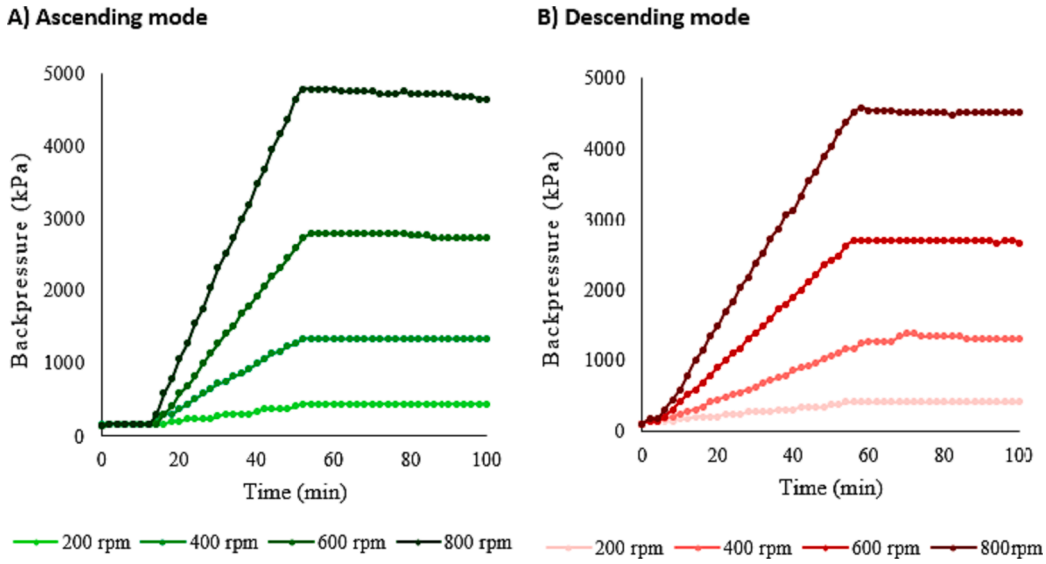


**Fig. 4.** Influence of $\omega$ on the backpressure (kPa) for the Ethanol/$K_2HPO_4$ ATPS at $F = 2$ mL/min for **A)** ascending and **B)** descending operating modes. The lines serve only as eye guides.

identical hydrodynamic contributions, and consequently, similar backpressures. This might indicate that either operating mode can be used, but this conclusion is not completely demonstrated because it lacks an evaluation of the hydrostatic part of the system (namely $S_F$, because $\Delta\rho$ will be the same for both operating modes), which is crucial for optimizing the overall performance of the CPC [10]. Further clarifications regarding the selection of the best operating mode for this type of ATPS are provided in Section 3.3.

It is also noted in Fig. 4 that the backpressure shows a pronounced increase at higher $\omega$ values. When $\omega$ is increased from 200 to 400 rpm, from 400 to 600 rpm, and from 600 to 800 rpm, the backpressure increases by 896, 1448, and 1999 kPa, respectively, between each variation of $\omega$ for both operating modes. Although high rotational speeds have been reported to enhance the dispersion of the mobile phase and the resulting separation efficiency of CPC, a higher $\omega$ leads to a higher hydrostatic pressure drop and an increase in the backpressure[7]. Thus, the backpressure is a very important operating parameter of the CPC, as the equipment rotor and its peripheral equipment will have a maximum limitation in pressure drop, consequently limiting the scalability of the separation process [7]. Fig. 5 shows the relationship between the backpressure values obtained for an increase in $\omega$ at a constant $F = 2$ mL/min and in the ascending mode.

By plotting the backpressure constant value of each $\omega$, the power trend line provided the best fit to the data by plotting the backpressure constant value of each $\omega$ ($R^2 = 0.9984$) (Fig. 5). The obtained results demonstrated that for the given conditions and equipment, $\omega$ higher than 800 rpm should be carefully evaluated for the Ethanol/$K_2HPO_4$ ATPS, as it can lead to the pump's overpressure (*i.e.*, backpressure over 5861 kPa). This conclusion is also valid for other biphasic systems with properties similar to those of the studied ATPS and the tested CPC equipment. Considering the above information, and because it has been previously reported that an increase in rotational speed leads to more efficient separations, the CPC equipment should operate at the maximum backpressure possible, with the separation process occurring at the highest $\omega$ possible, which is always limited by the maximum pressure drop allowed by the equipment [16].

The backpressure of the most stable polymer-based ATPS studied by Schwienheer et al. [16], composed of 20 wt% PEG-100/ 10.9 wt% $K_2HPO_4$/69.1 wt% $H_2O$ and named "increased amount," was 28 bar (approximately 2800 kPa) for $F = 2$ mL/min and $\omega = 1000$ rpm. Using the equation reported in Fig. 5, it is possible to estimate that the pressure drop for the ethanol-based system under these operating conditions would be near 6771 kPa (almost 68 bar), leading to a pump overpressure ($> 5861$ kPa). Although the alcohol-based ATPS presents lower viscosities and higher IFT than the polymer-based ATPS, which tends to improve the hydrodynamics of the separation process, the higher backpressure in comparison to the polymer-based ATPS can be related to the higher $\Delta\rho$ of the ethanol-based ATPS [16]. Owing to the versatility of ATPS and the possibility of adapting their physicochemical properties and extraction efficiencies by adjusting the amount of each phase-forming component, $\Delta\rho$ can be reduced by decreasing the amount of $K_2HPO_4$ in ethanol-based ATPS, while maintaining high IFT and low phase viscosities [13]. However, such a difference cannot be fully justified by the increase in $\Delta\rho$ alone, as the physicochemical properties of the Ethanol/$K_2HPO_4$ system (*i.e.*, lower phase viscosity and higher IFT) appear to be closer to those required for a viable and optimal CPC process than those reported for polymer-based ATPS. The main reason for this is that the compared results are highly influenced by the type of CPC equipment used in each work, such as the operation characteristics and design (*e.g.*, chamber geometry), which have a major impact on the hydrodynamics of the studied biphasic systems [6,7].

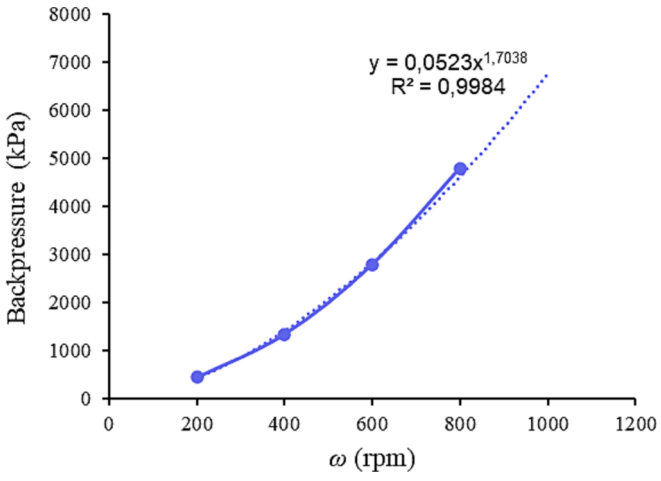


**Fig. 5.** Effect of $\omega$ on CPC backpressure (kPa) for the Ethanol/$K_2HPO_4$ ATPS at a constant $F = 2$ mL/min and ascending mode.

The influence of $F$ on the backpressure of the ethanol/salt system was also determined, with the last being recorded for flow rates varying between 2 and 10 mL/min in both operating modes and at a constant $\omega$ of 400 rpm (Fig. 6).

As shown in Fig. 6, increasing $F$ to 8 mL/min had a minor effect on the backpressure. For all evaluated $F$ values from 2 to 8 mL/min, the maximum backpressure achieved was approximately 1379 kPa in both operating modes. This effect is related to the sum of the hydrostatic and hydrodynamic contributions to the overall backpressure, with an increased hydrodynamic contribution ($F$) resulting in a lower $S_F$ (as can be seen in Section 3.3), and the decreasing $S_F$ (hydrostatic contribution) leading to lower backpressure in the CPC. Thus, the increase in hydrodynamic contribution due to the increase in $F$ is cancelled out by the decrease in $S_F$ and hydrostatic contribution, leading to less dependence of the backpressure on $F$ [10]. However, for $F$ above 8 mL/min, the backpressure decreased sharply, reaching values of 655 kPa and 862 kPa in the ascending and descending modes, respectively. This occurs because a high $F$ can cause a very fast passage of the mobile phase through the stationary phase, which does not allow sufficient time for the phases to interact and separate properly, thereby dragging the stationary phase out of the column [7,10]. This occurs because the mobile phase enters the coalescence zone with high velocities or as a stable sheet, limiting the mass transport of the solutes from the inner part to the surface of the sheet [2]. On the other hand, very low $F$ can also have detrimental effects in the hydrodynamics and separation efficiency of the CPC process, with the mobile phase entering in the chambers in the form of large droplets, leaving large parts of the chambers free of mobile phase due to uneven spread of this phase within the column and resulting in an inefficient convective transport in the stationary phase [2]. Ideally, the mobile phase should enter in the coalescence zone of the column in the form of small droplets and in a flow rate sufficient enough to provide a good mass transport of the solute from the stationary phase to the mobile phase, while maintaining the maximum volume possible of stationary phase within the columns [2,7,10].

An increase in $F$ resulted in an inversely proportional decrease in the time required for the phases to reach the steady state, as shown in Fig. 6, when the backpressure reached its maximum value for each $F$, as previously reported by Peng et al. [27]. Therefore, the use of a relatively high $F$ decreases the time required to reach a steady state and hence the process separation time, without causing a significant increase in the CPC backpressure. Nevertheless, an increase in $F$ can also induce a reduction in $S_F$, promoting bleeding in the stationary phase and significantly lowering the separation efficiency [7]. It should be noted that an increase in $F$ (between 2 and 8 mL/min) did not increase the backpressure (*i.e.*, the backpressure of approximately 1379 kPa obtained for $F = 2$ mL/min at 400 rpm in Fig. 4 was maintained to increase $F$ in Fig. 6), which indicates that 8 mL/min is the maximum $F$ that can be used to obtain good hydrodynamic performance of Ethanol/$K_2HPO_4$ ATPS in this CPC equipment. Analysis of all process parameters in both the hydrodynamic and hydrostatic parts is necessary to select the best

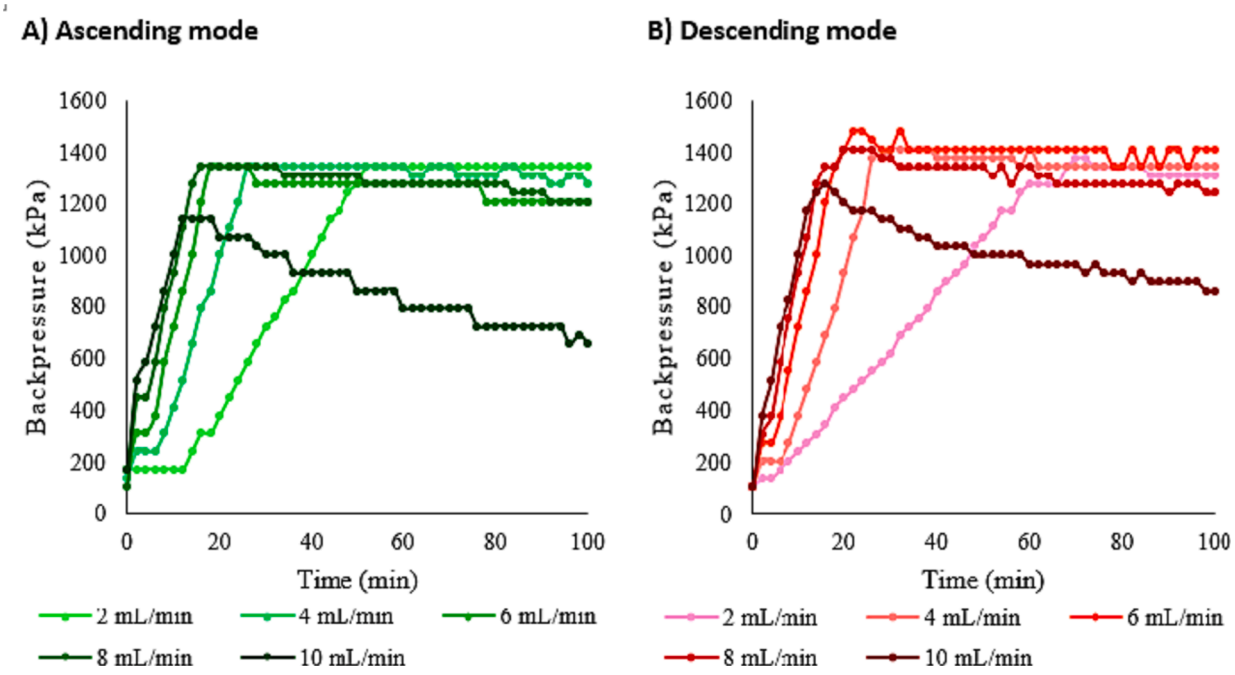


**Fig. 6.** Influence of $F$ on backpressure (kPa) for the Ethanol/$K_2HPO_4$ ATPS at a constant $\omega$ of 400 rpm for **A)** ascending and **B)** descending operating modes. The lines are only guides to the eyes.

operating conditions and optimize the CPC-based separation process.

### 3.4. Effect of operating parameters on $S_F$ using Ethanol/$K_2HPO_4$ ATPS

Stationary phase retention is of utmost importance for the selection of the best operating parameters and optimization of CPC, as it is dependent on $F$ and $\omega$ and influences the hydrodynamics and separation efficiency of the required separation process. Fig. 7 presents the $S_F$ values for $F$ between 2 and 10 mL/min in both the ascending and descending operating modes, and a constant $\omega$ of 400 rpm.

In the Quattro LabPrep$^{TM}$ CPC, $S_F$ decreases with increasing F (Fig. 7); this decreasing tendency of $S_F$ with increasing $F$ has been widely reported for CCC and CPC equipment [7,10]. However, while in the CCC the $S_F$ is linearly related to the square root of the $F$, in the CPC the relationship between the $S_F$ and the $F$ is not well established [10].

Roehrer and Minceva [34] investigated the influence of $F$ on the $S_F$ for various CCC and CPC equipment. The authors pointed out that, especially for CPC, the correlation between $S_F$ and $F$ is linear up to a certain value of $F$, with $S_F$ exhibiting an abrupt decrease when the $F$ value is exceeded. In Fig. 7, similar behavior is observed in this study. Increasing $F$ from 1 to 6 mL/min resulted in an 11.41% decrease in $S_F$ in the ascending mode (green curve) and a 5% decrease in the descending

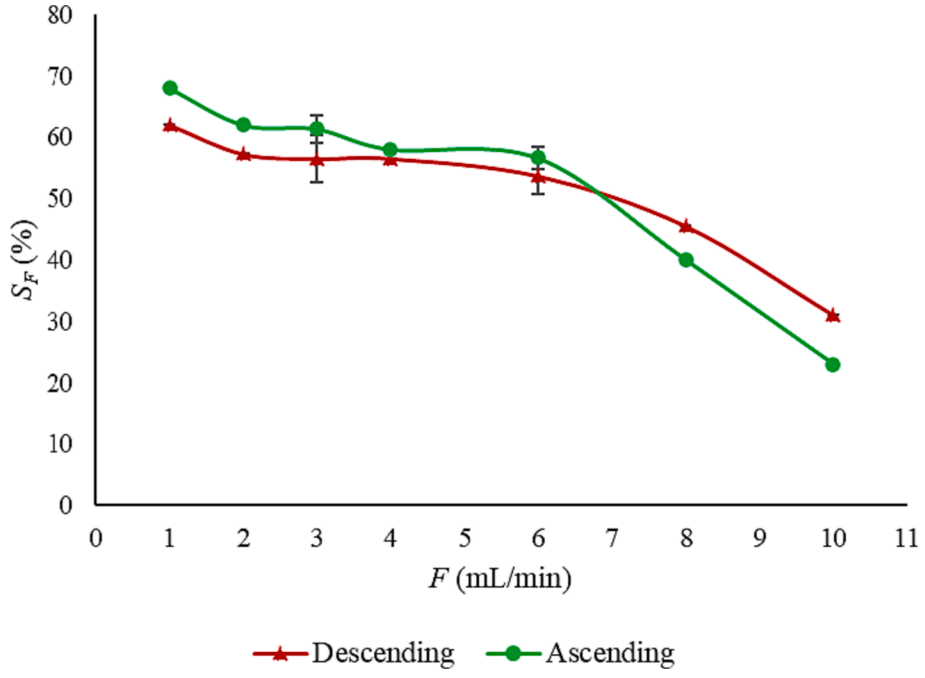


**Fig. 7.** Effect of $F$ on $S_F$ using Ethanol/$K_2HPO_4$ ATPS at a constant $\omega$ of 400 rpm for ascending and descending operating modes. The lines are only guides to the eyes.

mode (red curve). In contrast, $F$ above 6 mL/min led to a more pronounced decrease in $S_F$. Increasing $F$ from 6 to 10 mL/min caused a reduction in $S_F$ by 34% and 26% in the ascending and descending modes, respectively. The same trend was observed for the polymer/salt ATPS used by Schwienheer et al. [16], where the "increased amount" ATPS presenting a more stable hydrodynamic behavior than the "commonly used" ATPS (see composition in Table 1). The authors attributed those tendencies to the higher interfacial tension and density difference of the ATPS with the "increased amount" of phase-forming constituents (IFT = 2.44 mN/m and $\Delta\rho = 218.7$ kg/m$^3$) when compared to the "commonly used" ATPS (IFT = 0.06 mN/m and $\Delta\rho = 73.7$ kg/m$^3$), as it is possible to achieve a lower dispersion of mobile phase and faster coalescence of the stationary phase [16].

It was expected that the ethanol-based biphasic system would present a more stable trend of $S_F$ than the "increased amount" ATPS, as it possesses a lower phase viscosity, high $\Delta\rho$, and almost 3-fold higher IFT than the polymer-based ATPS reported in that work, which should improve the hydrodynamic performance of the alcohol-based system [16]. Although the studied alcohol-based ATPS presented better stability than the "commonly used" ATPS, it also recorded lower values of $S_F$ and stability when compared to the "increased amount" of ATPS. To understand the reason behind these results, it is important to highlight the main parameters of CPC that can influence the hydrodynamics of the separation process, which are divided into three distinct groups: i) parameters of the equipment design (chamber geometry), ii) the operation parameters (volume flow, rotational speed, or operation mode), and iii) biphasic system properties between the two studies [6]. Because the operation parameters were similar in both works ($F$ was the same; $\omega$ was higher for the polymer-based ATPS but had a small influence on the obtained $S_F$), the physicochemical properties of the ethanol-based ATPS appear to be more favorable for CPC application than the ATPS studied in another study. The alcohol-based system presented a higher $\Delta\rho$, lower phase viscosity, and higher IFT, as recommended for a good CPC-based separation process [16], but the hydrodynamics performance of the latter was superior to that of the ethanol-based system, and it can be hypothesized that are characteristics of the CPC equipment used in each work the cause for the results obtained. Once again, this is in accordance with the results discussed in Figs. 2 and 3 regarding the impact of polymers, ethanol, and phosphate salts using the Quattro LabPrep$^{TM}$ CPC, where it was proven that the use of different equipment has a major impact on the process hydrodynamics performance.

The physicochemical properties of the ethanol-based ATPS appear to be ideal for CPC applications, overcoming the low biocompatibility of aqueous-organic systems and presenting much higher IFT, lower phases' viscosity and higher density difference than the polymer-based ATPS. Moreover, considering that ethanol and phosphate salts aqueous solutions presented better hydrodynamics than aqueous mixtures of polymers using Quattro LabPrep$^{TM}$ CPC (*cf.* Fig. 3), we believe that ethanol/salt systems can present similar or better hydrodynamics than the ones obtained for polymer-based ATPS or even conventional aqueous-organic systems, when the same CPC equipment and operating parameters are used. The dependence of the physicochemical properties of the ATPS on the biphasic mixture composition also enables the optimization of the CPC-based process, making it possible to adjust the operating parameters, phase hydrodynamics, retention of the stationary phase, and separation efficiency of the target molecules according to the CPC equipment characteristics and easing its industrial implementation.

In addition, for the biphasic system studied in this work, it was observed that, up to $F = 6$ mL/min, the descending mode presented lower $S_F$ values than the ascending mode. In descending mode, the use of a denser and more viscous mobile phase resulted in a high drag force on the stationary phase (ethanol-rich top phase). This drag force, which is higher than the centrifugal force (which maintains a stable stationary phase in the column), drives the stationary phase out of the chambers, thus decreasing the $S_F$ [10]. Regardless of the operating mode, $S_F$ remained above 55% for $F$ up to 6 mL/min, which is above the 50% value and is considered satisfactory for promoting efficient separation in countercurrent chromatography [8]. Furthermore, it is important to note that because CPC is a preparative technique, the equipment should be operated with the aim of purifying products in a reduced time and using as little solvent as possible. Therefore, even though $F \geq 6$ mL/min results in decreased $S_F$, when aiming to save time and reagents in large-scale separation processes, the use of a higher $F$ can be advantageous [33].

To further analyze the effect of the simultaneous increase in $F$ and $\omega$ in the $S_F$ of the Ethanol/$K_2HPO_4$ system, Fig. 8 presents the $S_F$ values for $F$ varying between 1 and 6 mL/min and $\omega$ between 200 and 800 rpm.

In contrast to the increase in $F$, the increase in the centrifugal field intensity owing to the increase in $\omega$ did not cause a major change in $S_F$ for either operating mode (Fig. 8). This behavior is in line with previous studies, which have shown that $\omega$ has nearly no influence on $S_F$ for a variety of biphasic systems [2,6,9,10].

The effect of $\omega$ on the $S_F$ was analyzed for $F$ values of 1, 2, 3, and 6 mL/min. For $F$ from 1 to 3 mL/min, the increase in $\omega$ caused variations in $S_F$ of <9%. This variation in $S_F$ was not linear with an increase in $\omega$; that is, increasing $\omega$ does not necessarily generate an increase or decrease in $S_F$ values. In ascending mode, the maximum $S_F$ occurred at 400 rpm (for $F = 1$, 3, and 6 mL/min) and 600 rpm (for $F = 2$ mL/min). The same behavior was observed in the descending mode, where the maximum $S_F$ occurred at different values of ω for each applied $F$. However, the lowest $S_F$ was obtained for the highest $F$ (6 mL/min), regardless of $\omega$ applied, thus confirming the major influence of $F$ on $S_F$ compared to $\omega$ [10].

Although the use of the lowest and highest ω values (200 rpm and 800 rpm) resulted in an $S_F$ similar to that of the other $\omega$ values, other factors must be considered in the appropriate choice of $\omega$. It has been reported in the literature that the use of a very low $\omega$ can lead to flooding during the CPC run, that is, a constant leakage of the stationary phase from the column, causing a continuous decrease in $S_F$ [33]. However, two aspects must be considered when using a higher $\omega$. The application of higher centrifugal fields provides more stability to the stationary phase in the column over time and enables the creation of diffusion zones, thus increasing the number of theoretical plates [1]. Nevertheless, the heat generated in the equipment when high centrifugal fields are applied can disrupt the steady state at the column inlet [33]. Considering the lack of a temperature controller, higher energy consumption, and strong influence on the backpressure (shown in Fig. 4), 800 rpm was not considered as the best option for application in the Quattro LabPrep$^{TM}$ CPC.

Overall, from an operating performance point of view, the studied ethanol-based ATPS is a promising type of biphasic system for CPC-based processes owing to its high interfacial tension, low phase viscosities, and high-density difference, which are ideal for this type of separation technique [16]. However, this last parameter appears to be too high and can possibly be optimized to reduce its influence on the backpressure of the system. The versatility of ATPS and the possibility of controlling their physicochemical properties by changing the quantity of phase-forming compounds can be used to reduce this physicochemical property and consequently the impact of $\Delta\rho$ in backpressure, enhancing the performance of the entire separation process (*i.e.*, $\Delta\rho$ can be optimized or reduced by reducing the amount of $K_2HPO_4$ in the biphasic mixture, while maintaining high IFT and low viscosities in the coexisting phases).

### 3.5. Partitioning of model-solutes

Finally, after an evaluation of the performance and potential of ethanol-based ATPS for CPC assessment, the partitioning of several model solutes (*i.e.*, gallic acid and L-tyrosine) was studied. These experiments were performed at $\omega = 600$ rpm for both molecules, with $F =$ 2 mL/min for gallic acid and L-tyrosine in descending and ascending modes, respectively. To achieve a satisfactory separation efficiency of the target compounds in CPC, apart from the need for adequate

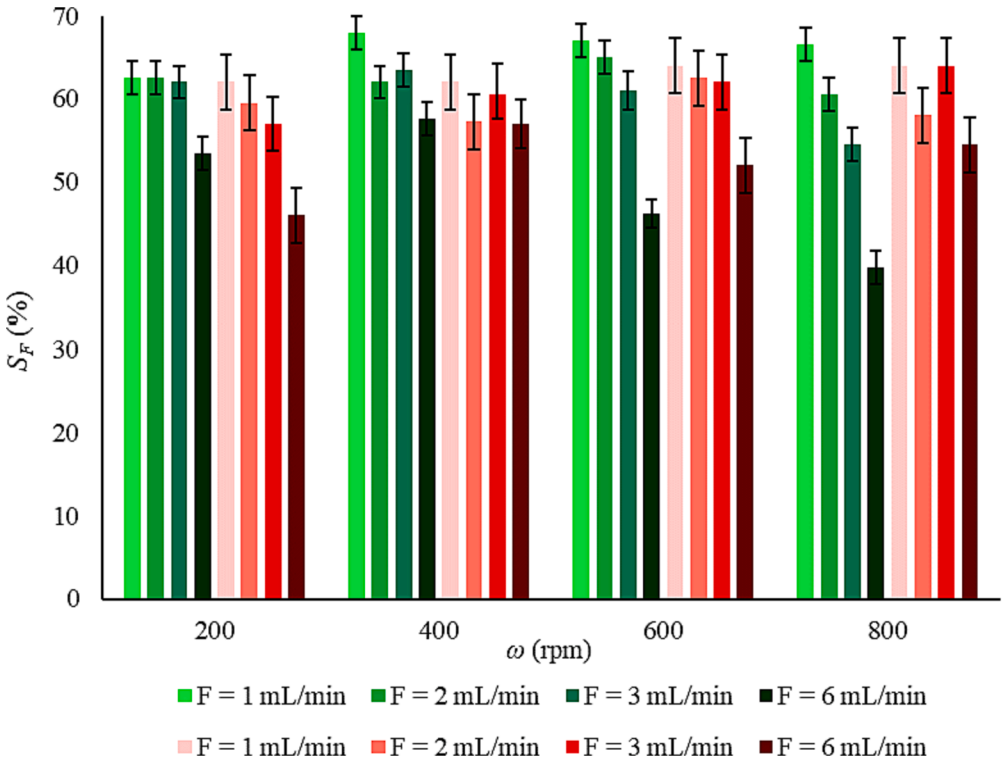


**Fig. 8.** Effect of $\omega$ on $S_F$ using Ethanol/$K_2HPO_4$ ATPS at $F$ = 1, 2, 3, and 6 mL/min in ascending (green shading) and descending (red shading) modes.

physicochemical properties of the used biphasic system, partition coefficients ($K_D$) between 0.25 and 16 and good solubility of the samples are required to obtain an adequate selectivity factor and mass transfer between the coexisting phases [2]. Moreover, in traditional on-directed modes, $K_D$ between 0.5 and 3 are desirable to obtain good resolution peaks [1]. Using an ATPS composed of Ethanol/$K_2HPO_4$, the $K_D$ of gallic acid and L-tyrosine was determined to infer the affinity of each compound for the ethanol-rich or salt-rich phase of the biphasic system. The obtained values were 0.51 ± 0.01 and 2.29 ± 0.03 for gallic acid and L-tyrosine, respectively, being in the range of operation required for CPC and making possible the evaluate the separation efficiency of these model-solutes using an Ethanol/$K_2HPO_4$ ATPS.

For gallic acid, three different $F$ values were used ($F$ = 1, 2, and 4 mL/min) at $\omega$ = 600 rpm. The chromatograms obtained are presented in

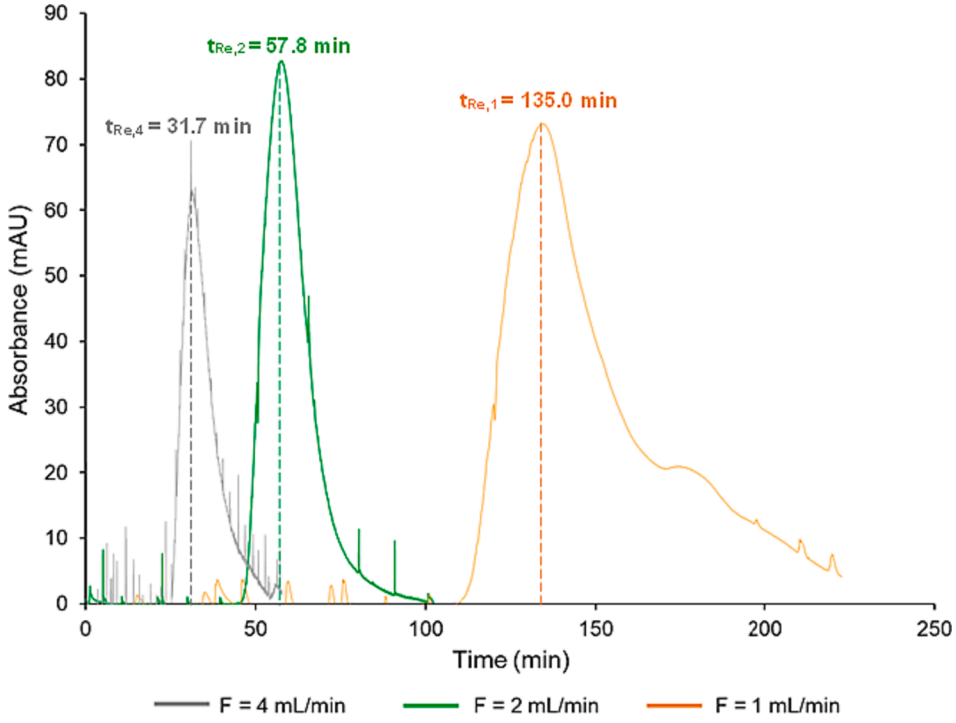


**Fig. 9.** Chromatograms of gallic acid partitioning for Ethanol/$K_2HPO_4$ ATPS using CPC for $F$ = 1, 2, and 4 mL/min at $\omega$ = 600 rpm.

Fig. 9.

From the analysis of Fig. 9, and considering the equipment characteristics, the mobile phase within the column (equivalent to the volume of the stationary phase volume) ($V_M$), $S_F$, solute retention volume ($V_{RD}$), calculated retention time ($t_{Rc}$), and experimental retention time ($t_{Re}$) were determined for each operating condition and are displayed in Table 2**.**

Observing the data for each operation condition presented in Table 2, it can be observed that higher values of $S_F$ were obtained for lower *F*. Although these results are in accordance with the previously discussed hydrodynamic behavior, the $S_F$ values obtained for the partitioning of gallic acid were lower than those observed in the hydrodynamic assays for the same operating parameters (*i.e.*, $S_F$ varied between 40 and 52% for the partitioning tests, between 50 and 60% for the hydrodynamic tests, and between 1 and 4 mL/min for *F*). Both assays were operated at different $\omega$ values (400 rpm for the hydrodynamic tests in Fig. 7 and 600 rpm for the partitioning tests); however, it was previously stated that the differences in rotational speed did not have a significant influence on $S_F$ [16]. Thus, these variations can be related to the process variability of the equipment and the differences in the operating parameters between the previously discussed hydrodynamic studies and the partitioning of the model solutes. If this trend occurred after the injection of gallic acid into the equipment, the decrease in $S_F$ could be related to the presence of gallic acid in the ATPS, as solutes with $K_D < 1$ move slower through the rotor than those with $K_D > 1$, leading to lower hydrodynamic and separation efficiencies [10]. In addition, the presence of gallic acid could result in a reduction in IFT, which would have a major impact on the steady state between the mobile and stationary phases, causing bleeding [16]. If this was the case, another alternative would be to reduce the concentration of gallic acid used in the injected sample, thereby reducing the influence of the hydrodynamics of the system and improving the separation efficiency of the process. Because the recorded $S_F$ values were recorded before the injection of gallic acid and were lower than 50%, and considering that $\omega$ does not have a major influence on the obtained results, the use of a lower *F* is recommended to obtain $S_F$ within the minimum range of operation for efficient separations ($S_F > 50\%$) [8]. In addition, it should be noted that the selection of the best operating conditions must consider the techno-economic analysis of the process, as a higher *F* leads to a higher loss of the stationary phase but also reduces the retention time, which could be financially advantageous. For instance, comparing $F = 1$ and 4 mL/min, there is a reduction of 11.5% in $S_F$ for a higher *F*, but also a reduction of >100 min in the retention time of the solute, which can result in a significant improvement in the operation time of the CPC-based process and increase its economic viability. Comparing $t_{Rc}$ and $t_{Re}$ for the different operating conditions (Table 2), it can be stated that the first was superior to their experimental results, indicating an anticipated elution of solutes from the chambers. These differences can be associated with the $K_D$ of gallic acid, as the analyte tends to elute before elution of one column capacity volume when $K_D < 1$ (and *vice-versa*) [8]. A decrease in the $t_R$ values is also registered for the increase in *F* because the increase in the mobile phase velocity leads to faster elution of the solute [7,16].

In the chromatograms obtained (Fig. 9), the peaks for each operating condition also present interesting features. The peak corresponding to *F* = 1 mL/min was broader than those observed in the chromatograms of the other systems. This was associated with the very low flow rate of the mobile phase, which reduces the convective transport of the solutes dissolved in the stationary phase to the mobile phase due to a less efficient phenomenon, increases the residence time distribution of the solutes and induces peak broadening [2,10]. Since the main objective of this work was only to confirm the suitability of alcohol/salt ATPS for CPC application (*i.e.*, the adequacy of this type of biphasic system's physicochemical properties and influence on the operating parameters for future CPC-based separation processes), the peaks broadening are not very important for this study. The obtained chromatograms in Fig. 9 confirm the effective separation of gallic acid using this type of ATPS and CPC equipment, which was the main goal of this experiment.

This broadening phenomenon was also observed in the other two recorded peaks, but not to such an extent as for $F = 1$ mL/min. It can also be observed that the peak heights have values between 60 and 80 mAU. Because the concentration of gallic acid in all three samples was the same (≈ 0.30 g/L), the registered differences in the obtained values resulted from the *F* variation in each separation, with $F = 2$ mL/min presenting the highest and most well-defined peak. These results indicate the effective partitioning of gallic acid using ethanol/salt ATPS and CPC equipment.

The separation studies were continued using another model solute considered in this study, l-tyrosine. To separate this compound, only one mobile phase flow rate was tested ($F = 2$ mL/min). To calculate the separation efficiency of this biomolecule using the ethanol-based ATPS and CPC process, the chromatogram of this operation presented the experimental retention time of L-tyrosine in the column, and the process fraction was collected closest to that time. The obtained chromatogram is shown in Fig. S2 (Supporting Information), where it can be observed that $t_{Re}$ for L-tyrosine was 66.8 min. Thus, a fraction of the eluted mobile phase was collected from CPC in a new separation process, and the concentration of L-tyrosine in the sample was determined by measuring its absorbance at 275 nm. Since the concentration of L-tyrosine in that fraction was 0.18 ± 0.03 g/L and the initial concentration of L-tyrosine was approximately 0.21 g/L, a separation efficiency of 85.7% was recorded for this compound using this type of ATPS and CPC equipment. This procedure was made in duplicate, being attained the following process parameters mean values: $V_M = 100.0 \pm 8.0$ mL, $S_F = 61.0 \pm 4.0$ %, $V_{RA} = 143.7 \pm 4.51$ mL and $t_{Rc} = 71.83 \pm 2.25$ min. The obtained $S_F$ and chromatogram (Fig. S2) for L-tyrosine indicate an effective partitioning of this compound using the tested ethanol/salt systems, with the CPC process with this ATPS presenting acceptable $S_F$ ($S_F > 50\%$). Furthermore, the calculated and experimental retention times were very close to each other, indicating good hydrodynamic predictability for the partitioning of this solute using this equipment.

Overall, the partitioning of gallic acid and L-tyrosine from different extracts rich in each of these compounds was possible using ethanol-based ATPS and Quattro LabPrep™ CPC equipment. However, it is necessary to consider the characteristics of solutes and their influence on the hydrodynamics and separation efficiency of the system. This is one of the most important steps in the design of chromatography-based separation processes because the partition coefficient, solubility, polarity, stability, UV absorption, and pH influence the performance of the system [10]. All these data must be gathered in the literature for the target compounds or experimentally determined by the researchers to select the operating conditions, biphasic system, and type of CPC equipment that provides the best possible trade-off between the operating parameters and enables a viable separation process [10]. Table 3 presents a summary of the critical parameters studied in this work and a comparison of the obtained results for the ethanol-based ATPS with the polymer-based ATPS and the conventional aqueous-organic systems used in CPC. This ranking was based on the results obtained in this study for the ethanol/salt ATPS and those presented in previous studies for the other two types of biphasic systems [2,6,16,30].

As indicated in Table 3, the ethanol/salt ATPS is a biphasic system

**Table 2**
The mobile phase within the column ($V_M$), stationary phase retention ($S_F$), solute retention volume ($V_{RD}$), calculated retention time ($t_{Rc}$), and experimental retention time ($t_{Re}$) for gallic acid using the Ethanol/$K_2HPO_4$ ATPS in CPC for $F = 1$, 2, and 4 mL/min and $\omega = 600$ rpm. The $V_{RD}$ and $t_{Rc}$ were obtained using **Equations 3** and **4**, respectively. $V_C$ and $V_D$ were 200 and 22 mL, respectively.

| *F* (mL/min) | $V_M$ (mL) | $S_F$ (%) | $V_{RD}$ (mL) | $t_{Rc}$ (min) | $t_{Re}$ (min) |
|---|---|---|---|---|---|
| 1 | 118 | 52.0 | 159.8 | 159.8 | 135.0 |
| 2 | 128 | 47.0 | 164.8 | 82.4 | 57.8 |
| 4 | 141 | 40.5 | 171.2 | 42.8 | 31.7 |

**Table 3**
Comparison between the main critical parameters of different types of biphasic systems for CPC application, with the systems ranked according to their suitability for this type of process.

| Critical parameters | Ethanol/Salt ATPS | Polymer-based ATPS | Aqueous-organic systems |
|---|---|---|---|
| Density difference | 1 | 3 | 2 |
| Interfacial tension | 1 | 3 | 2 |
| Phases' viscosity | 2 | 3 | 1 |
| Settling time | 2 | 3 | 1 |

with better density difference and interfacial tension, whereas the aqueous/organic system presented a better classification of the phases' viscosity and settling time. It should also be noted that the settling times of the ethanol/salt ATPS phases are very small and in the range of the values recorded for aqueous-organic systems, thus not being a limitation for future industrial implementation. Polymer-based ATPS were ranked as the least suitable for CPC application from a hydrodynamic and equipment operation point of view because of their lower density difference and interfacial tension, and the very high phase viscosity and settling time, when compared with the other two types of biphasic systems. However, polymer-based ATPS have the main advantage of being much more biocompatible with a larger number of molecules than the remaining biphasic systems with which they were compared, even though the purification of the extracted biomolecules with polymer-based systems usually requires complex and expensive processes, such as ultrafiltration or membrane processes [35].

Another very important part of a possible CPC industrial implementation is the recycling and reuse of the biphasic systems' phase-forming compounds. Several processes can be used to recover and reuse ATPS phases, and the selection of the most suitable one for each specific system is always dependent on the characteristics of the recovered phase-forming compounds and the impact of the regeneration process operation conditions on the extracted biomolecules. Some of the processes that can be used are membrane filtration, dialysis, ultrafiltration, or density-based recirculation [35]. In the case of Ethanol/$K_2HPO_4$ ATPS, besides all possible regeneration processes, the recovery and recycling of ethanol can also be achieved by the simple evaporation of ethanol and its reuse in ATPS formation. To choose the best type of regeneration process, a techno-economic analysis should also be conducted, and the more financially beneficial option is selected.

Therefore, ethanol/salt ATPS appear to possess ideal properties for the effective implementation of CPC-based processes in the purification of alcohol-stable biomolecules, presenting physicochemical properties that enable a better hydrodynamic performance than polymer-based ATPS for the CPC equipment in this study. Furthermore, the versatility of ATPS (*i.e.*, the possibility of controlling their physicochemical properties by adjusting the biphasic mixture composition) is another advantage in the industrial implementation of CPC processes using ethanol-based ATPS, always taking into consideration the influence of the biphasic system composition on the hydrodynamic performance of the equipment and partitioning of the target molecules. The use of this type of ATPS in CPC is very promising, and future studies are necessary to evaluate their performance under different equipment, operating conditions, and analytes to design an effective CPC-based separation process.

## 4. Conclusions

This work presents the hydrodynamic and separation performances of ethanol/salt ATPS in CPC. The ethanol/salt ATPS presented more suitable physicochemical properties for CPC implementation than polymer-based ATPS, and similar or even better properties than the aqueous-organic systems owing to their high IFT (6.40 mN/m), high $\Delta\rho$ (353 kg/m$^3$), and low phase viscosity (2.5–3.8 mPa.s). The better hydrodynamic performance of the ethanol-based system compared to the polymer-based ATPS was confirmed by the higher backpressures registered for aqueous solutions of polymers compared to aqueous solutions of ethanol or phosphate salt. The increases in $F$ and $\omega$ led to a reduction in $S_F$ and an increase in backpressure, with the ethanol-based system presenting good hydrodynamic stability ($S_F$ was almost constant and higher than 50 % for $F \leq 6$ mL/min). It was also proven that the design of each CPC equipment affected the performance of each biphasic system, demonstrating that the comparison of different types of solvent systems must occur for similar operating parameters in equipment with identical designs and characteristics. Regarding the partitioning of two model solutes (gallic acid and L-tyrosine), the ideal physicochemical properties for CPC good hydrodynamic performance and the $K_D$ of biomolecules indicate that ethanol/salt ATPS has enormous potential for CPC implementation. Further studies are required to facilitate the design of this type of separation process at the industrial scale.

## CRediT authorship contribution statement

**Ariane A. Oshiro:** Investigation, Software, Validation, Visualization, Writing – original draft. **Alexandre M.S. Jorge:** Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft. **Valéria C. Santos-Ebinuma:** Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Jorge F.B. Pereira:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

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