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Extraction of tetracycline from fermentation broth using aqueous two-phase systems composed of polyethylene glycol and cholinium-based salts

Jorge F.B. Pereira[a], Filipa Vicente[a], Valéria C. Santos-Ebinuma[b], Janete M. Araújo[c], Adalberto Pessoa[b], Mara G. Freire[a], João A.P. Coutinho[a,*]

[a] Departamento de Química, CICECO, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] Departamento de Tecnologia Bioquímico-Farmacêutica, Universidade de São Paulo, Avenida Prof. Lineu Prestes 580, Bl. 16, 05508-900 São Paulo, SP, Brazil
[c] Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Departamento de Antibióticos. Rua Prof. Artur de Sá, Cidade Universitária, 50670-420 Recife, PE, Brazil

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ABSTRACT

Aiming at developing not only cheaper but also biocompatible and sustainable extraction and purification processes for antibiotics, in this work it was evaluated the ability of aqueous two-phase systems (ATPS) composed of polyethylene glycol (PEG) and cholinium-based salts to extract tetracycline from the fermented broth of *Streptomyces aureofaciens*. Conventional polymer/salt and salt/salt ATPS were also studied for comparison purposes. The novel systems here proposed are able to extract tetracycline directly from the fermentation broth with extraction efficiencies higher than 80%. A tailored extraction ability of these systems can also be achieved, with preferential extractions either for the polymer- or salt-rich phases, and which further depend on the cholinium-based salt employed. The gathered results support the applicability of biocompatible ATPS in the extraction of antibiotics from complex matrices and can be envisaged as valuable platforms to be applied at the industrial level by pharmaceutical companies.

1. Introduction

Antibiotics are chemical compounds that can be totally or partly synthesized by living microorganisms that either inhibit the growth or even kill other microorganisms. Therefore, they are currently used worldwide for an effective control of the levels of pathogenic bacteria in humans and animals. At a global level, the antibiotic market reached US$42 billion (≈34 billion €) in 2009 [1]. Nevertheless, in the past few years, a number of patents regarding the synthesis and applications of antibiotics and other drugs have expired and they fall into the public domain [2]. The pharmaceutical industry is facing the growth of generic drugs and there is a crucial need to minimize operational costs by optimizing the antibiotics' production and their purification steps. The production cost of antibiotics derives, by a large extent, from the extraction and associated purification processes. In this context, it is imperative to find and evaluate new extractive/purification techniques which could be scaled-up by biopharmaceutical companies.

There are several classes of antibiotics such as β-lactams, aminoglycosides, macrolides and tetracyclines (TCs). Tetracyclines are bacteriostatic antimicrobials produced by *Streptomyces aureofaciens* or *Streptomyces rimosus*. They are a broad-spectrum antibiotic since they can be used against Gram positive and negative bacteria, *Coccidian*, *Trichomonas*, *Mycoplasma*, *Chlamydia* and *Rickettsia*. Tetracyclines inhibit the synthesis of bacterial proteins by binding to the small unit (30 S) of bacterial ribosome while preventing the access of aminoacyl tRNA to the acceptor site on the mRNA-ribosome complex [3–6]. Besides their antibiotic properties, tetracyclines also possess anti-inflammatory, anti-apoptotic and anti-neurodegenerative properties [6]. Due to all their benefits tetracyclines are currently added to animal feed in order to prevent diseases as well as a feed additive to accelerate growth [7].

Chromatographic techniques (particularly ion exchange chromatography), liquid–liquid or solid–liquid extractions are generally used for the extraction and purification of common antibiotics from the fermentation broth [8–10]. Among these techniques, the most used is the liquid–liquid extraction which has been carried out using organic solvents, namely ethyl acetate, acetonitrile and methanolic trichloroacetic acid (TCA) [11]. This type of liquid–liquid extraction is a useful technique which involves low costs and leads to a high purity level. However, these organics compounds present some drawbacks since they are volatile and hazardous to human health [8]. Taking into account the sustainability and biocompatibility of extraction processes, the use of aqueous two-phase systems (ATPS) can represent a viable option.

* Corresponding author. Tel.: +351 234 401507; fax: +351 234 370084.
E-mail address: jcoutinho@ua.pt (J.A.P. Coutinho).

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ATPS are considered a low cost, gentler and biocompatible alternative to other extraction techniques since they are mainly composed of water [12–14]. In the last decade, novel ATPS composed of ionic liquids (ILs) + water + organic/inorganic salts, amino acids, polymers or carbohydrates have also been proposed in literature [15]. The main advantages of polymer–salt or polymer–IL ATPS, when compared to polymer–polymer systems, relay on their lower viscosities and possibility of changing the polarities of the coexisting phases. Moreover, they usually display a quick phase separation and high extraction efficiencies which can be easily manipulated by a proper selection of the ions composing a given IL or salt [15]. Indeed, IL-based ATPS have been successfully used in the separation, concentration and purification of proteins, antioxidants, metal ions, alkaloids and antibiotics [15].

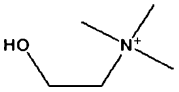
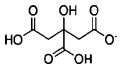
Most hydrophilic ILs exhibit unique properties that make them environmentally friendly solvents such as negligible vapor pressures, non-flammability and high thermal and chemical stabilities [16]. Their chemical diversity offers unique opportunities to develop solvents for specific purposes with tailored characteristics by the combination of proper ions. Due to their attractive physicochemical properties, ILs have additionally been applied for bio-purification and bio-extraction processes [15,17], in enzymatic catalysis [18], bioprocess operations [19] and in biofuel production [20]. However, most ILs are poorly biodegradable and of low biocompatibility. In this context, ionic liquids and salts based on the cholinium ion can be a valuable alternative; yet, poorly studied. Cholinium chloride is an essential nutrient, as considered by the Institute of Medicine in 1998, due to its role in the human body (used for neurotransmitter synthesis, cell-membrane signaling, etc.) [21]. It is of easy preparation, relatively cheap, stable in water and air, biocompatible and biodegradable. The cholinium-based salts, or ionic liquids (ILs) when their melting point is below 100 °C, are constituted by the 2-hydroxyethyl-*N*,*N*,*N*-trimethylammonium cation combined with anions as diverse as chloride, bicarbonate, acetate, levulinate, malate, glycolate, among others. Hence, the cholinium-based salts/ILs are a feasible option to be used in the formation of ATPS. We demonstrated recently the possibility of creating aqueous two-phase systems of the type polymer–salt or salt–salt either by the combination of PEG and cholinium-based salts or by the addition of an inorganic salt to mildly hydrophobic cholinium-based ILs [22,23]. Aiming at exploring the applicability of those novel ATPS, this work is focused on the extraction of tetracycline directly from the fermentation broth of *S. aureofaciens*. Systems composed of polyethylene glycol 600 and cholinium-based salts were investigated by means of the partition coefficients and extraction efficiencies obtained for tetracycline. To further ascertain on the enhanced ability of these novel systems to extract tetracycline, further experiments were carried out with conventional polymer/salt and salt/salt ATPS for comparison purposes.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG) with an average molecular weight of 600 g mol^{-1} (abbreviated as PEG 600) and tetracycline (TC), ≥98 wt% pure, were supplied by Fluka. The cholinium-based salts were acquired at Sigma–Aldrich: cholinium chloride, [Ch]Cl, cholinium bicarbonate, [Ch]Bic, and cholinium dihydrogencitrate, [Ch]DHcit, with purities of ≥98 wt%, 80 wt% (in aqueous solution), and 99 wt%, respectively. The cholinium acetate, [Ch]Ac, and cholinium dihydrogenphosphate, [Ch]DHph, were purchased from Iolitec with purity levels of 98 wt% and ≥98 wt%, respectively. These last two cholinium-based salts fall within the category of ILs since they present melting temperatures below 100 °C. Nevertheless, to avoid any ambiguity, all these materials will be thereinafter referred as cholinium-based salts. The chemical structures of the investigated cholinium-based salts are presented in Table 1. ^{1}H and ^{13}C NMR spectra were performed to evaluate the purity of each sample. All samples are of high purity and agree with the purity levels indicated by the suppliers. The inorganic salts K_3PO_4 (≥98 wt%) and Na_2SO_4 (>99.0 wt%) were acquired from Sigma–Aldrich and LabSolve, respectively. All the other reagents are of analytical grade and were used as received.

Table 1
Chemical structures of the studied cholinium-based salts.

Cholinium-based salts	Cation	Anion
[Ch]Bic		
[Ch]Cl		Cl^-
[Ch]Ac		
[Ch]DHcit		
[Ch]DHph		

2.2. Microorganism maintenance and fermentation processes

S. aureofaciens was kindly provided by the Microorganism Collection of the Department of Antibiotics from Federal University of Pernambuco, Recife, PE, Brazil. The frozen microorganism was maintained at −70 °C with glycerol in a cryotube. According to the procedure described by Darken et al. [24], a thick spore suspension contained in the cryotube was transferred to 25 mL of reactivation medium in 250 mL-Erlenmeyer flasks. After an incubation period of 24 h in an orbital shaker at 30 °C and 200 rpm, 5 mL of this suspension were added to 45 mL of the fermentation medium and re-incubated under the same conditions of reactivation during 48 h (at this condition the pH value was 4.50 ± 0.04). For the fermentation processes, 5.0 mL of the resulting cell suspension were added to 45 mL of fermentation medium in 500 mL-Erlenmeyer flasks and incubated during 120 h at the same operational conditions. At the end of the fermentation process, the fermented broth was filtrated through a Whatman N. 4 paper and then centrifuged at 3720 × *g* for 15 min at 5 °C. The supernatant obtained from this process presented a final pH of 4.27 ± 0.09 and a TC concentration of 0.175 g/L (quantified as described below). The supernatant was frozen and stored in an ultrafreezer at −70 °C and further used in all the partitioning studies.

2.3. Media composition

A modified liquid ISP-2 medium [25] with a constitution of 4.0 g L^{-1} of yeast extract and 10.0 g L^{-1} of malt extract was used for reactivation. The medium proposed by Darken et al. [24] for the preparation of the *S. aureofaciens* inoculum was used and it is composed as follows (g L^{-1} in deionized water): sucrose (30.0), soybean meal (5.0), $Na_3C_6H_5O_7{\cdot}5H_2O$ (1.0), $(NH_4)_2SO_4$ (3.3), $MgSO_4{\cdot}7H_2O$ (0.25), KH_2PO_4 (0.10), K_2HPO_4 (0.10), $CaCO_3$ (1.0), $MnSO_4{\cdot}4H_2O$ (0.01), $ZnSO_4{\cdot}7H_2O$ (0.04), $K_2Cr_2O_7$ (0.000016) and CH_3COOH (0.40). The fermentation medium was prepared according to the description given by Darken et al. [24] and it is composed of: $H_3C_6H_5O_7{\cdot}H_2O$ (12.8), sucrose (40.0), $(NH_4)_2SO_4$ (6.0), $MgSO_4{\cdot}7H_2O$ (0.25), KH_2PO_4 (0.15), $CaCO_3$ (11.0), $MnSO_4{\cdot}4H_2O$ (0.01), $ZnSO_4{\cdot}7H_2O$ (0.04) and $K_2Cr_2O_7$ ($0.016.10^{-3}$). All media were autoclaved at 121 °C for 15 min.

3. Methods

3.1. Phase diagrams and tie-lines

Phase diagrams for each ternary system composed of PEG600 + cholinium-based salt + H_2O at 25 °C were previously determined by us [23] using the cloud point titration method. Other ATPS used for comparison purposes were taken from literature [22,26]. The knowledge of these phase diagrams allows the choice of mixture points which correspond to a liquid–liquid two-phase system.

3.1.1. Optimization of the TC partitioning in ATPS composed of PEG and cholinium-based salts

In order to optimize the experimental conditions and phase compositions to be applied in the extraction of TC from the fermented broth, several model systems were initially investigated making use of commercial TC of high purity. Three different mixture compositions at the biphasic region, and for each ATPS, were selected. Small amounts of commercial TC, 0.25–0.45 mg, were added to glass tubes containing the appropriate weights of PEG, [Ch]-salt and water to form a system with a total mass of 5 g. After the equilibration time, 12 h at 25 (± 1) °C, the phases were carefully separated and the quantification of TC in both phases was carried out. The quantification of tetracycline is described below. All the assays were performed in triplicate and the respective standard deviations were determined.

The partition coefficient of TC, K_{TC}, is the ratio between the antibiotic concentration in the PEG-rich phase ($[TC]_{PEG}$) to that in the cholinium-rich phase ($[TC]_{Ch}$) and as described by Eq. (1):

$$K_{TC} = \frac{[TC]_{PEG}}{[TC]_{ch}} \quad (1)$$

3.1.2. Partitioning of TC in conventional PEG/Na_2SO_4 and [Ch]Cl/K_3PO_4 ATPS

For comparison purposes and to ascertain on the potential of PEG-cholinium-based ATPS as improved extraction routes, the K_{TC} data were also determined in two mixture points (biphasic systems) of the PEG600 + Na_2SO_4 + water system and for three mixture compositions of the [Ch]Cl + K_3PO_4 + water ATPS. The partitioning studies were carried out in triplicate and at 25 (±1) °C, and in accordance to the procedure previously described.

The partition coefficient of TC for the PEG-rich phase (K_{TC_PEG}) in the PEG/Na_2SO_4 system and for the cholinium-rich phase (K_{TC_Ch}) in the [Ch]Cl/K_3PO_4 system were determined according to Eqs. (2) and (3), respectively:

$$K_{TC\_PEG} = \frac{[TC]_{PEG}}{[TC]_{salt}} \quad (2)$$

$$K_{TC\_Ch} = \frac{[TC]_{Ch}}{[TC]_{salt}} \quad (3)$$

where $[TC]_{PEG}$, $[TC]_{salt}$ and $[TC]_{Ch}$ correspond, respectively, to the TC concentration in the PEG-rich, in the inorganic-salt-rich and in the cholinium-rich phase.

3.1.3. Recovery of TC from the fermented broth

The novel PEG/[Ch]Bic and PEG/[Ch]Cl ATPS and the conventional PEG/Na_2SO_4 and [Ch]Cl/K_3PO_4 aqueous systems were also used in the investigation of the recovery/extraction of TC from the fermentation broth of *S. aureofaciens*. The corresponding systems were prepared by choosing the mixture composition for which the highest partition coefficient was obtained in the optimization step with the model systems previously addressed. The partitioning studies were performed adding directly the liquid supernatant of the fermented broth to known amounts of polymer and cholinium-based salt. The partitioning behavior of TC was quantified in terms of the respective partitioning coefficients in the several ATPS, namely K_{TC} (for the PEG600/[Ch]-based salts ATPS), K_{TC_PEG} (for the PEG/Na_2SO_4 ATPS) and K_{TC_Ch} (for the [Ch]Cl/K_3PO_4 ATPS), as described above.

The percentage extraction efficiency of TC ($\%EE_{TC}$) for a given phase, e.g. the phase with higher TC concentration, was also evaluated through Eq. (4):

$$\%EE_{TC} = \frac{m_{TC-rich\ phase}}{m_0} \times 100 \quad (4)$$

where $m_{TC\text{-}rich\ phase}$ is the mass of TC in the phase with higher TC concentration and m_0 is the total mass of tetracycline in each system.

3.1.4. Quantification of tetracycline

The TC concentration in each ATPS phase was determined using an UV–vis spectrophotometer (SHIMADZU UV-1700, Pharma-Spect Spectrometer). The UV–vis spectrum of TC was firstly obtained and calibration curves were properly established at a neutral and alkaline pH since diverse salts can induce different pH values (cf. Supporting Information Data, Figs. A1–A4). TC shows two distinct wavelengths were maxima in absorbance are observed: (i) at neutral pH the biomolecule has no charge and these maxima occur at 276 nm and 357 nm; (ii) at an alkaline pH, the molecule is negatively charged, and these maxima occur at 269 nm and 381 nm. In the partitioning studies using the fermented broth of *S. aureofaciens* it was not possible to quantify the TC at 276 nm and 269 nm since the proteins present in the broth also contribute to the absorption observed at these wavelengths [27]. Therefore, the TC was measured using the calibration curves established at 357 and 381 nm, for the neutral and alkaline systems, respectively. The validation of the TC quantification using the calibration curves determined at 357 and 381 nm was additionally confirmed taking into account the difference between the total protein concentration ([TP]) present in the fermented broth, which was determined using the bicinchoninic acid method (BCA) [28], and the protein concentration ($[P_{BSA}]$) obtained by a calibration curve making use of bovine serum albumin (BSA) at 280 nm. The difference between the total absorbance and the BSA absorbance at 280 nm gives the TC absorbance. The TC concentration of the fermented broth measured by the second set of calibration curves is similar (within ±5%) to that obtained by the measurement of the absorbance in the first maximum wavelength peaks for the UV region (cf. Supporting Information, Table A1) and validate the experimental procedure adopted.

3.1.5. pH and conductivity measurements

After the separation of the coexisting phases of each system, the pH values (±0.02) and conductivity (±0.01 mS cm^{-1}) were measured at 25 (±1) °C using a SevenMultiTM equipment (Mettler Toledo Instruments). The conductivity measurements were used to identify the polymer and the salt-rich phases since they are colorless in most of the cases.

4. Results and discussion

4.1. Optimization of the TC partitioning in ATPS composed of PEG and cholinium-based salts

In order to optimize the TC partitioning, different cholinium-based salts were used in combination with PEG600 to form an ATPS. In this study, the cholinium-based salts investigated were [Ch]Cl, [Ch]Bic, [CH]DHcit, [Ch]Ac and[Ch]DHph. The initial mixture compositions were selected so that the liquid–liquid systems could be formed taking into account previous literature data with their phase diagrams [23]. The initial mixture compositions, the partition coefficients of tetracycline and the pH values of the phases are presented in Table 2. Based on the initial mixture compositions and the respective ternary phase diagrams [23], the composition of each phase was further determined, e.g. the corresponding tie-lines are presented in Supporting Information, Table A2. The tie-line length (TLL) corresponds to the length of each tie-line and indicates the difference in composition of the two phases. The values of the TLLs are presented in Table 2.

It should be remarked that for the systems based on [Ch]Bic, [Ch]DHcit and [Ch]DHph, the bottom phase is the salt-rich phase whereas the top phase corresponds to the PEG-rich phase. However, some inversions on the phases' densities were observed depending on the composition and constituents of the system. Particularly, an inversion on the top and bottom phases was observed with the systems composed of [Ch]Cl and [Ch]Ac. Since both phases are liquid and non-colored, the coexisting phases were identified by conductivity measurements at 25 °C (the conductivity values are provided in Supporting Information, Table A3).

This first optimization step aimed at understanding the dependence of K_{TC} with the cholinium-based salt and respective TLLs. The values of K_{TC} shown in Table 2 suggest that, in most cases, the antibiotic preferentially migrates for the PEG-rich phase. Only one exception was observed with the system composed of [Ch]Cl. In this system the K_{TC} value is always close to unity indicating that TC distributes equally by the two phases. Moreover, the pH values of the phases, which range between 5 and 11, seem to have no significant influence concerning the preferential migration of the antibiotic for the polymer-rich phase. Albeit tetracycline has preference for more hydrophilic phases, as displayed by its octanol–water partition coefficient ($\log K_{ow} = -1.19$) [29,30], in this study, tetracycline migrates preferentially for the most hydrophobic and polymeric phase. Therefore, this migration pattern could be a consequence of the salting-out effect of the cholinium-based salts over the antibiotic which forces its migration toward the PEG-rich phase.

To facilitate the comparison between the cholinium-salt effect and the composition of the system through the partitioning of TC, the logarithmic function of K_{TC} as a function of the TLL is depicted in Fig. 1.

An increase in the amount of the [Ch]-based salt or an increase in the polymer content lead to an increase in the TLL, i.e. to a higher difference in the composition of the coexisting phases of a particular system. According to Fig. 1, the most relevant effect of the TLL is observed with the system constituted by [Ch]Bic where an increase in the TLL leads to an increase in the K_{TC} value. In the remaining ATPS the K_{TC} values are approximately constant, taking into account the associated standard deviations, and independent of the TLL (in the composition range evaluated).

At a similar TLL, when comparing the different cholinium-based salts, the TC tends to migrate for the PEG-rich phase according to the following tendency: [Ch]Cl < [Ch]DHcit < [Ch]DHph ≈ [Ch]Ac < [Ch]Bic. Besides the preferential migration for the PEG-rich phase in most cases, there are differences between the diverse cholinium-based salts which depend on the pH values of the phases.

Table 2
Weight fraction composition of the initial mixture, TLL, pH value of each phase and partition coefficient of TC at 25 °C.

Ternary system	Weight fraction composition (wt%)		TLL (wt%)	$pH_{PEG\text{-}rich\ phase}$	$pH_{IL\text{-}rich\ phase}$	K_{TC}
	PEG600	Salt				
[Ch]Cl	54.94	34.93	115.65	7.12	7.09	0.98 ± 0.03
	54.93	29.98	95.93	7.05	6.72	0.92 ± 0.03
	54.91	24.95	57.58	7.03	6.81	1.02 ± 0.04
[Ch]Bic	49.88	34.93	117.85	11.22	10.65	10.07 ± 1.01
	49.81	29.96	102.38	10.93	10.55	6.72 ± 0.62
	49.87	24.99	73.18	10.83	10.51	5.68 ± 0.96
[Ch]DHcit	45.00	39.92	81.48	5.29	5.25	1.45 ± 0.17
	45.85	37.80	67.52	5.27	5.03	1.30 ± 0.06
	56.89	29.94	95.57	5.39	5.44	1.14 ± 0.05
[Ch]Ac	49.75	39.82	123.64	10.12	10.06	2.69 ± 0.27
	49.55	34.77	109.67	9.44	9.35	2.73 ± 0.40
	49.59	29.81	95.84	8.93	8.90	2.57 ± 0.21
[Ch]DHph	49.90	29.95	111.00	7.65	7.37	2.69 ± 0.43
	40.20	29.91	96.19	6.16	5.97	2.62 ± 0.15
	34.98	29.97	86.35	6.56	5.75	2.60 ± 0.19

TCs are amphoteric compounds and show three dissociation constants or pK_a values: 3.30, 7.68 and 9.69 [31]. Thus, in aqueous solutions of different pH, TCs can appear in one of the three forms: cationic when pH < pK_{a1} (3.30), zwitterionic when pK_{a1} (3.30) < pH < pK_{a2} (7.68) and anionic when pH > pK_{a2} (7.68). The anionic species can be monovalent or divalent when the pH is between the pK_{a2} and the pK_{a3} (9.68) or when the pH > pK_{a3}, respectively [32]. The dissociation curves of TC as a function of the pH are shown in Supporting Information, Fig. A5. Looking at the data in Table 2 it can be gauged that in the systems with [Ch]Cl, [Ch]DHcit and [Ch]DHph, TC is mainly in a neutral form (3.30 < pH < 7.68); in the ATPS composed of [Ch]Ac and [Ch]Bic, with pH values above the pK_{a2} of TC, the antibiotic is in a charged form (anionic); for the systems with 50 wt% of PEG600 and 40 wt% of [Ch]Ac and all the PEG600/[Ch]Bic ATPS the pH values are above the pK_{a3} (9.68) of TC and in these cases TC is present as a divalent anion.

In the PEG/[Ch]Cl ATPS there was no preferential affinity of TC for a given phase. This behavior is in close agreement with the weak ability of the chloride anion to induce the salting-out of other species [33,34]. Indeed, in a previous work [27] where a series of cholinium-based salts was evaluated, it was observed that [Ch]Cl was the compound with the weakest ability to form two liquid phases. In addition, in the [Ch]Cl systems with pH values around 7, TC is mainly in its neutral form (with only a small fraction being negatively charged) and has no preference for the highly charged salt-rich phase. When [Ch]DHcit is used to promote the TC partitioning, the antibiotic shows a slight affinity for the polymeric phase. In the [Ch]DHcit system, with pH values between 5 and 6, TC is mainly in its neutral form although some small fraction of positively charged TC is also present. Even though the citrate anion is usually considered a good salting-out agent, according to the Hofmeister series [33,34], the observed biomolecule partitioning for the PEG-rich is not significant possibly due to dissociation effects of the citrate anion. At a pH value around 6 the citrate anion is also dissociated in a divalent species as discussed before by Pereira et al. [23].

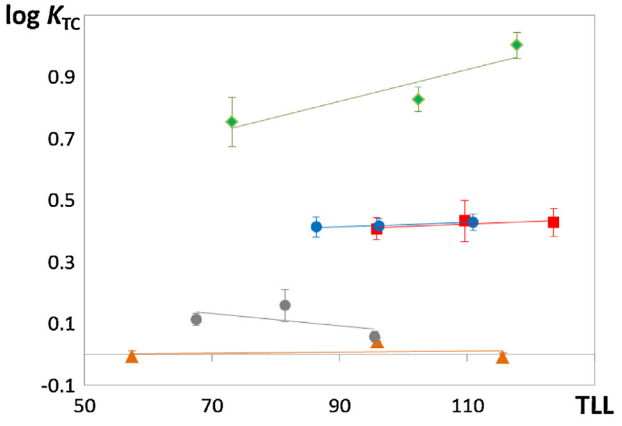


Fig. 1. Logarithm of K_{TC} versus TLL, at 25 °C, for the systems composed of PEG600 + [Ch]-based salt + water: (♦) [Ch]Bic; (■) [Ch]Ac; (●)[Ch]DHph; (●)[Ch]DHcit; (▲) [Ch]Cl.

Following the study of different anions in the cholinium-based salts, similar K_{TC} for the PEG600/[Ch]DHph and PEG600/[Ch]Ac systems were identified. For the system with [Ch]DHph the antibiotic partitioning for the polymer-rich phase can be a direct consequence of the strong salting-out ability of the salt. On other hand, in the [Ch]Ac-based ATPS, despite acetate anion having a lower ability to promote the salting-out of solutes when compared with the other salts, the alkaline pH of both phases seems to favorably induce the partitioning of TC for the PEG-rich phase. This trend is in accordance with the data obtained with the [Ch]Bic-based system where the highest partition coefficients were observed.

Since it was observed that the pH is a relevant feature in the TC partitioning, it was also studied the effect of the pH change within a system. As observed with the systems constituted by PEG600 and [Ch]Ac or [Ch]Bic the presence of TC in its anionic form leads to higher K_{TC} values. Thus, the effect of increasing the pH value was further studied in the PEG600/[Ch]Cl system. Here a change of the pH with addition of 0.5 wt% of NaOH from 7 to 12 was achieved. This change leads to an increase in the partition coefficient of tetracycline from 0.98 ± 0.03 to 3.69 ± 0.32 attesting the importance of the pH on the partition of TC on the studied systems.

In summary, the optimization tests using the commercial TC indicate that improved partition coefficients are obtained with ATPS composed of [Ch]Bic. Therefore, this cholinium-based salt was chosen, along with the [Ch]Cl salt, to conduct the extractions from the fermented broth described below.

4.2. Partitioning of TC in conventional PEG/Na_2SO_4 and [Ch]Cl/K_3PO_4 ATPS

ATPS composed of PEG600, [Ch]-based-salts and water are very recent [23]. Most of the studies previously published have been dealing with conventional ATPS. Hence, two polymer/salt and salt/salt ATPS were also studied in the TC extraction in order to

Table 3
Weight fraction composition of the initial mixture, TLL, pH value of each phase and partition coefficient of TC at 25 °C in the conventional systems.

Weight fraction composition (wt%)				TLL (wt%)	$pH_{topphase}$	$pH_{bottomphase}$	K_{TC}
K_3PO_4	[Ch]Cl	PEG600	Na_2SO_4				
–	–	40.04	7.48	59.22	6.18	5.30	16.61 ± 0.12
–	–	40.02	10.01	65.11	6.25	6.08	13.00 ± 1.08
35.02	20.04	–	–	71.97	14.16	13.66	30.05 ± 1.40
29.97	20.08	–	–	52.67	13.65	13.49	28.75 ± 6.51
27.51	20.10	–	–	35.46	13.51	13.39	22.82 ± 2.21

compare with the performance of the systems here studied. Two ATPS composed of PEG600 and Na_2SO_4, and K_3PO_4 and [Ch]Cl were also investigated. All assays were prepared in triplicate and the respective weight fraction compositions, K_{TC}, TLL, and the pH of the coexisting phases are presented in Table 3. For the PEG600/Na_2SO_4 aqueous system, K_{TC} is the ratio between the TC concentration in the polymer-rich phase to that in the salt-rich phase whereas for the [Ch]Cl/K_3PO_4 ATPS it is the TC concentration ratio between the [Ch]Cl- and K_3PO_4-rich phases.

From the pH values displayed in Table 3, it can be anticipated that TC is in different forms in the two conventional ATPS: in the PEG600/Na_2SO_4 system, TC is mainly in its neutral form ($5.30 < pH < 6.25$) whereas in the [Ch]Cl/K_3PO_4 system it is completely deprotonated (pH of the phases > pK_a3). In the polymer/salt system TC is recovered in the top phase (PEG-rich phase) with K_{TC} values higher than 13. In the [Ch]Cl–K_3PO_4 system the antibiotic preferentially migrates for the [Ch]Cl-rich phase (with $K_{TC} > 22$). Both examples suggest that, independently of the pH, the salt used or the salting-out species employed have a major influence on the partitioning of tetracycline. In the PEG-salt systems it is clear that Na_2SO_4, being a stronger salting-out agent than the cholinium-based salts, enhances the partitioning of TC for the polymer-rich phase. On the other hand, when an inorganic salt is used in combination with [Ch]Cl, TC migrates for the [Ch]Cl-rich phase – a consequence of the strong K_3PO_4 salting-out aptitude [33,34]. In summary, the choice of the main constituents of the ATPS has a crucial impact in the preferential partitioning of TC. Indeed, according to these results, TC can be recovered in the polymer- or in the cholinium-rich phase depending on the second species added to the medium. Considering that in the PEG/[Ch]Cl ATPS at an alkaline pH the TC partitions for the polymeric phase and that in the [Ch]Cl/K_3PO_4 systems TC migrates for the [Ch]Cl-rich phase, these two systems can be used sequentially to carry out the back extraction of the antibiotic. Some experiments regarding back extraction procedures were conducted and the results are presented in Supporting Information, Fig. A6.

The behavior observed with the PEG/Na_2SO_4system is in close agreement with literature data for other biomolecules such as L-trypthophan, which tends to partition for the polymer-rich phase in the presence of inorganic salts [26]. Recently, Shahriari et al. [22] have also applied more hydrophobic cholinium-based salts and K_3PO_4 ATPS to separate three antibiotics forms, and among them, TC. The results provided by the authors [22] showed that the salting-out effect exerted by the phosphate anion is a dominant effect in the partitioning phenomenon.

In summary, although the polymer/cholinium-based ATPS here studied exhibit lower TC extraction capabilities as compared with PEG/$Na_2SO_4$and [Ch]Cl/K_3PO_4 systems, it should be remarked that both the cholinium-based salts and PEG are biocompatible and biodegradable highlighting and supporting their applicability in a fermentation broth to recover an antibiotic for human consumption.

Table 4
pH value of each phase, $\%EE_{TC}$ and partition coefficient of TC from the fermented broth at 25 °C.

System	K_{TC}	$\%EE_{TC}$	$pH_{topphase}$	$pH_{bottomphase}$
PEG600/[Ch]Cl	0.21 ± 0.03	81.63 ± 5.26	5.03	4.43
PEG600/[Ch]Bic	3.98 ± 0.41	82.54 ± 11.03	9.31	9.48
[PEG600]/Na_2SO_4	17.78 ± 1.25	54.67 ± 6.55	4.86	4.66
[Ch]Cl/K_3PO_4	45.95 ± 1.66	92.42 ± 2.84	13.92	13.71

4.3. Recovery of TC from the fermented broth

Based on the previous results, the PEG600/[Ch]Cl and PEG600/[Ch]Bic ATPS were selected for the studies on the extraction of TC from the fermented broth of *S. aureofaciens*. The conventional PEG/Na_2SO_4 and [Ch]Cl/K_3PO_4 systems were also considered for comparison purposes. All these systems were studied as a pre-purification step capable of concentrating the antibiotic in one aqueous-rich phase while the contaminants would be retained in the other phase. The corresponding systems (at the same concentrations presented in Tables 2 and 3 with the model systems) were prepared in triplicate, and the values of K_{TC} and respective standard deviations, extraction efficiencies ($\%EE_{TC}$), and pH values of the coexisting phases are presented in Table 4.

The results obtained are compared in Fig. 2 where the logarithmic function of K_{TC} and the $\%EE_{TC}$ are presented.

The comparison of the K_{TC} values obtained from the fermented broth (Fig. 2 and Table 4) and with the model systems (Table 3) shows that the ATPS formed by PEG and cholinium-based salts can be applied in real extraction routes. As expected some deviations were observed when compared with the results obtained from the model systems. For the [Ch]Bic-based ATPS it was observed a decrease in the K_{TC} values when applying the system directly to the fermented broth (from 10.07 with the commercial TC to 3.98 in the real system). This behavior can be explained by the complexity of

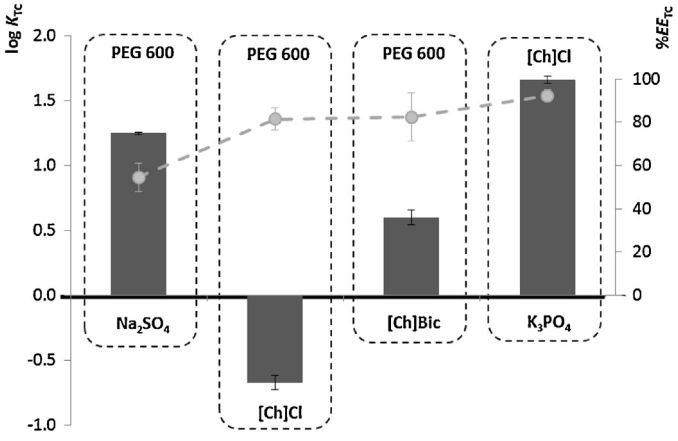


Fig. 2. Logarithm of K_{TC} (■) and $\%EE_{TC}$ (- ● -) obtained in several ATPS formed with the fermented broth.

the fermented broth that either interferes with the partitioning of TC or changes the pH of the medium. In the real system with [Ch]Bic it was observed a decrease in the pH (from 11.22 to 9.48) which, as previously discussed, changes the TC speciation. On other hand, an interesting result was observed with the PEG/[Ch]Cl system, where the TC was majorly concentrated in cholinium-rich phase ($\log K_{TC} < 0$) – an inverse trend to that observed with the model systems. This inversion of the TC partitioning can be a result of both the system acidification or due to the presence of residuals salts from the fermented medium.

With the conventional systems, namely with the PEG600 + Na_2SO_4 ATPS, it was also observed a lower extraction efficiency than with model systems. In fact, the extraction efficiency is even lower than that observed for the PEG-cholinium-based ATPS. However, with the [Ch]Cl/K_3PO_4 system almost all TC migrates for the cholinium rich-phase with extraction efficiencies higher than 92%. With this system it is verified an intensification of the partitioning behavior as observed in other works using IL/salt ATPS with complex matrices [35].

Although the [Ch]Cl/K_3PO_4 ATPS presents the highest ability to extract TC it should be remarked that other advantages are inherent to ATPS composed of cholinium-based salts and PEG. First of all, the ATPS composed of PEG and cholinium-based salts are biocompatible and biodegradable and avoid the use of inorganic salts of high ionic strength. Moreover, depending on the salt employed the extraction of TC can be tailored either for the PEG- or salt-rich phase and with single step extraction efficiencies higher than 80%. The combination of several PEG-cholinium-based systems can be used as back-extraction approaches for the recovery of TC. The extraction of TC to the polymer-rich phase can be envisaged as an economically viable pre-purification step to remove most part of the fermented broth contaminants, such as proteins, cell-debris and other amphiphilic impurities. This polymeric phase can be further applied in chromatography using fluidized beds processes. Albeit a search on literature data was carried out for comparison purposes, no results concerning the extraction of TC obtained from the *S. aureofaciens* fermented broth were found. Thus, this work represents a novel contribution regarding the definition of alternative strategies to perform the pre-concentration and pre-purification of TC directly from the fermentation broth.

5. Conclusions

The extraction of tetracycline using cheaper and biocompatible ATPS as alternative liquid–liquid extraction platforms was evaluated. Both model systems using commercial tetracycline and systems employing the fermentation broth of *S. aureofaciens* were investigated. In general, the cholinium-based salt nature and the pH of the medium largely contribute to the differences observed with the TC partitioning. For the systems composed of PEG600, cholinium-based salts and the fermented broth, extraction efficiencies of the antibiotic higher than 80% were achieved. Moreover, depending on the cholinium-based salt, the recovery either occurs at the polymer- or at the salt-rich phase. These novel results support a tailoring ability of the cholinium-based ATPS which can be further combined and applied as back extraction routes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2013.02.025.

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