



Recovery of biomolecules in sustainable ATPSs (298.15 K) and solubility determination in pure water and ethyl lactate (283.15, 298.15 and 313.15 K)

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ABSTRACT

Biowaste is considered to hold an immense potential as a source of bioactive compounds, such as vitamins and antioxidants, which constitute important value-added chemicals for the food and pharmaceutical industries. Moreover, the valorisation of biowaste could help fight resource depletion and forward a more circular economy. Therefore, the study of green extraction techniques is crucial for the effective retrieval of these biomolecules, with Aqueous Two-Phase Systems (ATPSs) standing out due to their high scale-up potential and use of harmless materials.

In this work, sustainable ATPSs composed of water, ethyl lactate and the organic salts disodium succinate ($\text{Na}_2\text{Succinate}$), tripotassium citrate ($\text{K}_3\text{Citrate}$) and trisodium citrate ($\text{Na}_3\text{Citrate}$) were used in a lab scale extraction of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA), at 298.15 K and 0.1 MPa. To assess extraction performance, the partition coefficient (K) and the extraction efficiency (E) were calculated, with catechin presenting the most promising results: a maximum K of $(5 \pm 1) \cdot 10^1$ and maximum E of $(94.2 \pm 0.9)\%$, for the longest tie-line of the ATPS composed of $\text{Na}_3\text{Citrate}$. Conversely, the ATPSs based on $\text{Na}_2\text{Succinate}$ presented the worst results, only achieving a maximum K of 7.33 ± 0.04 and a maximum E of $(75.8 \pm 0.6)\%$, for the longest tie-line in the extraction of cyanocobalamin.

Considering the promising results for the extraction of cyanocobalamin observed in several ATPSs, as reported in previous works of the research group, an additional study was conducted on the limiting effect of the solubility of this biomolecule in water or ethyl lactate on its partitioning in ATPSs. The solubilities in water and ethyl lactate were found to be extremely higher (over 40 and 300 times, respectively) than the concentration of cyanocobalamin in the phases of the ATPS, indicating that solubility was not a limiting factor.

1. Introduction

Despite the growing efforts on waste recycling, a significant fraction of the produced waste is still mismanaged through open dumping or burning [1], leading to increased greenhouse gas emissions [2] and waste of valuable materials [3]. This issue further highlights the importance of circular economy as a means of recycling useful substances and reducing the environmental footprint [4], contributing to a more sustainable future.

In what concerns biowaste, the extraction of vitamins and antioxidants has been increasingly studied for their potential use in the

production of food supplements [5,6] and medicinal drugs [7,8]. In this work, the extraction of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) was conducted, with their main sources and health benefits being presented in Fig. 1.

Cyanocobalamin or vitamin B12 is a biomolecule associated with numerous essential reactions in mammals [9]. This vitamin, which is mainly found in fish, meat, eggs and dairy products [10], has a key role in the development and function of the central nervous system, red blood cell formation and deoxyribonucleic acid (DNA) synthesis [11]. Therefore, this cobalt-containing compound is essential in the human health, aiding in the prevention of anaemia, neurologic disorders and

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cardiovascular diseases, especially in pregnant women and the elderly [9].

Catechin (Cat) is a polyphenolic compound with the basic structure of flavan-3-ol [12], which is commonly found in green tea, beans and cocoa berries [13]. Its antioxidant and anti-inflammatory properties are highly beneficial, promoting cardiovascular protection and possible cancer prevention [14].

Nicotinic acid (NiA), a pyridine carboxylic acid, can be obtained from meat, fish, nuts, legumes and grains [15]. This biomolecule has an important role in the treatment and prevention of cardiovascular diseases. Particularly, dyslipidaemia (*i.e.*, unbalance of lipids in the bloodstream) and atherosclerosis (*i.e.*, thickening or hardening of the arteries) are two of the main illnesses in which NiA is primarily applied [16].

To effectively extract and valorise these bioactive compounds from biowaste, it is imperative to study new green extraction techniques. Aqueous Two-Phase Systems (ATPSs) have emerged as a simple, highly biocompatible and easy to scale-up liquid–liquid extraction technique [17–20]. Furthermore, ATPSs are considered cost-effective, not requiring complex technology or high energy consumption [21–23]. This method involves mixing water, a salting out agent and an additional component to form two distinct phases [24]. The biomolecule partitions between the two phases, typically favouring one phase over the other. Variables such as hydrophobicity, polarity, biospecific affinity, electronic charge, molecular size and molecular conformation may have a significant influence in the partitioning of the biomolecule [25,26], with the balance of interactions determining how the migration of the target species occurs.

Over the years, ATPSs composed of two polymers, a polymer and a salt or an ionic liquid and other salt have been mainly used for the extraction of biomolecules [27–29]. However, the use of organic solvents has been investigated as an effective option, with particular focus on ethyl lactate (EL). Derived from the fermentation of corn, this organic solvent is very well-known for its biodegradability and non-toxic nature, standing out as a novel green alternative [30,31]. Moreover, organic salts, which have the same eco-friendly traits as EL, have also been increasingly used in place of inorganic salts [32]. The implementation of these sustainable compounds highlights the value of ATPSs.

Given the expanding application of ATPSs for biomolecule extraction, there is a rising need to optimize this technique. For example, the study of solubility as a potential limitation to the biomolecule partitioning is a path that has not been deeply explored in literature and that could promote a more extensive discussion and better understanding of the partition results.

In this work, the extraction of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) was conducted in ATPSs composed of water, ethyl lactate (EL) and organic salts, namely disodium succinate

(Na₂Succinate), tripotassium citrate (K₃Citrate) and trisodium citrate (Na₃Citrate). Although the mentioned ATPSs and biomolecules have been studied in previous works, these combinations of biomolecules and ATPSs have not yet been addressed, so it is important to determine the effectiveness of the individual ATPSs in the extraction of each biomolecule. This allows process optimization and recovery maximization. Furthermore, the solubility of B12 in pure water and EL was studied at 283.15, 298.15 or 313.15 K.

2. Experimental

2.1. Materials

Table 1 summarises the chemicals used in this work, as well as their respective chemical formulas, supplying companies, purities, Chemical Abstracts Service (CAS) numbers and abbreviations. These compounds

Table 1

List of chemicals, with respective chemical formula, supplier, purity, CAS number and abbreviation.

Chemical	Supplier	Purity / % ^a	CAS	Abbreviation
Acetic acid (C ₂ H ₄ O ₂)	Merck	> 99.8	64-19-7	AA
Catechin monohydrate (C ₁₅ H ₁₄ O ₆ ·H ₂ O)	Sigma	> 98.0	225937-10-0	Cat
Cyanocobalamin (C ₆₃ H ₈₈ CoN ₁₄ O ₁₄ P)	Thermo Scientific	> 98.0	68-19-9	B12
Disodium succinate hexahydrate (C ₄ H ₄ Na ₂ O ₄ ·6H ₂ O)	Tokyo Chemical Industry	> 95.0	6106-21-4	Na ₂ Succinate
Ethanol (C ₂ H ₆ O)	Sigma-Aldrich	> 99.0	64-17-5	EtOH
(–)-ethyl L-lactate (C ₅ H ₁₀ O ₃)	Sigma-Aldrich	> 98.0	97-64-3	EL
Nicotinic acid (C ₆ H ₅ NO ₂)	Sigma-Aldrich	> 99.0	59-67-6	NicoA
Purified water (H ₂ O)	VWR chemicals	^b	7732-18-5	W
Sodium hydroxide (NaOH)	Merck	> 99.0	1310-73-2	NaOH
Tripotassium citrate monohydrate (C ₆ H ₅ K ₃ O ₇ ·H ₂ O)	Sigma-Aldrich	> 99.0	6100-05-6	K ₃ Citrate
Trisodium citrate dihydrate (C ₆ H ₅ Na ₃ O ₇ ·2H ₂ O)	VWR chemicals	> 99.0	6132-04-3	Na ₃ Citrate

^a Provided by the supplier in mass percentage.

^b The electrical conductivity (κ) of pure water was found to be 5·10^{−6} S·m^{−1}.



Fig. 1. Natural sources and potential health benefits of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA).

were used as provided by the suppliers, with no additional purification steps being performed. Additionally, the chemical structures of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) are shown in Fig. S1, in the Supplementary Materials.

2.2. Equipment

Mass (m) was quantified using an Adam Equipment AAA 250L balance, with a standard measurement uncertainty of 10^{-4} g, while pH was measured with a VWR pH 1100L device, with standard measurement uncertainties of 0.01 in pH and 0.1 K in temperature (T). To confirm the water content of the used biomolecules and organic salts, a Mettler Toledo C20 Coulometric Karl Fisher titrator, with standard measurement uncertainty of 5 ppm, was employed, with hydrate water being accounted for during solution preparation. The electrical conductivity (κ) of pure water was measured with a Hanna EDGE EC conductivity meter, coupled with a platinum conductivity cell HI763100, with a relative measurement uncertainty of 0.01. Furthermore, a ThermoScientific Varioskan Flash ultraviolet–visible (UV–Vis) spectrophotometer was used to measure the UV–Vis absorbance, having a standard measurement uncertainty of 10^{-4} . Liquid density (ρ) was quantified using an Anton Paar DSA-4500 M oscillating densimeter, with standard measurement uncertainties of $3 \cdot 10^{-5}$ g cm $^{-3}$ and 0.01 K. To monitor the temperature of the assays, an OVAN Therm H TH100E thermostatic bath with standard measurement uncertainty of 0.1 K was used, with temperature being checked with a glass thermometer with a standard measurement uncertainty of 0.01 K. Both the densimeter and the pH meter were calibrated according to their manuals before conducting any experiments.

2.3. Influence of pH on the biomolecules

Variations in pH can trigger protonation reactions (release of H^+) in the studied biomolecules, affecting the mean electrical charge (q) of these compounds [33,34]. This, in turn, may alter the hydrophobicity of the target species, affecting their partitioning, while also causing variations in the UV–Vis absorbance spectra. Thus, to prevent inaccurate results, it is crucial to study the influence of pH on the spectra and assess its stability throughout the experimental procedure.

To assess the effect of pH on the mean electrical charge of the studied biomolecules, the relative fraction (x_A) of ionised species, also known as stages [35], must be calculated. Based on the definition of acid dissociation constant (K_a), Eq. (1) can be used to relate the negative base-10 logarithm of the acid dissociation constants (pK_a) of each biomolecule with the concentration ratios of different stages, at a given pH. This leads to the expression for x_A , as shown in Eq. (2) [35].

$$\frac{[A^{q_0-i+1}]}{[A^{q_0-i}]} = 10^{pH-pK_a^i} \quad (1)$$

$$x_{A^{q_0-i+1}} = \frac{[A^{q_0-i+1}]}{[A^{q_0-1}]} / \left(\frac{[A^{q_0}]}{[A^{q_0-1}]} + 1 + \sum_{j=2}^{i_{\max}} \left[\prod_{k=2}^j \frac{[A^{q_0-k}]}{[A^{q_0-(k-1)}]} \right] \right) \quad (2)$$

where q_0 is the electrical charge of the biomolecule A at pH = 0, i is the number of donated protons, $[A^{q_0-i+1}]$ and $[A^{q_0-i}]$ are the mole concentrations of the stages with electrical charges equal to $(q_0 - i + 1)$ e and $(q_0 - i)$ e, respectively, with e referring to the elementary charge ($1.602 \cdot 10^{-19}$ C), $x_{A^{q_0-i+1}}$ is the fraction of species with electrical charge equal to $(q_0 - i + 1)$ e, and $i_{\max}$ is the maximum number of protons that can be donated.

With the relative fraction of ionised species, the mean electrical charge (q) can be determined through a weighted arithmetic mean, as shown in Eq. (3).

$$q = \sum_{i=1}^{i_{\max}} [x_{A^{q_0-i+1}} \cdot (q_0 - i + 1)] + \left[1 - \sum_{i=1}^{i_{\max}} (x_{A^{q_0-i+1}}) \right] \cdot (q_0 - i_{\max}) \quad (3)$$

Considering the formation of stages at distinct pH values, the effect of structural and intermolecular alterations on the UV–Vis absorbance spectra must be investigated. For this reason, a study was conducted by preparing biomolecule solutions at different pH values and comparing their spectra.

Stock solutions of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) were prepared with concentrations of $3.12 \cdot 10^{-2}$, $3.25 \cdot 10^{-2}$ and $2.50 \cdot 10^{-2}$ % in mass, respectively. Afterwards, the pH was adjusted using 0.5 M aqueous solutions of acetic acid (CH_3COOH) and sodium hydroxide (NaOH), by gradually adding droplets of the appropriate pH adjuster. The pH values were chosen according to the pK_a values for each biomolecule, so as to characterize the UV–Vis absorbance spectra for each stage. The spectra measurements for all samples were conducted in the 200–600 nm range at 298.15 K and 0.1 MPa. Furthermore, considering the dilutions due to the addition of the pH adjusters, a normalisation of the spectra was performed. Thus, Eq. (4) was used, with this approximation being applicable for minimal differences in concentration. Finally, the spectra were rechecked after 3 days, to ensure they were stable for the duration of the experiment.

$$A' = A \cdot \frac{C_{pH \approx 7}}{C_{pH=k}} \quad (4)$$

where A and A' are the measured and normalised UV–Vis absorbances, respectively, $C_{pH \approx 7}$ is the reference concentration (chosen according to the characteristic pH of the studied ATPSs, which is approximately 7) and $C_{pH=k}$ is the concentration of the biomolecule solution at pH = k .

2.4. Calibration curve determination

After identifying the characteristic values of wavelength for each biomolecule (B12: 363 nm, Cat: 279 nm and NiA: 264 nm), calibration curves were determined to relate UV–Vis absorbance with biomolecule concentration. Stock solutions of the biomolecules were prepared with concentrations close to their solubility limit or the upper threshold of the useful measurement range of the UV–Vis spectrophotometer, maximizing the applicability of the calibration curve. Additionally, the absorbances of water and the plate were subtracted to obtain solely the absorbance of the biomolecules. Triplicate absorbance measurements at pH $\approx$ 7, P = 0.1 MPa and T = 298.15 K were conducted for solutions of known concentration and adjusted to a first-order expression, as demonstrated by Eq. (5).

$$A = \alpha C + \beta \quad (5)$$

where A is absorbance, α is the slope of the calibration curve (absorptivity), C is the biomolecule concentration (in g·mL $^{-1}$) and β is the y-intercept.

2.5. Liquid-liquid equilibria data

To conduct the partition experiments, the liquid–liquid equilibrium (LLE) data (tie-line composition) of the studied systems were needed. Therefore, the LLE data of ATPSs with disodium succinate ($Na_2Succinate$), tripotassium citrate ($K_3Citrate$) and trisodium citrate ($Na_3Citrate$) were taken from previous works [36,37], as displayed in Table 2. Additionally, the respective phase equilibria diagrams can be found in Fig. S2, in the Supplementary Materials.

2.6. Biomolecule partitioning

To determine how the target species partition within each ATPS, two

Table 2Tie-line compositions for the ATPSs {Ethyl lactate (EL) (1) + Na₂Succinate [36] or K₃Citrate [37] or Na₃Citrate [37] (2) + Water (3)}, at 298.15 K and 0.1 MPa ^{a,b}.

No. TL	Feed		Top phase			Bottom phase			TLL / %
	$w_{EL,feed}$	$w_{salt,feed}$	$w_{EL,top}$	$w_{salt,top}$	pH	$w_{EL,bot}$	$w_{salt,bot}$	pH	
{EL (1) + Na ₂ Succinate (2) + Water (3)} [36]									
TL1	0.280	0.126	0.602	0.030	7.25	0.189	0.153	7.25	43.10
TL2	0.300	0.130	0.687	0.019	7.45	0.145	0.174	7.37	56.40
TL3	0.320	0.135	0.742	0.014	7.37	0.106	0.196	7.34	66.20
{EL (1) + K ₃ Citrate (2) + Water (3)} [37]									
TL1	0.355	0.126	0.579	0.039	7.21	0.150	0.203	7.39	45.91
TL2	0.375	0.130	0.619	0.034	7.22	0.115	0.232	7.41	54.02
TL3	0.392	0.135	0.674	0.021	7.19	0.091	0.258	7.37	62.96
TL4	0.411	0.139	0.704	0.016	7.23	0.075	0.282	7.41	68.28
TL5	0.430	0.143	0.736	0.011	7.12	0.060	0.310	7.39	73.92
TL6	0.446	0.148	0.758	0.009	7.22	0.052	0.331	7.43	77.66
{EL (1) + Na ₃ Citrate (2) + Water (3)} [37]									
TL1	0.300	0.110	0.517	0.030	7.00	0.160	0.157	6.98	37.58
TL2	0.320	0.114	0.575	0.020	6.98	0.123	0.185	6.96	48.18
TL3	0.343	0.117	0.615	0.014	6.98	0.098	0.207	6.97	55.17
TL4	0.365	0.121	0.650	0.010	7.00	0.079	0.230	7.00	61.17
TL5	0.385	0.123	0.677	0.007	6.98	0.068	0.247	6.97	65.44
TL6	0.406	0.126	0.701	0.005	6.98	0.055	0.266	7.00	69.68

^a w_i stands for the mass fraction of species i .^b The reported standard measurement uncertainties (u) are: $u(T) = 0.01$ K, $u(P) = 2$ kPa, $u(w_i) = 10^{-3}$ and $u(pH) = 10^{-2}$.

sets of assays were carried out: the blanks and the partitions. The blanks consisted of ATPSs composed of water, ethyl lactate (EL) and organic salt, while the partitions substituted 1 mL of water by 1 mL of biomolecule stock solution. These mixtures were stirred for 6 h and left to settle overnight at 298.15 K and 0.1 MPa, with two phases being formed. The phases were then separated, and their mass, liquid density, pH and UV–Vis absorbance were measured. Afterwards, by subtracting the absorbance of the blanks from the partitions, the biomolecule absorbance could be obtained. It is worth noting that the biomolecule stock solutions were prepared at concentrations about four times higher than those corresponding to absorbances within the useful measurement range (B12: $12.6 \cdot 10^{-2}$ % in mass, Cat: $14.8 \cdot 10^{-2}$ % in mass, NiA: $20.8 \cdot 10^{-2}$ % in mass), ensuring proper quantification after dilution in the phases. Furthermore, as it is commonly done in the literature [17,38,39], the presence of biomolecule is assumed not to alter the ATPS phase composition, with the relative composition between top and bottom phases remaining unchanged.

To evaluate the biomolecule partitioning in each system, two performance indicators were analysed: the partition coefficient (K) and the extraction efficiency (E). The partition coefficient (K), given by Eq. (6), refers to the ratio between the biomolecule concentration in the top and bottom phases of the same tie-line, indicating how the solute distributed itself between the two phases.

$$K_i = \frac{C_{T,i}}{C_{B,i}} \quad (6)$$

where i is the tie-line number, C is the biomolecule concentration (in $\text{g} \cdot \text{mL}^{-1}$), and T and B refer to the top and bottom phases, respectively. The concentrations can be obtained through the measured UV–Vis absorbances and the previously determined calibration curves.

Regarding the extraction efficiency (E), it quantifies the percentage of biomolecule, in mass, that migrated to the top phase in each tie-line and was determined using Eq. (7).

$$E_i = \frac{C_{T,i}V_{T,i}}{m_{SF,i}} \cdot 100 \quad (7)$$

where m_{SF} is the feed mass of biomolecule and V is the phase volume, which was calculated through Eq. (8).

$$V_{f,i} = \frac{m_{f,i}}{\rho_{f,i}} \quad (8)$$

where f refers to the top (T) or bottom (B) phase, m is phase mass and ρ is the liquid density of the phase. The extraction efficiency is sensitive to the relative size of the phases, being affected by the chosen feed composition within the same tie-line. Therefore, this parameter should be analysed simultaneously with the partition coefficient to secure an adequate assessment of the biomolecule partitioning.

To validate the performance indicators, and in accordance with previous works of the research group [39,40], mass losses in phase separation (L_m) and quantification (L_s) were determined using Eqs. (9) and (10).

$$L_{m,i} = \frac{(m_{T,i} + m_{B,i}) - m_{F,i}}{m_{F,i}} \cdot 100 \quad (9)$$

where i is the tie-line number, m_F refers to the feed mass, and m_T and m_B refer to the mass of the top and bottom phases, respectively.

$$L_{s,i} = \frac{m_{SQ,i} - m_{SF,i}}{m_{SF,i}} \cdot 100 \quad (10)$$

where m_{SF} and m_{SQ} are the feed and quantified masses of biomolecule. In turn, the quantified mass was calculated using Eq. (11).

$$m_{SQ,i} = C_{T,i}V_{T,i} + C_{B,i}V_{B,i} \quad (11)$$

where C is the biomolecule concentration (in $\text{g} \cdot \text{mL}^{-1}$), V is the phase volume and T and B refer to the top and bottom phases, respectively.

2.7. Solubility studies

To further validate the obtained results and address solubility as a limitation to the biomolecule partitioning, the solubility of cyanocobalamin (B12) was determined in pure ethyl lactate and in pure water. This experiment was conducted using jacketed glass cells with magnetic stirrers, which were connected to a peristaltic pump and to a water bath at the established temperature, with the solubility being determined at 283.15, 298.15 or 313.15 K and 0.1 MPa. The temperature of 298.15 K was chosen as it represents the approximate ambient temperature and aligns with the conditions used in ATPS extractions. Additionally, 313.15 K was selected because it is close to the average human body temperature, relevant for applications of the studied biomolecules as food supplements. Finally, a lower temperature of 283.15 K was chosen to broaden the analysis of temperature effects on solubility and ensure

consistent temperature intervals.

Saturated solutions of B12 in each solvent were prepared by adding solvent and excess solute, leading to the formation of precipitate. The solutions were stirred for about 24 h and then were left to settle for about 48 h to allow the existent solid to deposit at the bottom of the cells. The experimental setup is illustrated in Fig. 2.

Afterwards, the saturated liquid solutions were extracted using a syringe with a VWR filter (polypropylene membrane with 0.45 μm pores). Due to the high concentration of the solutions, a dilution step was necessary to enable the quantification of B12 through the UV–Vis spectrophotometer. Solubility was then calculated using the previously determined calibration curve for water and by employing a new calibration curve for ethyl lactate (EL). Three consistent measurements were carried out for each temperature and solvent. The assays were done independently for each temperature.

3. Results and discussion

3.1. Influence of pH on the biomolecules

Using the values of acid dissociation constant (pK_a) taken from literature (B12: 3.28 [41]; Cat: 8.64, 9.41, 11.26 and 13.26 [42]; NiA: 4.95 [43]), and following Eqs. (1) to (3), the relation between the mean electrical charge and pH could be described and plotted, as seen in Fig. 3.

The studied ATPSs have a characteristic pH of approximately 7, at which all the biomolecules exhibited mean electrical charge values close to integer (B12: $q = -1.00$ e; Cat: $q = -0.01$ e; NiA: $q = -0.99$ e). This is advantageous for the experiment, as the lack of differently charged species provides more stability and allows for more accurate results. The complete information for the variation of the electrical charge with pH can be found in Tables S1 to S3, in the [Supplementary Materials](#).

Considering the pK_a values and the mean electrical charge plot, specific pH values were selected to analyse the characteristic spectra of each differently charged species. However, for Cat, given the proximity of its pK_a values, it was impossible to precisely define all the different stages. Regardless, pH values that characterised the stages as close as possible were chosen. In Fig. 4, the UV–Vis absorbance spectra of B12, Cat and NiA at different values of pH can be observed. Moreover, Eq. (4) was applied to normalize the spectra and account for dilutions due to pH adjustment.

For B12, pH was found to have a negligible effect on the UV–Vis absorbance spectra, with the intensity and wavelength of the characteristic peak (at 363 nm) being the same for all pH. On the other hand, the same cannot be said for Cat or NiA. Significant changes occurred in the spectra of Cat for higher values of pH, with a shift in wavelength and intensity of the characteristic peak (at 279 nm) being observed. For NiA, only a change in the intensity of the characteristic peak (at 264 nm) was

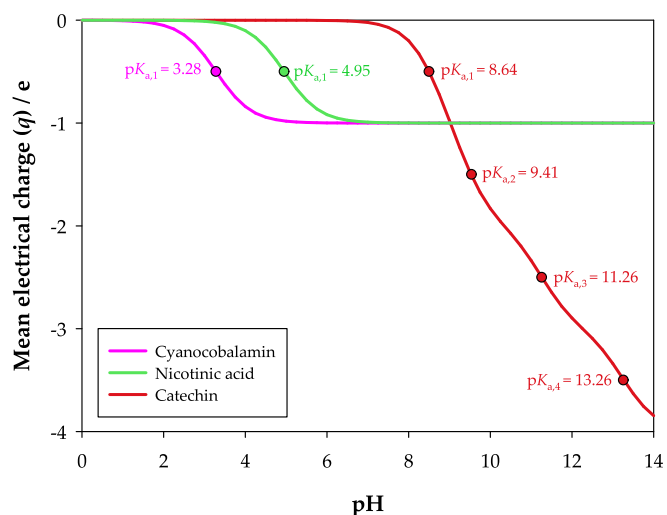


Fig. 3. Calculated mean electrical charges (q) with pH for cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA).

observed. With B12 being significantly larger and more complex than the remaining two biomolecules, protonation reactions may have minimal effect on the UV–Vis absorbance spectra. The conformation and interactions, both with water and between the biomolecules themselves, are unlikely to undergo substantial changes, resulting in close to no modifications in the spectra. The same may not be true for Cat and NiA, with alterations being more pronounced and resulting in a slightly altered spectra.

To assess the stability of the UV–Vis absorbance spectra for the duration of the procedure (~ 24 h), the absorbance measurements were repeated after 3 days. In Figs. S3 to S5, the spectra comparison can be observed, with B12 and NiA remaining stable. Cat, on the contrary, only showed stability at acidic or neutral pH values, being unstable for $\text{pH} = 10.4$ and $\text{pH} = 12$. Nevertheless, since the partition assays were conducted at approximately neutral values of pH, the spectra were stable in the context of this work, with no signs of degradation being shown.

3.2. Calibration curve determination

To relate UV–Vis absorbance with the concentration of biomolecules in solution and enable their quantification after the phase separation, calibration curves were determined at the wavelengths of 363 nm, 279 nm and 264 nm for B12, Cat and NiA, respectively. In Fig. 5 the calibration curves for each biomolecule are represented.

3.3. Partition of biomolecules

In this work, the extraction of B12 and NiA in the ATPS based on disodium succinate ($\text{Na}_2\text{Succinate}$) was performed and compared with the previously investigated tripotassium and trisodium citrate-based ATPSs [39–41]. Moreover, as a novel study in the research group, the extraction of Cat was studied in tripotassium and trisodium citrates ATPSs, while also being compared with the extraction of both B12 and NiA in these systems.

Having conducted the extraction of the biomolecules and separated the phases, the mass (m), liquid density (ρ) and pH of each phase were measured, and, following Eq. (9), the mass losses in phase separation (L_m) were calculated, with the results being exhibited in Table 3.

As expected, liquid density was always lower in the top phase than in the bottom phase for each tie-line. Moreover, the mass losses in phase separation were found to be minimal, being smaller than 1 %. Regarding pH, nearly neutral values were registered for all assays, as anticipated for the studied ATPSs. Having confirmed the validity of the measured properties, the biomolecule content in each phase was determined

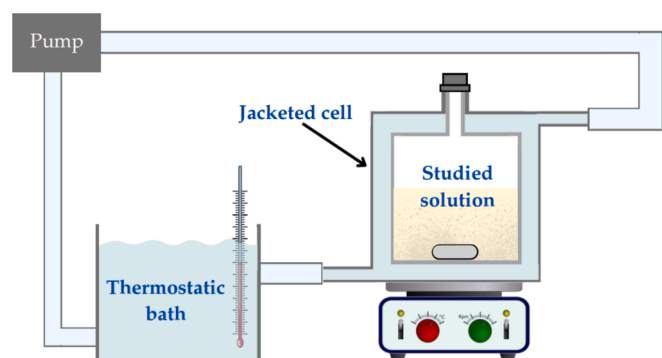


Fig. 2. Representation of the experimental setup for the biomolecule solubility studies.

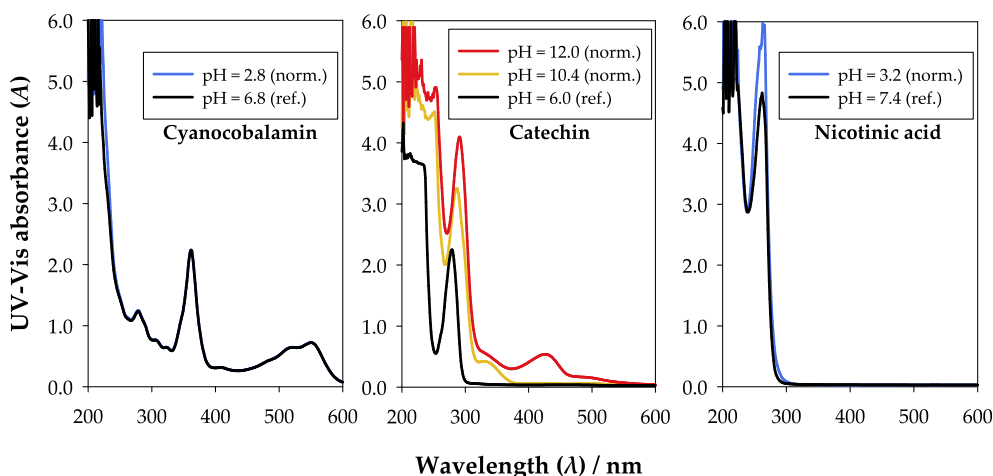


Fig. 4. Effect of pH on the UV–Vis absorbance spectra of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA).

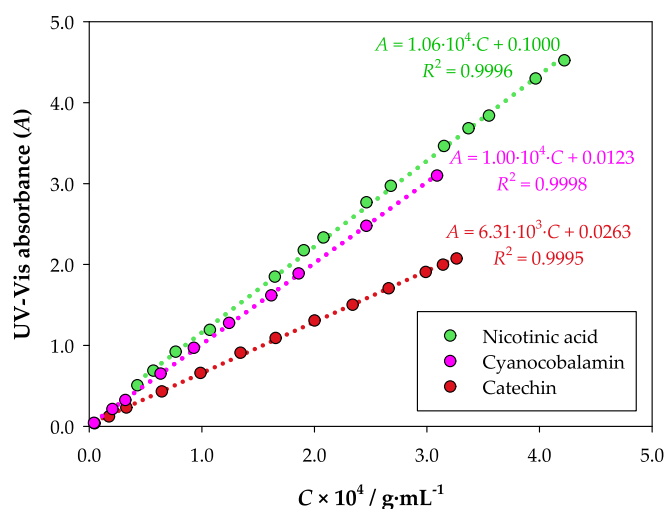


Fig. 5. UV–Vis absorbance calibration curves for cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) in water, at 298.15 K and 0.1 MPa [40].

through the respective calibration curves. Afterwards, the mass losses in quantification (L_s) were measured, with values lower than 5 % being observed for all tie-lines, validating the employed analytical method. At last, the two performance indicators – partition coefficient, K , and extraction efficiency, E – could be calculated for each tie-line using the equations shown in section 2.6. The results for the mass fractions of biomolecule ($w_{\text{biomolecule}}$), mass losses in quantification (L_s) and the two performance indicators are shown in Table 4.

The relation between the natural logarithm of the partition coefficients (K) and tie-line length is represented graphically in Fig. 6, with a close to linear relationship being observed. Overall, among all biomolecules, Cat presented the most promising results, particularly for the ATPS containing $\text{Na}_3\text{Citrate}$, in which K reached a maximum value of $(5 \pm 1) \cdot 10^1$ for the longest tie-line. In contrast, when analysing the various ATPSs, $\text{Na}_2\text{Succinate}$ -based systems presented much lower partition coefficients, with the highest value of K being 7.33 ± 0.04 . Nevertheless, all studied partitions presented values of K higher than unity, demonstrating a stronger affinity of the biomolecules to the top phase (EL-rich phase) rather than the bottom phase, favouring the use of the studied ATPSs.

A comparison of the obtained data with previous works [39–41] was also carried out, starting with the extraction of Cat in $\text{K}_3\text{Citrate}$ and $\text{Na}_3\text{Citrate}$ ATPSs in relation to the remaining biomolecules. For these

Table 3

Experimental phase mass (m), mass loss percentage in phase separation (L_m), liquid-density (ρ) and pH for the extraction of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) in the ATPSs {EL (1) + $\text{Na}_2\text{Succinate}$ or $\text{K}_3\text{Citrate}$ or $\text{Na}_3\text{Citrate}$ (2) + Water (3)}, at 298.15 K and 0.1 MPa.

Tie-line	Phase	$m/\text{ g}$	$L_m/\text{ \%}$	$\rho/\text{ kg}\cdot\text{ m}^{-3}$	pH
B12 in {EL (1) + Na ₂ Succinate (2) + Water (3)}					
1	Top	1.9532	−0.22	1053.51	7.56
	Bottom	8.0364		1119.23	8.05
2	Top	2.6123	−0.20	1049.62	8.56
	Bottom	7.3923		1130.71	8.26
3	Top	3.0661	−0.41	1051.69	8.64
	Bottom	6.9142		1126.80	8.14
NiA in {EL (1) + Na ₂ Succinate (2) + Water (3)}					
1	Top	2.1270	−0.40	1048.61	8.65
	Bottom	7.8500		1120.70	8.61
2	Top	3.1082	−0.41	1051.40	8.53
	Bottom	6.8500		1130.71	8.34
3	Top	3.2357	−0.72	1047.12	8.43
	Bottom	6.7000		1140.31	8.30
Cat in {EL (1) + K ₃ Citrate (2) + Water (3)}					
1	Top	4.4631	−0.31	1061.83	7.94
	Bottom	5.5470		1154.35	7.94
2	Top	4.6246	−0.18	1052.94	7.96
	Bottom	5.3401		1168.24	7.98
3	Top	4.8245	−0.27	1051.73	7.98
	Bottom	5.1303		1185.68	8.05
4	Top	4.9685	−0.30	1047.78	7.99
	Bottom	4.9989		1204.09	8.11
5	Top	5.1760	−0.17	1046.45	8.01
	Bottom	4.7950		1220.97	8.04
6	Top	5.2725	−0.34	1045.49	8.09
	Bottom	4.6998		1061.83	8.10
Cat in {EL (1) + Na ₃ Citrate (2) + Water (3)}					
1	Top	3.4081	−0.55	1052.08	7.32
	Bottom	6.5402		1124.75	7.35
2	Top	4.1342	−0.26	1049.76	7.41
	Bottom	5.8499		1143.61	7.75
3	Top	4.4956	−0.34	1047.56	7.67
	Bottom	5.4501		1158.01	7.68
4	Top	4.6236	−0.21	1044.31	7.71
	Bottom	5.3201		1171.46	7.90
5	Top	4.9297	−0.35	1041.95	7.71
	Bottom	5.0299		1187.96	7.76
6	Top	4.9592	−0.20	1042.01	7.42
	Bottom	4.6998		1201.47	7.34

^a The standard measurement uncertainties (u) are: $u(m) = 10^{-4}$ g, $u(\rho) = 0.03$ kg·m⁻³, $u(\text{pH}) = 10^{-2}$, $u(T) = 0.01$ K and $u(P) = 2$ kPa.

Table 4

Tie-line length (TLL), mass fraction of biomolecule ($w_{\text{biomolecule}}$), mass loss percentage in quantification (L_s), partition coefficient (K) and extraction efficiency (E) for cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) in the ATPSs {EL (1) + Na₂Succinate or K₃Citrate or Na₃Citrate (2) + Water (3)}, at 298.15 K and 0.1 MPa.

Tie-line	TLL / %	Phase	$w_{\text{biomolecule}}$	L_S / %	K	E / %
B12 in {EL (1) + Na₂Succinate (2) + Water (3)}						
1	43.10	Top	$3.33 \cdot 10^{-4}$	−1.08	$4.18 \pm$	51.4 ± 0.3
		Bottom	$7.48 \cdot 10^{-5}$		0.02	
2	52.13	Top	$3.27 \cdot 10^{-4}$	−1.04	$5.69 \pm$	67.7 ± 0.5
		Bottom	$5.33 \cdot 10^{-5}$		0.03	
3	60.15	Top	$3.12 \cdot 10^{-4}$	−2.48	$7.33 \pm$	75.8 ± 0.6
		Bottom	$3.97 \cdot 10^{-5}$		0.04	
NiA in {EL (1) + Na₂Succinate (2) + Water (3)}						
1	43.10	Top	$2.35 \cdot 10^{-4}$	−2.83	$1.08 \pm$	$23.09 \pm$
		Bottom	$2.04 \cdot 10^{-4}$		0.02	0.05
2	52.13	Top	$2.42 \cdot 10^{-4}$	−2.89	$1.16 \pm$	35.2 ± 0.1
		Bottom	$1.94 \cdot 10^{-4}$		0.03	
3	60.15	Top	$2.48 \cdot 10^{-4}$	−4.28	$1.23 \pm$	37.6 ± 0.1
		Bottom	$1.85 \cdot 10^{-4}$		0.03	
Cat in {EL (1) + K₃Citrate (2) + Water (3)}						
1	45.91	Top	$2.60 \cdot 10^{-4}$	−4.41	5.0 ± 0.3	77.8 ± 0.6
		Bottom	$4.78 \cdot 10^{-5}$			
2	54.02	Top	$2.73 \cdot 10^{-4}$	−3.76	8.0 ± 0.6	85.1 ± 0.8
		Bottom	$3.10 \cdot 10^{-5}$			
3	62.96	Top	$2.62 \cdot 10^{-4}$	−4.36	7.7 ± 0.6	85.2 ± 0.8
		Bottom	$3.03 \cdot 10^{-5}$			
4	68.28	Top	$2.65 \cdot 10^{-4}$	−2.05	10 ± 1	90.0 ± 0.9
		Bottom	$2.32 \cdot 10^{-5}$			
5	73.92	Top	$2.64 \cdot 10^{-4}$	−3.69	15 ± 2	91.6 ± 0.9
		Bottom	$1.48 \cdot 10^{-5}$			
6	77.66	Top	$2.62 \cdot 10^{-4}$	−1.93	18 ± 3	94.1 ± 0.9
		Bottom	$1.23 \cdot 10^{-5}$			
Cat in {EL (1) + Na₃Citrate (2) + Water (3)}						
1	37.85	Top	$3.23 \cdot 10^{-4}$	−1.40	5.1 ± 0.2	72.9 ± 0.6
		Bottom	$5.93 \cdot 10^{-5}$			
2	48.18	Top	$3.03 \cdot 10^{-4}$	−4.05	9.8 ± 0.9	84.7 ± 0.8
		Bottom	$2.85 \cdot 10^{-5}$			
3	55.17	Top	$2.92 \cdot 10^{-4}$	−4.50	17 ± 3	89.9 ± 0.9
		Bottom	$1.52 \cdot 10^{-5}$			
4	61.17	Top	$2.88 \cdot 10^{-4}$	−4.83	23 ± 5	91.1 ± 0.9
		Bottom	$1.12 \cdot 10^{-5}$			
5	65.44	Top	$2.76 \cdot 10^{-4}$	−4.37	31 ± 7	93.0 ± 0.9
		Bottom	$7.74 \cdot 10^{-6}$			
6	69.68	Top	$2.90 \cdot 10^{-4}$	−4.02	$(5 \pm 1) \bullet$	94.2 ± 0.9
		Bottom	$5.33 \cdot 10^{-6}$		10^1	

^a The standard measurement uncertainties (u) are: $u(T) = 0.01$ K and $u(P) = 2$ kPa.

systems, the following order in partition coefficients for the longest tie-line could be observed: B12 (78.6 ± 0.6) > Cat (18 ± 3) > NiA (6.5 ± 0.4) in K₃Citrate ATPS; B12 (55.4 ± 0.4) > Cat (5 ± 1) $\cdot 10^1$ > NiA (3.6 ± 0.1) in Na₃Citrate ATPS. Therefore, Cat was seen to be effectively extracted using the organic salt-based ATPSs, yielding favourable partition coefficients. Furthermore, when comparing Na₂Succinate ATPSs with K₃Citrate and Na₃Citrate for the studied biomolecules, the partition coefficients were arranged in this sequence: K₃Citrate (78.6 ± 0.6) > Na₃Citrate (55.4 ± 0.4) > Na₂Succinate (7.33 ± 0.04) for B12; K₃Citrate (6.5 ± 0.4) > Na₃Citrate (3.6 ± 0.1) > Na₂Succinate (1.23 ± 0.03) for NiA. Hence, Na₂Succinate systems did not present such satisfactory results as other organic salt-based ATPSs.

In what concerns extraction efficiencies (E), the relation with the tie-line length is illustrated in Fig. 7, with similar conclusions being drawn from these results. Higher values were observed for the extraction of Cat, with the extraction efficiencies for the longest tie-lines being (94.1 ± 0.9) % and (94.2 ± 0.9) % for K₃Citrate and Na₃Citrate ATPSs, respectively. Moreover, among the studied ATPSs, systems containing Na₂Succinate presented extraction efficiencies lower than expected, with the longest tie-line values being (75.8 ± 0.6) % for B12 and ($37.6 \pm$

0.1)% for NiA.

Comparing with systems assessed in previous works [39–41], the extraction efficiency of B12, Cat and NiA in K₃Citrate and Na₃Citrate systems followed the same pattern as seen for the partition coefficients. Thus, in light of the obtained performance indicators, the application of ATPSs for the extraction of Cat was successful. Additionally, when comparing ATPSs based on K₃Citrate and Na₃Citrate with Na₂Succinate-containing systems, a similar trend was observed for the partition coefficients. Therefore, although ATPSs composed of Na₂Succinate can be used to effectively extract the biomolecules under study, the results for these systems indicate a poorer performance compared to the remaining organic salt based-ATPSs.

This work is crucial in determining the performance of various ATPSs in the extraction of B12, Cat and NiA. However, with this being a laboratory-scale simulation of a biowaste treatment process, it omits several other steps that would be required in an industrial setting. One of the key considerations of such a process would be the recovery of the biomolecules from the extraction solvents. Considering the effectiveness of distillation as a recovery technique and the thermosensitive nature of these compounds, vacuum distillation, as proposed in a previous work [39], is a possible path of recovery of the target species. Additionally, it allows for the reuse of the extraction solvents, ethyl lactate and water, in earlier steps of the process.

3.4. Solubility studies

To address solubility as a limitation to the biomolecule partitioning, and considering the favourable results that were observed in several previous studies with B12 [40,41,44], the solubility of this biomolecule was determined in pure water and ethyl lactate (EL). An UV–Vis calibration curve for B12 in EL was determined, with the calibration curves being represented in Fig. 8. These curves were nearly identical, indicating that the differences in solute–solvent interactions for B12 in water and EL did not influence the UV–Vis absorbance spectra.

The solubility of B12 was measured at 0.1 MPa and 3 different temperatures, namely 283.15, 298.15 and 313.15 K, with the results being displayed in Table 5. Additionally, the representation of the results was carried out following two different approaches: the dependence of the biomolecule mass fraction (w) on the temperature, as illustrated in Fig. 9(a), and the dependence of the natural logarithm of the molar concentration (x) on the inverse of temperature, as seen in Fig. 9 b). The second graphical representation aimed to determine whether the solubility followed the van't Hoff equation [45], which relates a component's solubility with temperature in an ideal solution, as shown in equation (12).

$$\ln x = -\frac{\Delta H}{R} \cdot \frac{1}{T} + \frac{\Delta S}{R} \quad (12)$$

where ΔH and ΔS are the enthalpy and entropy of dissolution, R is the ideal gas constant and T is absolute temperature.

In Fig. 8(a) the solubility of B12 in EL and water in terms of mass fraction can be observed. B12 was significantly more soluble in EL than in water, favouring the use of the studied ATPSs, as the extraction is carried out through an EL-rich phase. Moreover, considering the mass fractions obtained in each assay of the partition studies (as seen in Table 4) and comparing with the determined solubilities at 298.15 K, a substantial difference could be observed, with the solubility being over 40 times higher in water and over 300 times higher in EL. In light of the above, the solubility does not present a limitation to the partitioning of B12, validating the results for the extraction of this biomolecule.

By analysing Fig. 9(b), despite using only three points, the data was found to align well with the linear regression, with a significantly high determination coefficient ($R^2 > 0.995$) being obtained. This suggests that B12 solutions exhibit a close to ideal behaviour and the van't Hoff equation is suitable to describe the solubility of this biomolecule. With

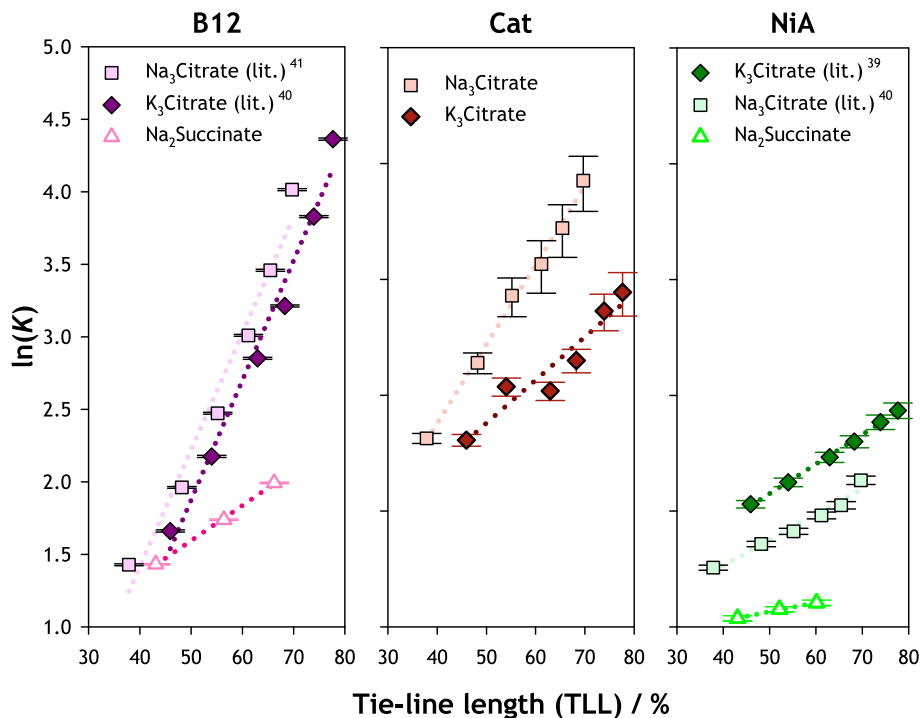


Fig. 6. Natural logarithm of the partition coefficients (K) with tie-line length (TLL) for the extraction of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) in the ATPSs {Ethyl lactate (1) + $\text{Na}_2\text{Succinate}$ or $\text{Na}_3\text{Citrate}$ or $\text{K}_3\text{Citrate}$ (2) + Water (3)}, at 298.15 K and 0.1 MPa. Data for B12 and NiA in ATPSs composed of $\text{K}_3\text{Citrate}$ and $\text{Na}_3\text{Citrate}$ were reported in previous works of the research group [39–41].

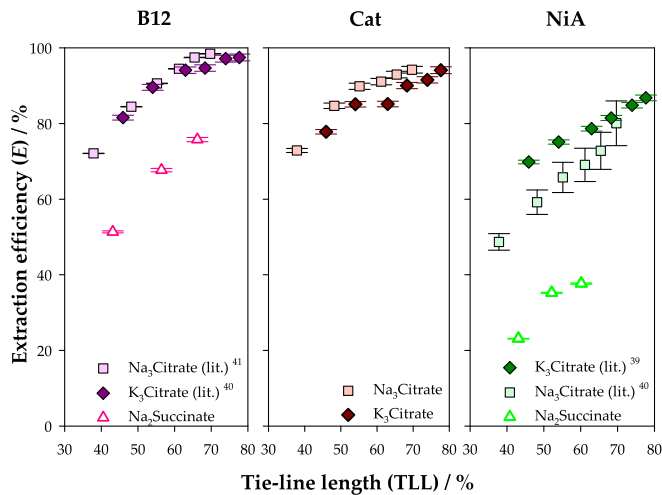


Fig. 7. Extraction efficiencies (E) with tie-line length (TLL) for the extraction of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) in the ATPSs {Ethyl lactate (1) + $\text{Na}_2\text{Succinate}$ or $\text{Na}_3\text{Citrate}$ or $\text{K}_3\text{Citrate}$ (2) + Water (3)}, at 298.15 K and 0.1 MPa. Data for B12 and NiA in ATPSs composed of $\text{K}_3\text{Citrate}$ and $\text{Na}_3\text{Citrate}$ were reported in previous works of the research group [39–41].

this, the enthalpy and entropy of dissolution could be obtained through the slope and y-intercept of the linear regression equations, respectively. Hence, the enthalpy of dissolution ($\left(\frac{\Delta H}{R}\right)$) of B12 would be $-1.78 \cdot 10^{-3}$ K for ethyl lactate and $-2.9 \cdot 10^{-3}$ K for water and the entropy of dissolution ($\left(\frac{\Delta S}{R}\right)$) would be 1.6 for EL and 1.4 for water, respectively.

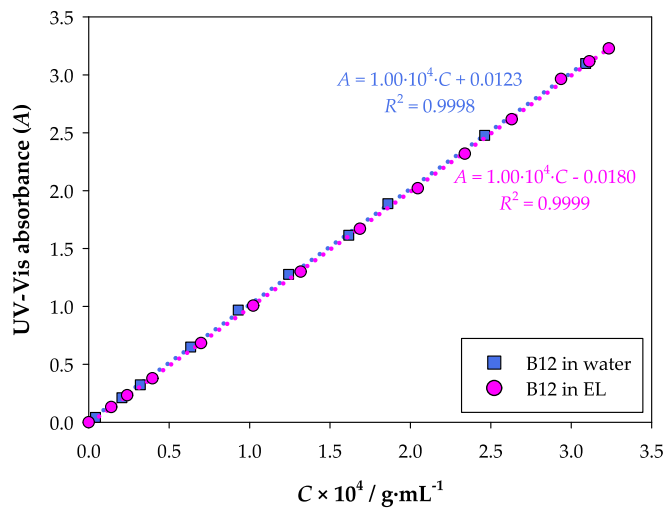


Fig. 8. UV-Vis absorbance calibration curves for B12 in water and ethyl lactate, at 298.15 K and 0.1 MPa.

Table 5

Solubility of cyanocobalamin (B12) in water or ethyl lactate at 283.15, 298.15 and 313.15 K and 0.1 MPa, in mass fraction and molar fraction.

Solubility in mass fraction (w)			
	$T = 283.15 \text{ K}$	$T = 298.15 \text{ K}$	$T = 313.15 \text{ K}$
Water	$(9.6 \pm 0.1) \times 10^{-3}$	$(1.52 \pm 0.01) \times 10^{-2}$	$(2.55 \pm 0.02) \times 10^{-2}$
EL	$(9 \pm 1) \times 10^{-2}$	$(1.26 \pm 0.08) \times 10^{-1}$	$(1.607 \pm 0.05) \times 10^{-1}$
Solubility in molar fraction (x)			
	$T = 283.15 \text{ K}$	$T = 298.15 \text{ K}$	$T = 313.15 \text{ K}$
Water	$(1.28 \pm 0.02) \times 10^{-4}$	$(2.06 \pm 0.01) \times 10^{-4}$	$(3.47 \pm 0.03) \times 10^{-4}$
EL	$(9 \pm 1) \times 10^{-3}$	$(1.25 \pm 0.08) \times 10^{-2}$	$(1.64 \pm 0.05) \times 10^{-2}$

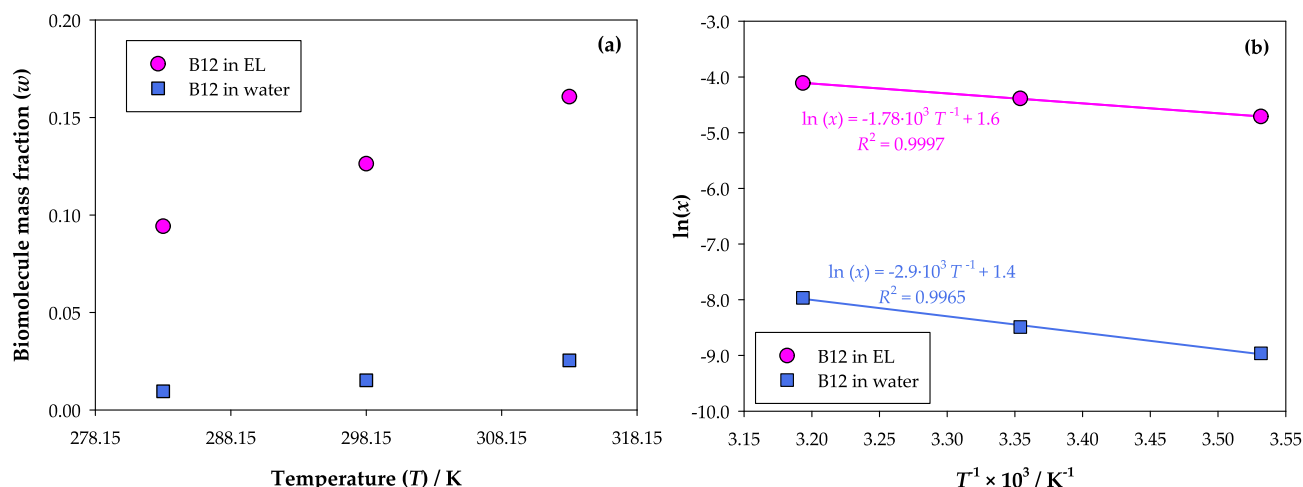


Fig. 9. Representation of the solubility of cyanocobalamin (B12) in water or ethyl lactate at 283.15, 298.15 and 313.15 K and 0.1 MPa in terms of mass fraction (a) and natural logarithm of molar fraction (b).

4. Conclusions

In this work, the extraction of cyanocobalamin (B12), catechin (Cat) and nicotinic acid (NiA) was conducted using the Aqueous Two-Phase Systems (ATPSs) {Ethyl lactate (1) + $\text{Na}_2\text{Succinate}$ or $\text{Na}_3\text{Citrate}$ or $\text{K}_3\text{Citrate}$ (2) + Water (3)}, at 298.15 K and 0.1 MPa. Favourable results were obtained for Cat, particularly for the ATPSs composed of $\text{Na}_3\text{Citrate}$, which achieved a partition coefficient (K) of $(5 \pm 1) \cdot 10^1$ and an extraction efficiency (E) of $(94.2 \pm 0.9) \%$ for the longest tie-line. Nevertheless, all systems presented partition coefficients higher than unity, indicating a preferential migration of the biomolecules to the top phase. On the other hand, the ATPS based on $\text{Na}_2\text{Succinate}$ presented the least promising results, with a maximum partition coefficient of 7.33 ± 0.04 and extraction efficiency of $(75.8 \pm 0.6) \%$ for the extraction of B12.

Considering the favourable results for the extraction of B12 reported in several works of the research group, the solubility of this biomolecule was assessed in water or ethyl lactate to better discuss the partitioning results. The observed solubility of B12 was over 40 times higher in water and over 300 times higher in ethyl lactate than the mass fractions of B12 in the partition studies, indicating that solubility was not limiting the partitioning of B12, which would imply an incorrect assessment of the performance indicators of extraction. Additionally, the results were closely aligned with a first-order linear regression, indicating that the solubility of B12 followed the van't Hoff equation.

CRediT authorship contribution statement

Gonalo Perestrelo: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Pedro Velho:** Investigation, Methodology, Writing – review & editing, Conceptualization, Supervision. **Eugénia A. Macedo:** Writing – review & editing, Conceptualization, Supervision.

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.

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50020/2020) and UIDP/50020/2020 (DOI: 10.54499/UIDP/50020/2020); and ALiCE, LA/P/0045/2020 (DOI: 10.54499/LA/P/0045/2020). Gonalo Perestrelo acknowledges a research grant funded through FCT/MCTES (PIDDAC): LSRE-LCM, UIDB/50020/2020 (DOI: 10.54499/UIDB/50020/2020) and UIDP/50020/2020 (DOI: 10.54499/UIDP/50020/2020); and ALiCE, LA/P/0045/2020 (DOI: 10.54499/LA/P/0045/2020). Pedro Velho is grateful for the funding support from FCT [2021.06626.BD].

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.seppur.2024.130987>.

Data availability

Data will be made available on request.

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