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Separation of natural compounds using eutectic solvent-based biphasic systems and centrifugal partition chromatography

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ABSTRACT

A study on the formation of ternary biphasic systems composed of heptane, 1-butanol or ethyl acetate and type III or type V deep eutectic solvents based on levulinic acid and choline chloride or thymol was carried. Binodal curves and densities and phase compositions of phases in equilibrium for seven systems are reported. The partition coefficients of six natural compounds, namely quercetin, apigenin, coumarin, β-ionone, retinol, and α-tocopherol, in these systems were measured. Results show that the influence of choline chloride on the partition coefficients is more significant in systems with 1-butanol or ethyl acetate than previously reported for ethanol, and that the separation of natural compounds is worst when using DES containing thymol instead of choline chloride. Based on these partition coefficients, one system composed of heptane, 1-butanol and the DES choline chloride:levulinic acid at molar ratio 1:3 was selected to be applied in centrifugal partition chromatography, and the results obtained confirmed that it allows a good separation of apigenin, coumarin, β-ionone and α-tocopherol.

1. Introduction

The need to achieve a sustainable development has led to an intense activity in the scientific community in order to propose more sustainable alternatives to current existing processes. One key aspect of such processes is related to the reduction of energy consumption, and another to the use of environmentally friendly and sustainable solvents. In this search for alternative solvents, deep eutectic solvents (DES) have drawn a strong interest [1–3]. DES are mixtures of at least two compounds that exhibit a low melting point allowing the formation of liquid solvents from solid compounds [4], thus expanding the number of potential candidates to use in solvent design and formulation. Physical chemical properties of DES have been extensively studied in the past decade and DES were applied to many fields [5,6] such as electrochemistry,[7] electrochemical energy storage,[8] liquid-liquid extraction [9–12], catalysis [13,14], and biomass processing.[15,16] In the field of liquid-liquid extraction of high value natural compounds, DES have also been used with organic solvents such as ethanol and heptane in order to form biphasic systems.[17–20] Such systems opened the way to new and very diverse biphasic mixtures based on sustainable solvents that exhibit phases with specific solvent properties, such as polarity or hydrogen bonding abilities. These systems can be finely tuned to selectively separate natural compounds and have been shown to be a promising class of systems in centrifugal partition chromatography (CPC) for the preparative separation of naturals compounds [17,21,22].

Centrifugal partition chromatography (CPC) is a counter-current chromatographic technique introduced for the first time in 1982 [23]. It is based on the principles of liquid/liquid partition chromatography. In this chromatographic technique, a column containing a large number of extraction cells is set up in a rotating cylinder. Rotation results in a centrifugal force applied to the column. One phase of the biphasic system is introduced in the column, remaining within and playing the role of a stationary phase. The other phase, pumped through the column is the mobile phase and flows through the extraction cells. Upon injection of a solute, dissolved in one of the phases, into the CPC, it flows through all extraction cells and elutes at a given speed related to its partition coefficient towards the phases of the biphasic system used [24].

In the last decade, this chromatographic technique drew a considerable attention in particular for natural products separation and purification. CPC has been applied to the separation of phenolics [25], anthocyanins [26], terpenoids [22], or proteins [27]. A

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large number of organic solvents were used in CPC [28], and more recently alternative solvents such as terpenes [29], ionic liquids [30–32] or deep eutectic solvents [17,33]. In particular, in 2016, Roehrer et al. successfully separated a mixture of three to four hydrophobic compounds using centrifugal partition chromatography and a biphasic system containing heptane, ethanol and a hydrophilic deep eutectic solvent, so-called DES, based on choline chloride [17]. In 2019, the same group also proposed an alternative solvent system based on a hydrophobic DES containing L-menthol, to be used in CPC [33].

Recently, our group has investigated the influence of choline chloride on the formation of biphasic systems containing heptane and ethanol and on the partition of several natural compounds present in vegetal raw material and used as additives for cosmetics or nutraceutics [34]. Nevertheless, most systems reported in the literature were based on heptane, ethanol and choline chloride-based DES. To the best of our knowledge, data on other biphasic systems are very scarce, only one team having studied systems with methanol replacing ethanol and DES based on thymol or menthol instead of choline chloride [33,35].

Following our previous work dealing with the study of binodal curves of heptane/ethanol/DES biphasic systems and application thereof to the separation of natural compounds such as quercetin, coumarin, apigenin, β-ionone or α-tocopherol, this work proposes to study new biphasic systems based on more hydrophobic solvents or DES. To that end, systems based on heptane as a first solvent, 1-butanol or ethyl acetate as a second solvent, and levulinic acid, or the corresponding DES based on choline chloride or thymol as a third solvent were studied. 1-butanol was selected to evaluate the influence of the alcohol alkyl chain length on the phase separation and partition, while ethyl acetate was selected as a hydrophobic compound with a different structure. Because levulinic acid exhibits a good solubility in these two organic solvents, it was preferred over glycerol or ethylene glycol and fixed in this study. Preliminary attempts at measuring binodal curves using DES based on glycerol or ethylene glycol and butanol or ethyl acetate led to the formation of precipitate, a phenomenon that is not acceptable when such systems are expected to be used with CPC. Finally, another family of DES based on thymol, so-called type V DES, was selected. Because such DES are generally immiscible in water, thus often called “hydrophobic DES”, they are expected to interact with natural compounds differently than choline chloride-based type III DES. Binodal curves for new organic biphasic systems based on **i)** heptane, **ii)** 1-butanol or ethyl acetate and **iii)** levulinic acid or corresponding choline-chloride or thymol-based DES, were measured. The compositions of selected biphasic mixtures were measured experimentally using GC-FID and HPLC-DAD-ELSD. Densities and mass ratios for both phases of a range of biphasic mixtures were also obtained. Finally, the partition coefficients (K_D) of six natural compounds, namely quercetin, apigenin, coumarin, β-ionone, retinol and α-tocopherol on these systems were measured. These compounds, that are not expected to be found together in a raw material extract, were selected as model of natural compounds, exhibiting different polarity values ranging from a low octanol water partition coefficient value of 1.41 for coumarin, to a high octanol water partition coefficient value of 12.000 for tocopherol [1,34]. Furthermore, these compounds were used for comparison reasons as they are classically used as model compounds in the literature [17,34,36]. Based on the K_D values obtained for the biphasic systems studied here, separation of coumarin, apigenin, β-ionone and α-tocopherol was studied with the help of CPC using a biphasic system composed of heptane, 1-butanol and a DES based on choline chloride and levulinic acid. To the best of our knowledge, this is the first time that this biphasic system is applied in CPC and that moderately apolar compounds, such as coumarin and apigenin are separated with this technique.

Table 1
Compositions of five DES studied.

Abbreviation	HBA	HBD	Molar ratio HBA:HBD
Ch:LevA (1:2)	Choline chloride	Levulinic acid	1:2
Ch:LevA (1:3)	Choline chloride	Levulinic acid	1:3
LevA:Thy (1:1)	Levulinic acid	Thymol	1:1
LevA:Thy (2:1)	Levulinic acid	Thymol	2:1

2. Experimental procedure

2.1. Chemicals

All chemicals including choline chloride (99% purity), ethylene glycol (99% purity), levulinic acid (99% purity), thymol (98.5% purity), quercetin (99% purity), apigenin (99% purity), coumarin (99% purity), retinol (95% purity), α-tocopherol (97% purity), β-ionone (99% purity) as well as ethanol (HPLC grade), 1-butanol (99.4% purity), ethyl acetate (HPLC grade) and heptane (HPLC grade) were purchased from Merck (Saint-Quentin-Fallavier, France) and used without further purification.

2.2. Preparation of deep eutectic solvents

Two type III DES based on choline chloride mixed with levulinic acid were prepared by mixing both compounds in a 30 mL vial, under continuous stirring at 70 °C until no further solid was observed. Two type V DES based on levulinic acid and thymol, playing respectively the roles of HBA and HBD were also prepared in a similar manner. For all DES the abbreviations and molar ratios of HBA:HBD are reported in Table 1. Typically, 3 to 5 g DES were prepared by weighing each compound with an analytical scale Pioneer from Ohaus (Readability 0.1 mg). For all DES, wt% and mol% are given with an uncertainty of ± 0.5%. Water content for each DES was measured using a Karl Fisher titrator T50 from Metler Toledo with a DM143-SC electrode. Values for the water contents of ChCl:LevA, is 0.8wt%. Ethylene glycol and levulinic acid contained below 0.2 wt% of water.

2.3. Binodal curves

Binodal curves for seven organic biphasic systems (OBS) containing heptane, 1-butanol or ethyl acetate, and levulinic acid or one of the DES detailed above were measured. Compositions and abbreviations for the OBS studied here are presented in Table 2. The system containing heptane, 1-butanol and choline chloride was not studied because the latter is not soluble enough in these organic solvents to allow the measurement of a binodal curve. The binodal curves were measured using the cloud point titration method. To that end, a sample of about 1 g, composed of 10 wt% 1-butanol or ethyl acetate and 90 wt% heptane was prepared in a vial sealed with a septum cap. Then, a solution composed of 90 wt% of the third component and 10 wt% 1-butanol or ethyl acetate, prepared in a sealed vial was added using a syringe into the sealed vial until a cloudy solution was obtained. After weighing the vial 1-butanol or ethyl acetate was added dropwise using a syringe until the solution became clear. The vial was then weighted again and subsequent addition of the third component and 1-butanol or ethyl acetate was then added to obtain a cloudy solution again. All experiments were carried out at a temperature of 20 °C. The clear points were then plotted on a phase diagram.

Additionally, system H/B/EG was measured for comparison purposes. The system H/EtOAc/Thy is not reported here because it is monophasic over the whole composition range. Systems H/B/Ch or H/EtAc/Ch were not reported due to the poor solubility of choline chloride in 1-butanol or ethyl acetate.

Table 2
Compositions of the seven organic biphasic systems studied.

Abbreviation	Solvent 1	Solvent 2	Solvent 3
H/B/LevA	Heptane	1-Butanol	Levulinic acid
H/B/Ch:LevA (1:2)	Heptane	1-Butanol	Choline chloride - Levulinic acid (1:2)
H/B/Ch:LevA (1:3)	Heptane	1-Butanol	Choline chloride - Levulinic acid (1:3)
H/B/EG	Heptane	1-Butanol	Ethylene glycol
H/EtAc/LevA	Heptane	Ethyl acetate	Levulinic acid
H/EtAc/LevA:Thy (1:1)	Heptane	Ethyl acetate	Levulinic acid - Thymol (1:1)
H/EtAc/LevA :Thy (1:2)	Heptane	Ethyl acetate	Levulinic acid - Thymol (1:2)

2.4. Density and volume ratio measurements

Density was measured using a densimeter DMA 4100 N from Anton Paar. The densimeter was calibrated with air and water prior to experiments. Values were measured in triplicates and are given with an uncertainty of ± 0.001 g.cm^{-3}.

Phase volumes of 10 g biphasic systems containing 37.5 wt% heptane, 25 wt% 1-butanol or ethyl acetate and 37.5 wt% DES (or HBD) were prepared in 20 mL cylindrical vials. Since the vials are cylindrical, the volume of each phase can be obtained by measuring the height of the interfaces with an electronic calliper (RS PRO electronic calliper, precision 0.03 mm). The set up was calibrated by measuring the height in the vial of 0.5 to 15 mL of water. Volumes were given with an uncertainty of ± 0.02 cm^3.

2.5. Density and volume ratio measurements

Settling time of biphasic mixtures were measured using a previously described procedure [37]. Briefly, each biphasic mixture containing 37.5 wt% heptane, 25 wt% ethanol, 1-butanol or ethyl acetate and 37.5 wt% DES (or HBD) were prepared in test tubes. Tubes were then shaken by hand with ten inversions and placed in a tube rack. The settling time was measured using a stopwatch and correspond to the time needed for full reestablishment of the meniscus. This measurement was performed in triplicate for each sample. Settling time were given with an uncertainty of ± 2 s.

2.6. Experimental determination of biphasic mixtures composition

For biphasic mixtures, each phase was assumed to contain up to four compounds: heptane, 1-butanol or ethyl acetate, choline chloride or thymol, and levulinic acid or ethylene glycol. For each compound the mass present in each phase was measured as follows.

For heptane, 1-butanol, ethyl acetate and ethylene glycol, distribution of these solvents between the upper and lower phases was obtained using the ratio of peak surfaces (S) from GC-FID analysis. The mass of heptane, 1-butanol, ethyl acetate or ethylene glycol in the upper phase was then calculated according to Eq. (1):

$$m_{up} = m_{total} \times \frac{V_{up}}{V_{up} + V_{low} \times \frac{S_{low}}{S_{up}}} \quad (1)$$

with *up* and *low* referring to the upper and lower phases, respectively. *V* refers to the volume of a phase and m_{total} the total mass of a compound in the biphasic system.

The mass in the lower phase was obtained as the difference between its total mass in the mixture and its mass in the upper phase according to Eq. (2)

$$m_{low} = m_{total} - m_{up} \quad (2)$$

The amount of levulinic acid and choline chloride was obtained considering the peak surfaces (S) on chromatograms recorded using HPLC-DAD-ELSD analysis, at 280 nm and with ELSD, respectively. Masses in upper and lower phases were then calculated using Eq. (1) and (2).

2.7. HPLC analysis

Liquid chromatographic analyses were performed using an HPLC Agilent 1200 system equipped with a diode array detector (DAD) and an evaporative Light Scattering Detector (ELSD). Compounds were separated on Luna C18 column (Phenomenex, 5 μm, 250 × 4.60 mm) at 25 °C. The mobile phase pumped through the column at a flow rate of 1 mL/min was composed of three solvents: A, water + 0.1% formic acid; B, acetonitrile + 0.1% formic acid and C, isopropanol. The following mobile phase gradient was used: 0.00–10.00 min, 35–40% B; 10.01–12.00 min, 40%–0% B 0–100% C; and 12.01–18.00 min, 100% C. The column was then equilibrated under initial conditions during 10 min. For each compound, calibration was carried out by injecting different samples containing between 20 and 250 µg/mL.

2.8. GC analysis

Gas chromatographic analyses were performed with an Agilent 6890 gas chromatograph equipped with a flame ionization detector (FID) and connected to a multifunction automatic sampler (CombiPal, CTC Analytics). The column used was an HP-1 non-polar column (100% Dimethylpolysiloxane, 50 *m* × 200 μm, 0.33 μm film thickness, Agilent). Split ratio was 1/50 with an injection volume of 1µL. The inlet liner used was packed w/10% OV-1 on WHP and heated at a temperature of 250 °C. Helium was used as carrier gas (constant flow rate of 1 mL/min). The oven temperature was initially held at 40 °C for 10 min and then increased from 40 °C to 270 °C (15 °C/min) and finally held at 270 °C for 10 min.

2.9. Experimental determination of partition coefficients and phases composition

Partition coefficients (K_D) of quercetin, apigenin, coumarin, β-ionone, retinol and α-tocopherol and phases composition were determined for the OBS described above. To that end 2 to 3 mg of a natural compound was dissolved in 5 g of 1-butanol or ethyl acetate. Then circa 5 g of a biphasic system composed with 37.5 wt% heptane, 25 wt% natural compound solution in 1-butanol or ethyl acetate and 37.5 wt% of the third component were prepared. Each tube was sealed, mixed in a vortex for 1 min and although such a long time is not necessary, tube was left for equilibration overnight in order to be certain to have a biphasic system at equilibrium and avoid any variation in the final composition of both phases . Around 1 mL of upper and lower phases of each tube were separated using Pasteur pipettes. Each phase was diluted 4 times in methanol and analyzed by HPLC-DAD-ELSD. For each natural compound, the corresponding peak surfaces obtain using HPLC analysis (*S*) were then used to calculate the partition coefficient, K_D according to Eq. (3)

$$K_D = \frac{S_{up}}{S_{low}} \quad (3)$$

with *up* and *low* referring to the upper and lower phases, respectively.

2.10. Evaluation of the stability of natural compounds in the biphasic system H/B/Ch:LevA

Stability of quercetin, apigenin, coumarin, β-ionone, retinol and α-tocopherol in the biphasic system H/B/Ch:LevA (1:3) was assessed. To that end, a biphasic mixture was prepare with 8.925 g of Ch:LevA (1:3), 5.95 g of 1-butanol and 8.925 g of heptane. Mixtures were introduced into sealed tubes, vigorously shaken and left for equilibration during 15 h. Phases were then collected into separated containers. In a test tube, 2 to 3 mg of each natural compounds was dissolved in 500 µL of top phase and 500 µL of bottom phase. The tube was closed with a cap and stirred at 1000 rpm in an oil bath at 35 °C for 1 or 24 h. One hour was selected because it is approximately the duration of a CPC experiment. The temperature of 35 °C was selected as it is the temperature expected to be found within the CPC apparatus, due to the heat generated by moving parts and in particular the rotor motor. After stirring for 1 or 24 h, the mixture was diluted in 50 mL of methanol and analyzed by HPLC-DAD to determine the amount of each compounds remaining unchanged in the biphasic system. Experiments were performed in triplicate.

2.11. Separation of a multicomponent mixture using centrifugal partition chromatography

2.11.1. Equipment

Separation of selected natural compounds was carried on a fast centrifugal partition chromatography system, model CPC–C, from Kromaton Rousselet-Robatel (Annonay, France). The rotor consists of 13 associated stainless-steel disks, each containing 64 twin cells for a total of 832 twin-cells coated with PTFE suitable for applications involving biomolecules. The column has a total volume of 50 mL and could withstand a pressure drop of 70 bar. The maximum achievable rotational speed is 3000 rpm for a maximum centrifugal field of approximately 1500 g. The CPC system is connected to an analytical HPLC pump ECOM ECP2010, which can deliver flow rates from 0.02 to 10.00 mL/min with a maximum pressure of 400 bar, and a diode array detector ECOM Flash14 from ECOM Spol. S.r.o. (Czech Republic). Samples are injected through an injection port consisting of a Rheodyne valve model 3055–023 from IDEX Health & Science (Wertheim, Germany) fitted with a 10 mL PEEK sample loop. Finally, fractions were recovered using an ADVANTEC Super Fraction Collector CHF122SC from Advantec Toyo Kaisha, Ltd. (Tokyo, Japan).

2.11.2. Preparation of biphasic mixture

To prepare a biphasic mixture, 187.5 g of Ch:LevA (1:3) were first prepared as describe above. At room temperature 125 g of 1-butanol and 187.5 g of heptane were then added and the mixture was vigorously stirred under magnetic stirring for at least 10 min. The mixture was subsequently transferred to a separation funnel closed with a cap and left for equilibration overnight in order to ensure a complete phase separation. At the end, phases were split into separated glass bottles, filtered over HPLC mobile phase filtration membranes, and degassed using an ultrasonic bath. Water content for each phase was measured using a Karl Fisher titrator Metrohm KF Coulometer model 831. Values for the water content of bottom and top phase are 1.0 wt% and below 0.1 wt% respectively.

2.11.3. Centrifugal partition chromatography

To carry out purification of natural compounds using CPC, approximately 80 mL of liquid used as a stationary phase, corresponding to twice the volume of the extraction column, is pumped within the column at a speed of 2 mL/min. Then, using a rotational speed of 1500 rpm, the mobile phase is pumped through the column at a specific flow rate. After this step, the retention factor S_f, corresponding to the percentage of the column filled with stationary phase, is obtained by measuring the difference between the total volume of the column and the volume of the stationary phase eluted from the column according to the following equation:

$$S_f = \frac{V_s}{V_{column}} = \frac{V_{column} - V_e}{V_{column}} \quad (4)$$

where V_s is the volume of stationary phase in the column, V_{Column} the volume of the column and V_e the volume of stationary phase eluted.

The detector wavelength is set to 340 nm, at which the absorbance of analytes is sufficient and the background signal related to the solvent is low. A mixture of natural compounds diluted in 3 mL of top phase and 2 mL of bottom phase is then injected via the injection port. Fractions of the mobile phase coming out from the column are collected, each fraction representing 40 s of elution.

Dual mode elution has been previously described as a modified procedure to recover highly retained compounds [38]. Experiment in dual elution mode is carried out as follows. First, bottom and top phases are used as mobile and stationary phases, respectively. The column is set to descending mode and flow rate is set to 0.7 mL/min. Upon equilibration of the two phases, a mixture of 3.34 mg of α-tocopherol, 2.29 mg of β-Ionone, 2.48 mg of coumarin and 0.64 mg of apigenin is injected. Elution lasted 53 min until no more compound is observed coming out from the column, as monitored with the help of DAD. Then, the top phase is pumped into the systems as the mobile phase and the elution mode is set to descending mode. Flow rate is increased to 1.5 mL/min and elution occurs for 17 min until no more compound is observed coming out from the column, as monitored with the help of DAD.

Elution-extrusion procedure has been previously described as a modified elution procedure to recover highly retained compounds [39]. Experiments using elution-extrusion procedure were carried out as follows. Top and bottom phases are used as mobile and stationary phases, respectively. The column remains in ascending mode and the flow rate is set to 1.5 mL/min. Upon equilibration of the two phases, a mixture of 3.93 mg of α-tocopherol, 1.80 mg of β-Ionone, 2.01 mg of coumarin and 0.83 mg of apigenin is injected. Elution occurs for 50 min. After that, the bottom phase is pumped into the column as the mobile phase, resulting in an extrusion of the bottom phase initially present as a stationary phase in the column. Flow rate is then reduced to 0.7 mL/min to prevent column overpressure related to the viscosity of the bottom phase. Extrusion is carried out for 70 min until no more compound is observed coming out from the column, as monitored with the help of DAD.

2.11.4. CPC fractions analysis

To evaluate the separation and the amount of compound recovered, fractions were diluted four times in methanol, filtered on syringe filter (nylon, 0.45 µm) and analyzed in duplicates by HPLC-DAD using the method described above with slight modifications. The HPLC apparatus used in this part was a VWR HPLC system, Chromaster from Hitachi equipped a diode array detector model 5430. Compounds were separated on a C18 column (SunShell, 5 μm, 250 $\times$ 4.60 mm). The other parameters including oven temperature, flow rate, mobile phase composition and gradient were kept identical. The separation was not changed by these modifications. For each compound calibration curves were generated by injecting different concentrations of standard compounds in the range 20–250 µg/mL.

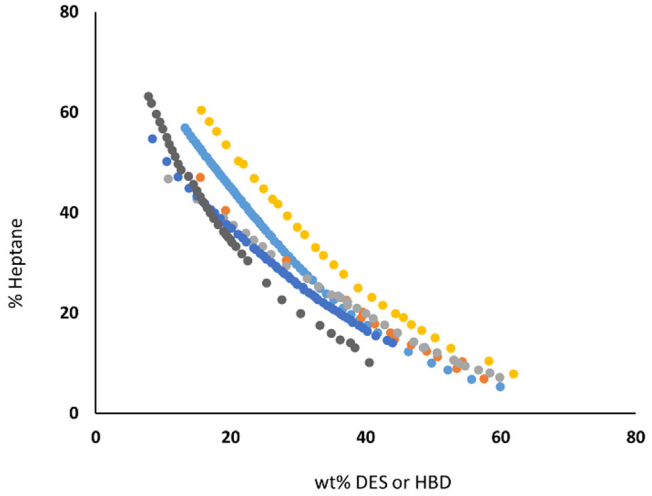

Fig. 1. Binodal curves for systems based on heptane and 1-butanol. H/B/EG: light blue; H/B/Ch:LevA 1:2: orange; H/B/Ch:LevA 1:3: gray; H/B/LevA: yellow; H/EtAc/LevA: dark blue. System H/E/LevA (black) is presented for comparison.

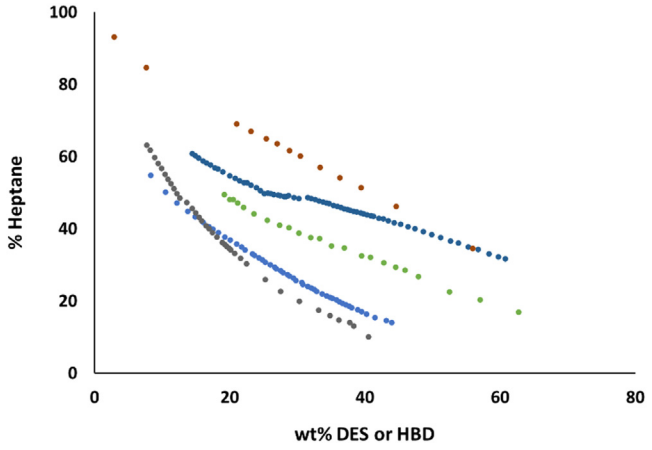

Fig. 2. Binodal curves for the systems: Blue: H/EtAc/LevA. Green: H/EtAc/LevA:Thy(2:1). Dark blue: H/EtAc/LevA:Thy (1:1). Brown: H/B/LevA:Thy (1:1). gray: H/E/LevA.

3. Results

3.1. Binodal curves

All binodal curves measured in this work are presented in Figs. 1 and 2. For comparison purposes, the binodal curve for heptane/ethanol/levulinic acid (H/E/LevA) reported previously [34] is also plotted. Among the systems of this type previously reported, H/E/LevA was the system exhibiting the largest biphasic domain. The decrease in the biphasic region observed when using butanol or ethyl acetate instead of ethanol is explained by the smaller differences in polarity and log K_{OW} (see Table 3) between heptane and 1-butanol or ethyl acetate, when compared to ethanol. These results also explain why the biphasic domain for H/B/EG is larger than that for H/B/LevA, in agreement to what was observed for H/E/EG and H/E/LevA[34]. This is due to the fact that the log K_{OW} for ethylene glycol is lower than that of levulinic acid. Ethylene glycol being more polar than levulinic acid, it induces more easily a phase separation when mixed with heptane than levulinic acid does.

The fact that 1-butanol leads to a larger biphasic domain than ethyl acetate might appear surprising as the values for the E_N^T polarity scale are 0.654, 0.586 and 0.228 for ethanol, 1-butanol and ethyl acetate, respectively [40]. But this result is in agreement with the log K_{OW} reported in Table 3, which can be explained considering that biphasic systems formation are related to the partition of solvents. Despite the lower polarity of ethyl acetate, the better miscibility of heptane, 1-butanol and a DES or HBD is most probably due to favourable interactions occurring between the alkyl chains of 1-butanol and heptane.

Table 3
Normalized polarity scale (E_T^N) and logarithm of the Octanol–Water partition coefficient (Log K_{ow}) for solvents used in this study.

	E_T^N	LogK_{ow}	Ref.
Heptane	0.012	4.66	[53]
Ethanol	0.654	−0.31	[50]
1-Butanol	0.586	0.88	[50]
Ethyl acetate	0.228	0.73	[50]
Levulinic acid	–	−0.49	[50]
Ethylene glycol	0.790	−1.36	[50]
Thymol		3.3	[50]

Binodal curves for ethanol or 1-butanol-based systems with EG or LevA are roughly parallel, which is consistent with the similar chemical nature of these two alcohols. However, adding choline chloride to levulinic acid has marked consequences. First, and in agreement with previous reports for ethanol-based OBS, the biphasic domain for H/B/Ch:LevA increases compared to that obtained for H/B/LevA. However, when using DES with a molar ratio HBA:HBD of 1:2, some choline chloride precipitation appeared when the DES percentage fell below 40 wt%. Therefore, the molar proportion of choline chloride was reduced to 1:3 with very little influence on the binodal curves. This suggests that the presence of choline chloride, above a certain concentration, no longer influences the size of the biphasic domain. Binodal curves for H/B/Ch:LevA are no longer parallel to those of systems H/E/LevA or H/B/LevA. Instead, binodal curves for H/B/Ch:LevA and H/B/LevA tend to merge at high DES concentrations.

When the proportion of DES is low, on the contrary, the binodal curve of H/B/Ch:LevA diverges significantly from that of H/B/LevA and even crosses the H/E/LevA binodal curve. This is easily explained by the fact that choline chloride, a salt hardly soluble in heptane or butanol, triggers a phase separation, even at very large concentrations of heptane and butanol.

Binodal curves obtained with LevA:Thy, a hydrophobic type V DES are presented in Fig. 2. These systems exhibit biphasic domains smaller than those presented in Fig. 1. H/B/LevA:Thy with an equimolar ratio of thymol and levulinic acid is monophasic over almost all the phase diagram. Biphasic mixtures appear only when the amount of 1-butanol in the system is below 10%. This shows that butanol acts as a solvent for both heptane, thymol and levulinic acid.

Because the size of the biphasic domain for H/EtAc/LevA:Thy is too small and a mixture composed of 37.5 wt% of heptane, 25 wt% of ethyl acetate and 37.5 wt%, of LevA:Thy with a molar ratio of 1:1 is monophasic, the binodal curve of H/EtAc/LevA:Thy with a molar ratio 1:2 in thymol and levulinic acid was studied. As it can be seen in Fig. 2, a higher amount of levulinic acid, the most hydrophilic compound of all chemicals present in the system, yields a larger biphasic domain, especially in the region of the phase diagram with high DES content. According to the binodal curve, in the case of LevA:Thy (2:1), the DES-rich phase is expected to contain more levulinic acid and less heptane than with LevA:Thy (1:1), hence yielding a larger number of biphasic mixtures. Mixture 37.5/25/37.5 of H/EtAc/LevA:Thy (2:1) is indeed in the biphasic domain, though very close to the binodal curve. Unlike choline chloride-based DES, LevA:Thy is based on a hydrophobic HBD, namely thymol, and a hydrophilic HBA, levulinic acid.

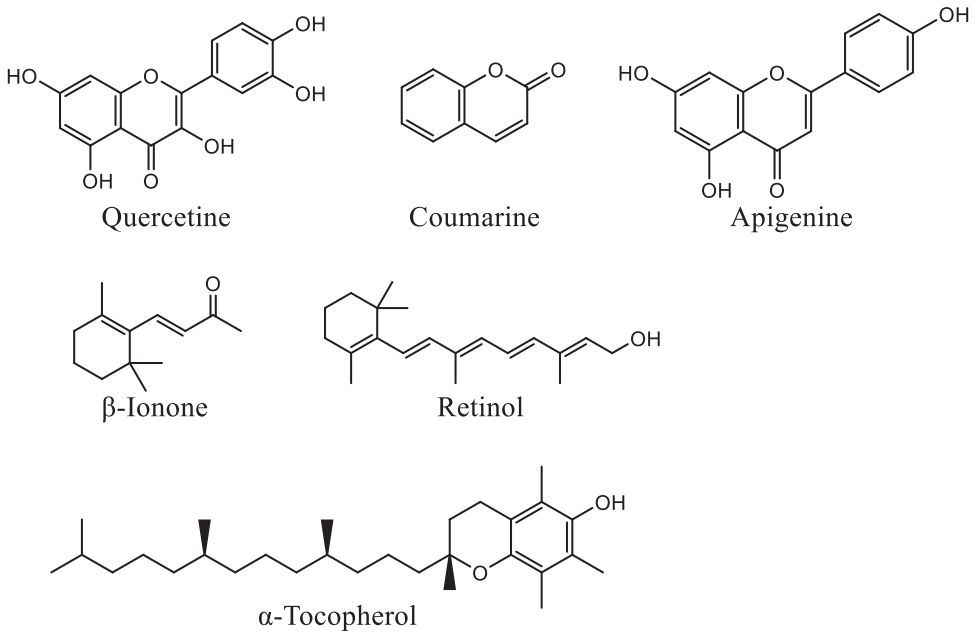

Fig. 3. Structures of natural compounds used in this work.

These compounds are thus expected to partition differently between the two phases, according to their octanol water partition coefficients (see Table 3). In this case, the cloud point titration method used here to obtain the binodal curve does not give any information on the partitioning of each component of the DES between the two phases. For LevA:Thy-based systems, phase diagrams as plotted in Fig. 3 only represent the amount of DES as a pseudo-component added to the mixture in order to obtain a biphasic mixture, regardless of the partition of HBA/HBD between the phases. No precise indication of the actual composition of the phases can therefore be obtained from the binodal curves for such systems. To get a better insight in the actual partitioning of all compounds in such OBS, phase compositions of selected biphasic mixtures were measured as detailed in the next subsection.

3.2. Phase composition

In order to gain better insight into the formation of these biphasic systems, phase compositions of selected biphasic mixtures were measured experimentally. To that end, biphasic mixtures composed with 37.5 wt% heptane, 25 wt% 1-butanol or ethyl acetate and 37.5 wt% of levulinic acid, ethylene glycol, Ch:LevA or Thym:LevA were prepared. These mixtures were selected because previous reports focused on systems with such compositions [17,18,33,34]. For simplicity, they will be hereafter mentioned as 37.5/25/37.5. Volumes and densities of each phase were measured, along with the resulting mass and volume ratio as well as the settling time necessary for obtaining clear separation of the two phases. Results for all seven systems studied in this work are detailed in Table 4. For comparison purposes, four systems based on ethanol were also studied.

Results show that densities for the upper phases increase when ethanol is replaced with 1-butanol or ethyl acetate, according to the following trend:

$$H/E/X < H/B/X < H/EtAc/X$$

where X stands for a HBD compound only or a DES. This result is in agreement with densities of neat ethanol, 1-butanol and ethyl acetate,[41] respectively of 0.789, 0.810 and 0.903 g.mL^{-1} and with the fact the upper apolar phase is composed mainly of heptane and ethanol, 1-butanol or ethyl acetate, as will be confirmed below. Moreover, for a given system based on ethylene glycol or levulinic acid, densities increase as well when choline chloride is added to the system.

Densities of lower phases vary, as expected, in a different way. Replacing 1-butanol by ethanol leads to a less dense phase when levulinic acid is used, whereas this density increases in presence of ethylene glycol. On the opposite, systems H/EtAc/LevA or H/EtAc/Ch:LevA exhibit densities for the lower phases significantly above those of any ethanol or 1-butanol-based systems. Again, adding choline chloride to levulinic acid or ethylene glycol yields an increase in density of the lower phase. Densities for Ch:LevA and Ch:EG are 1.140 [42] and 1.110 [43] g.mL^{-1},respectively, values similar to those of ethylene glycol or levulinic acid. As discussed below, this implies a significant modification of the composition for the lower phase upon addition of choline chloride.

For heptane/ethanol systems, adding choline chloride to levulinic acid or ethylene glycol does not significantly change the volume ratios, the latter remaining close to 1. On the opposite, when ethanol is replaced with 1-butanol or ethyl acetate, the addition of choline chloride to the system led to an increase in upper phase by a factor of nearly 2. For instance, the volume ratio for H/B/LevA is 0.96, while this value increases to 1.92 for H/B/Ch:LevA. Highest value for volume ratio, namely 2.60 was obtained for H/EthAc/Ch:LevA.

Biphasic systems exhibit settling times ranging between 8 and 45 s. Clear trends for settling times are not obvious. On the one hand, shortest settling time values of 8 and 11 s have been measured for H/E/LevA and H/EthAc/LevA, respectively. On the other hand, system H/EthAc/LevA:Thy exhibits the longest settling time, namely 40 s. This is most probably due to the fact that thymol, as shown later in the manuscript, partitions between the two phases. Its equilibration therefore requires more time compared to other systems in which HBD or DES partition mostly in the lower phase. The presence of ethanol or butanol does not appear to significantly change the settling time. When using ethyl acetate and ChCl:LevA, settling time is 14 s, approximately 10 s below that of ethanol or butanol-based homologue systems.

In order to get detailed insight into the composition of the phase compositions of these systems, chemical analysis

Table 4
Density of upper (ρ_{Up}) and lower (ρ_{Low}) phases, along with volume (R_V) mass ratio (R_M) and settling time (S_T) for the biphasic systems studied.

Solvent 1	Solvent 2	Solvent 3	ρ_{Up} / g.mL^{-1}	ρ_{Low} / g.mL^{-1}	R_V	R_M	S_T / s
H	E	EG	0.686*	0.956*	0.85	0.61	30
H	E	Ch:EG	0.687*	0.977*	0.96	0.68	22
H	E	LevA	0.689*	0.969*	0.81	0.58	8
H	E	Ch:LevA	0.689*	0.985*	0.97	0.68	21
H	B	EG	0,7027	0,9657	0,98	0,71	35
H	B	LevA	0,7099	0,9402	0,78	0.59	25
H	B	Ch:LevA (1 :2)	0,7260	-[a]	1.42	–	22
H	B	Ch:LevA (1 :3)	0.7166	0.9910	1.49	1.07	25
H	EtAc	LevA	0,7257	1,03,375	1,26	0,88	11
H	EtAc	Ch:LevA	0,75,165	1,1201	2,60	1,75	14
H	EtAc	Thym:LevA	0,8095	0,9421	2,08	1,78	43

* Taken from ref. [34]. a: Density not measured due to precipitate formation during density measurements.

Table 5
Comparison between phase compositions measured in this work and previously reported data for selected organic biphasic systems.

	H/E/LevA 37.5/25/37.5				H/E/Ch:EG 37.5/25/37.5				H/MeOH/LevA:Thy 50/20/30				H/MeOH/LevA:Thy 50/20/30			
	Upper EXP.	Upper LIT.	Lower EXP.	Lower LIT.	Upper EXP.	Upper LIT.	Lower EXP.	Lower LIT.	Upper EXP.	Upper LIT.	Lower EXP.	Lower LIT.	Upper EXP.	Upper LIT.	Lower EXP.	Lower LIT.
Heptane /wt%	96.5	98.9 [a]	6.1	6.2 [a]	96.2	98.7 [a]	1.7	2.1 [a]	88.9	86.7[b]	24.5	21.8 [b]	78.2	74.8 [b]	35.8	35.6 [b]
E or MeOH /wt%	2.4	1 [a]	37.0	37.2 [a]	3.8	1.2 [a]	37.9	38.7 [a]	2.3	5.0 [b]	31.6	30.0 [b]	7.3	9.6 [b]	26.7	25.3 [b]
Ch or Thy /wt%	–	–	–	–	0	0.0 [a]	32.0	59.2 [a]	5.5	6.6 [b]	19.7	22.0 [b]	9.9	12.0 [b]	24.6	25.8 [b]
LevA /wt%	1.1	0.1[a]	56.9	56.6 [a]	0		28.4		3.4	1.8 [b]	24.1	26.2 [b]	4.5	3.6 [b]	12.9	13.3 [b]

[a] COSMO-RS predictions taken from ref. [34].
[b] experimental data taken from ref. [35].

Table 6
Phase composition measured for selected organic biphasic systems.

	H/EtAc/LevA 37.5 / 25 / 37.5		H/EtAc/Ch:LevA 37.5 / 25 / 37.5		H/EtAc/LevA:Thy 37.5 / 25 / 37.5		H/B/LevA 37.5 / 25 / 37.5		H/B/Ch:LevA (1:3) 37.5 / 25 / 37.5	
	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Heptane /%wt	75.3	4.8	58.6	0.0	48.3	17.2	85.1	11.1	65.8	1.8
E, B or EtAc /%wt	20.6	28.8	38.1	1.7	23.5	27.8	9.7	33.5	29.1	19.8
HBA / wt%	–		0.0	53.4	18.5	25.8	–	–	0.0	24.3
HBD / wt%	4.1	66.4	3.3	44.9	9.7	29.2	5.3	55.4	5.1	54.2

for H/E/EG, H/E/LevA, H/E/Ch:EG, H/EtAc/LevA, H/EtAc/Ch:LevA, H/EtAc/LevA:Thy, H/B/LevA, and H/B/Ch:LevA with composition 37.5/25/37.5, was carried out using GC-FID and HPLC. To validate our experimental procedure, systems H/E/LevA, H/E/Ch:EG and H/MeOH/LevA:Thy, described in previous works were used [34,35]. Measured compositions of each phase are detailed and compared with reported values in Table 5.

The analytical method used here relies on a direct analysis of each phase using GC-FID and HPLC-DAD-ELSD. This strategy differs from that used in previous works that estimated phase compositions from experimental binodal curves [35]. The results obtained for H/E/LevA and H/E/Ch:EG are in good agreement with our previous results obtained using COSMO-RS model,[34] as well as the results obtained for H/MeOH/LevA:Thy with data available in the literature [35]. Discrepancy between experimental and estimated or reported values is always below 4 wt%. However, the analytical techniques used here, are more reliable than other approaches previously reported and provide direct experimental evidence for the phase compositions of these biphasic systems.

Analysis of the composition of each phase for mixtures H/EtAc/LevA, H/EtAc/Ch:LevA, H/EtAc/LevA:Thy, H/B/LevA, and H/B/Ch:LevA with initial compositions 37.5/25/37.5 are presented in Table 6. As expected, heptane is the major constituent of the upper phase. 1-butanol and ethyl acetate are also found in significant proportions in the upper phase, unlike what was previously observed for ethanol. This is due to the lower polarity of these two solvents that increases their affinity for heptane. Moreover, because of the ionic nature of choline chloride, the latter is not present in the upper phase in any of the systems studied. This result is in agreement with previous reports [34]. On the opposite, thymol is found in the upper phase, as expected from the low polarity and the hydrophobicity of this compound. Levulinic acid appears to partition slightly more significantly towards the upper phase in presence of 1-butanol or ethyl acetate than in presence of ethanol. This is most probably due to the fact that 1-butanol and ethyl acetate being more significantly present in the upper phase will act as co-solvent enhancing the extraction of levulinic acid towards this phase.

In the lower phase, heptane is generally present in negligible quantities. Only for H/EtAc/LevA:Thy and H/B/LevA, the lower phases have heptane present in concentrations of 17.1 and 11.1 wt%, respectively. This result, though surprising, can be accounted for by the fact that on one hand, thymol is a hydrophobic compound, and on the other hand, in the absence of choline chloride and in presence of 1-butanol, heptane finds good conditions to partition between the two phases.

As expected given their different polarities, the ratio of thymol:levulinic acid present in each phase of H/EtAc/Thym:LevA is significantly different from the initial ratio, namely 1:2 used here. In the upper and lower phases, mass ratios of approximately 2:1

Table 7
Partition coefficients ($\log(K_D)$) of the natural compounds studied using organic biphasic systems.

		Log(*Kd*)					
Solvent 1 / Solvent 2	DES or HBD	Quercetin	Apigenin	Coumarin	β-ionone	Retinol	α-tocopherol
H/E	EG	<−3	−2.91 ± 0.02	−1.19 ± 0.03	0.21 ± 0.03	–	1.38 ± 0.06
	Ch :EG	<−3	−2.91 ± 0.07	−1.19 ± 0.03	0.54 ± 0.03	–	1.49 ± 0.01
	AcLev	<−3	<−3	−1.49 ± 0.03	−0.24 ± 0.02	–	0.74 ± 0.02
	Ch:AcLev	<−3	<−3	−1.31 ± 0.02	0.24 ± 0.02	–	1.90 ± 0.03
H/B	EG	<−3	−2.10 ± 0.08	−0.87 ± 0.07	0.21 ± 0.08	0.13 ± 0.10	0.95 ± 0.25
	Ch:EG	<−3	−1.61 ± 0.01	−0.46 ± 0.01	1.27 ± 0.02	>2	>2
	AcLev	<−3	<−3	−1.16 ± 0.00	−0.25 ± 0.00	−0.36 ± 0.00	0.47 ± 0.010
	Ch:AcLev (1 :3)	<−3	−1.20 ± 0.02	−0.89 ± 0.003	0.28 ± 0.006	0.85 ± 0.01	1.08 ± 0.06
H/EtAc	AcLev	<−3	<−3	−1.18 ± 0.02	−0.13 ± 0.002	−0.21 ± 0.026	>2
	Ch:AcLev	<−3	<−3	−0.53 ± 0.01	1.47 ± 0.30	>2	>2
	Thy :AcLev (1 :2)	−1.12 ± 0.01	−0.97 ± 0.01	−0.45 ± 0.002	0.052 ± 0.01	0.03 ± 0.16	>2

and 1:1 respectively, are found. This shows that the DES partially dissociates during the phase separation process, each compound partitioning differently between the two phases [44].

Finally, a comparison of the phase compositions of H/EtAc/LevA with H/EtAc/Ch:LevA, and those of H/B/LevA with H/B/Ch:LevA, explains the variation of volume and mass ratios observed for these systems and reported in Table 4. It appears that in both cases the addition of choline chloride yields a nearly complete depletion of both organic solvents from the lower phase. For instance, in H/EtAc/LevA, the lower phase was composed by approximately 5 and 29 wt% heptane and ethyl acetate, respectively. Upon addition of choline chloride, no heptane and just 1.7 wt% ethyl acetate are present in the lower phase. Because these solvents represent a total of 62.5 wt% of the overall mixture and exhibit a density lower than that of choline chloride and levulinic acid, an upper phase with a significantly larger volume than that of the lower phase is formed.

Overall, this reveals that adding a co-solvent such as 1-butanol and ethyl acetate first enriches the upper phase in co-solvent and in HBD. Moreover, it shows that unlike choline chloride-based DES, for which HBA and HBD remain mostly together in the lower phase, a DES composed by a hydrophobic and a hydrophilic compound, such as thymol and levulinic acid, leads to a separation of its components in biphasic systems. Finally, the addition of choline chloride to an OBS containing a second solvent of relatively low polarity leads to a significant increase in the volume of the upper phase.

3.3. Partitioning of natural compounds in OBS

In order to apply biphasic systems presented here to the separation of natural compounds and to compare them with the ethanol-based systems previously reported, the partition of six natural compounds, namely quercetin, apigenin, coumarin, retinol, β-ionone and α-tocopherol, representative of the natural compounds families of polyphenols, terpenoids and vitamins, were measured. Moreover, these compounds exhibit significantly different polarity values and have previously been used in reports dealing with the study of the purification of natural compounds using biphasic systems and CPC [19]. To that end, mixtures with composition 37.5/25/37.5, as characterized in the preceding section were used. Such a composition was chosen since it is a composition located in the biphasic region of all systems, and was previously used in the literature.[17] Results are given in $\log(K_D)$ units in Table 7.

As shown in Table 7, partition coefficients measured for all six compounds follow the trend:

$$K_{Quercetin} < K_{Apigenin} < K_{Coumarin} < K_{\beta-ionone} \sim K_{Retinol} < K_{\alpha-tocopherol}$$

This trend is in agreement with previous literature data on the partition coefficients of such compounds in heptane/ethanol/DES biphasic systems [34]. Values obtained for retinol are close to those obtained for β-ionone, as reported elsewhere [33]. Nevertheless, in the absence of choline chloride, K_D values for retinol are systematically lower than those for β-ionone, a trend that is reversed by the introduction of choline chloride into the system. This suggests that the partition of retinol towards the upper phase is more influenced by choline chloride than that of β-ionone. This could be explained by the fact retinol presents a long alkyl chain and that when choline chloride is added to the system, it triggers a modification in the phase composition, replacing some of the neutral levulinic acid or ethylene glycol, and thus decreasing the favourable interactions between these compounds and retinol.

For apigenin and coumarin, replacing ethanol by 1-butanol leads to a general increase in the K_D values, i.e. an increase in the partition of natural compounds towards the upper phase. For example, using H/E/LevA and H/B/LevA, $\log(K_D)$ values of −1.19 and −0.89 respectively, are obtained for apigenin, or −1.49 and −1.16 for coumarin. This is further enhanced in presence of choline chloride. For instance, apigenin, though always partitioning preferentially towards the lower phase, exhibits $\log(K_D)$ values below −3 for H/E/Ch:LevA, compared to −1.82 obtained with H/B/Ch:LevA. Values for β -ionone remain surprisingly stable when ethanol is replaced by 1-butanol. For instance, $\log(K_D)$ values of 0.24 or 0.25 are obtained with systems H/E/LevA and H/B/LevA, respectively. Similar results are obtained in presence of ethylene glycol. Only for systems H/E/Ch:EG to H/B/Ch:EG, $\log(K_D)$ values for β -ionone vary by 0.8 log units. Finally, α-tocopherol seems to exhibit a behavior opposed to that of apigenin or coumarin. K_D values in 1-butanol-based systems are lower than those observed for ethanol-based systems. These results can be explained by the fact that unlike ethanol, 1-butanol is present in both phases. It thus increases the polarity of the top phase and decreases that of the bottom phase. Top and bottom phases are thus expected to exhibit more similar polarities in presence of 1-butanol. The affinity of a given natural compound for one specific phase will then be less marked. Compounds exhibiting very negative or very positive values for log (K_D) in presence of ethanol are thus expected to exhibit much lower values in presence of butanol.

1-Butanol and ethyl acetate appear to have a similar influence of partition coefficients as H/B/LevA and H/EtAc/LevA yields similar values for $\log(K_D)$ of all natural compounds. Again, this can be explained by the change in polarities of upper and lower phases triggered by the presence of ethyl acetate in both phases.

In our previous work, adding choline chloride to a biphasic system containing ethanol was found to increase the value of $\log(K_D)$. This observation is further confirmed in this study. In all cases, switching from H/B/EG or H/B/LevA to H/BCh:EG or H/B/Ch:LevA, yields an increase in $\log(K_D)$ values for all natural compounds reported here. The influence of choline chloride on $\log(K_D)$ is however more pronounced in presence of 1-butanol than in pres-

Table 8
Stability overtime of selected natural compounds in the biphasic system H/B/Ch:LevA (1:3) at 35 °C.

	Quercetin		Apigenin		Coumarin		β-ionone		Retinol		α-tocopherol	
Time	Yield	±	Yield	±	Yield	±	Yield	±	Yield	±	Yield	±
1 h	93%	5%	98%	3%	93%	2%	100%	2%	46%	6%	98%	2%
24 h	86%	5%	97%	1%	97%	4%	95%	5%	19%	5%	97%	3%

ence of ethanol. For instance, values for $\log(K_D)$ of β-ionone are 0.21 and 0.54 with systems H/E/EG and H/E/Ch:EG, respectively. Values are 0.19 and 1.23 with systems H/B/EG and H/B/Ch:EG, respectively. For retinol, an increase of 2 log units is observed when changing from H/B/EG to H/B/Ch:EG or from H/B/LevA to H/B/Ch:LevA. Furthermore, such an increase in $\log(K_D)$ values is even more significant in presence of ethyl acetate. The variation in $\log(K_D)$ obtained for coumarin and β-ionone using H/EtAc/LevA and H/EtAc/ChCl:LevA, respectively, is twice as high as that obtained with 1-butanol homologues.

Comparing results obtained with H/EtAc/LevA:Thy with those obtained using choline chloride-based systems, shows that all $\log(K_D)$ values, except for α -tocopherol, tend to a value of 0. $\mathrm{Log}(K_D)$ values for quercetin, apigenin and coumarin are higher than those obtained using H/EtAc/Ch:LevA. On the opposite, compounds such as β-ionone and retinol exhibit a decrease on $\log(K_D)$ values. As shown in Table 6, ethyl acetate and thymol are present in similar proportions in both phases of H/EtAc/LevA:Thy and 17% of heptane is present in the polar, bottom phase. It thus appears that despite the presence of 30 wt% levulinic acid in the lower phase, both phases contain mostly the same compounds, and are expected to exhibit similar polarities. When a given solute is added to such a biphasic systems, it will partition more or less equally between the two phases.

3.4. Application of biphasic systems to the separation of natural compounds

3.4.1. Selection of a suitable biphasic system for the separation of selected natural compounds

From the $\log(K_D)$ values obtained in this study, the most promising biphasic system to be applied in CPC was selected. From a theoretical point of view, in order to obtain good separation of natural compounds by CPC, compounds should exhibit $\log(K_D)$ values ranging between −0.3 and 0.5 approximately, and differ by at least 0.2.[24] Under such conditions, all systems containing ethanol as well as systems H/B/LevA and H/EtAc/LevA were excluded because $\log(K_D)$ values for quercetin, apigenin and coumarin were too low and close to each other, resulting in a lack of elution and separation of these compounds. Similarly, systems H/B/Ch:EG and H/EtAc/Ch:LevA were excluded because $\log(K_D)$ values for β-ionone, retinol and α-tocopherol are all above 1.2. Furthermore, H/EtAc/LevA:Thy was excluded because $\log(K_D)$ values of β-ionone and retinol are almost identical.

Values of $\log(K_D)$ for β-ionone and retinol obtained with system H/B/ChCl:LevA are very close to the range [−0.3; 0.5] for separating compounds and differ significantly. The α-tocopherol, exhibiting a $\log(K_D)$ value above 1, is expected to be easily separated from all other compounds. The value of the $\log(K_D)$ for coumarin is slightly below the optimum range but differs significantly from those of apigenin and quercetin. Therefore and despite the $\log(K_D)$ values for these two compounds being well below the preferred range, suggesting that these two compounds could not be easily separated from each other, the system H/B/ChCl:LevA was selected.

3.4.2. Selection of natural compounds

Because a CPC run lasts over one hour, the temperature within the apparatus is expected to exceed room temperature, and since some natural compounds, such as retinol, have already been reported to undergo some degradation or some reaction over time and temperature,[45] the stability of all natural compounds studied here was assessed. Stability tests were conducted by dissolving all natural compounds in biphasic system H/B/Ch:LevA, as detailed in the experimental section, and stirring for 1 h or 24 h at 35 °C. Results are presented in Table 8.

Results show that apigenin, coumarin and α-tocopherol do not undergo any significant degradation. The stability yield for these three natural compounds is 97% after 24 h. β-ionone also appears to be stable after 24 h stirring, since 95% of it was recovered. Some degradation was observed for quercetin, since only 86% was recovered after 24 h. A lower stability of quercetin was previously reported and assigned to oxidation due to atmospheric oxygen.[46] Retinol stability was poor in this biphasic system with recovery yields of 46% and 19% respectively after 1 h or 24 h. Two mechanisms explain this result. First, retinol degradation is induced by UV light and oxygen.[45] Second, some retinol esterification has been reported to occur under certain conditions.[47] Retinol and to a lesser extent quercetin instability constitutes a limitation to this system and in order to avoid any bias in the results obtained with CPC, retinol and quercetin were excluded from the following experiments to separate natural compounds with the help of CPC.

3.4.3. Separation of natural compounds using Centrifugal partition chromatography

Separation of α-tocopherol, β-ionone, apigenin and coumarin using H/B/Ch:LevA (1:3) was investigated with the help of CPC . Because of the low K_D values observed for apigenin and coumarin, and in order to avoid using a very large retention volume, separation of all compounds was studied using two distinct modified elution procedures, namely dual elution mode and elution/extrusion, as detailed in the experimental section. First, from an operational point of view, dual elution mode is advantageous because at the end of the separation procedure, the two phases remain in the column. This makes a follow up with a second injection easy, simply switching from ascending back to descending mode. On the contrary, at the end of the elution extrusion procedure the column is filled only with bottom phase. This implies that a second injection is possible only after a column filling step, using the top phase as the mobile phase.

Results obtained using dual elution mode are shown in Fig. 4 presenting the chromatogram obtained and Table 9, collecting data on retention times and yields for a given compound obtained with a 94 or 99% purity level. Using this mode, separation was achieved in 69 min elution. It appears that aiming at recovering a 99% pure natural compounds, less than half of apigenin and α-tocopherol injected initially were recovered. Furthermore, no pure coumarin was obtained in this way, due to the presence of a long tailing for apigenin. Only pure β-ionone was obtained with a satisfying yield, namely 98%.

Higher yields were achieved when collecting larger fractions of eluting solvent. In such a case, however, purity for each compound decreases because first coumarin and apigenin appear to be slightly contaminating each other due to a long tailing of apigenin and second α-tocopherol and β-ionone exhibit a slight coelution together. Aiming at a minimum purity of 94% for each natural

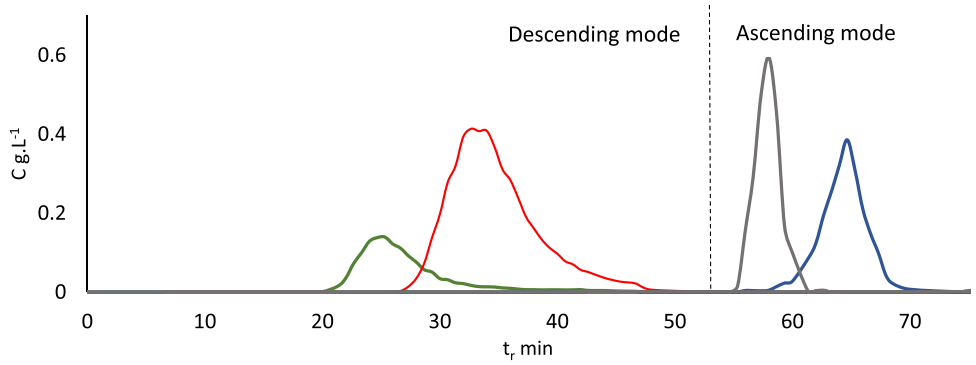

Fig. 4. Concentrations of apigenin (green curve), coumarin (red curve), α-tocopherol (gray curve) and β-ionone (blue curve) as a function of the retention time using CPC in dual elution mode. Biphasic system: H/B/Ch:LevA (1:3). Operating conditions for elution in descending mode: bottom phase as mobile phase; descending mode; rotational speed: 1500 rpm; flow rate: 0.7 mL.min^{-1}; Retention factor: Sf $\approx$ 50%. Operating conditions for elution in ascending mode: top phase as mobile phase; ascending mode; rotational speed of 1500 rpm; flow rate of 1.5 mL.min^{-1}.

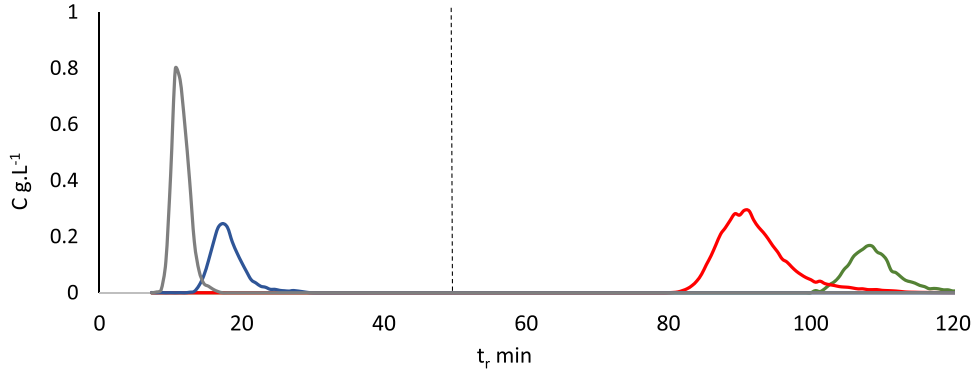

Fig. 5. Concentrations of α-tocopherol (gray curve), β-ionone (blue curve), coumarin (red curve) and apigenin (green curve) according to the retention time during CPC using elution/extrusion procedure. Biphasic system: H/B/Ch:LevA (1:3). Operating conditions for elution: top phase as mobile phase; ascending mode; rotational speed of 1500 rpm; flow rate of 1.5 mL.min^{-1}; Retention factor: Sf $\approx$ 50%. Operating conditions for extrusion: bottom phase as mobile phase; rotational speed of 1500 rpm; flow rate of 0.65 mL.min^{-1}.

Table 9
Natural compounds recovery yields according to the desired purity in the CPC experiment using dual elution procedure. The engaged mass of apigenin, coumarin, β-ionone and α-tocopherol were of 0.64 mg, 2.48 mg, 2.29 mg and 3.34 mg, respectively. *: No purity higher than 96% reachable.

	Apigenin		**Coumarin**		***β*-Ionone**		***α*-Tocopherol**	
	Retention times (min)	Yield (%)	Retention times (min)	Yield (%)	Retention times (min)	Yield (%)	Retention times (min)	Yield (%)
Purity $\geq$ 99%	20.0–26.7	47	–	0*	61.3–68.7	98	54.7–58.0	48
Purity $\geq$ 94%	20.0–28.0	57	29.3–46.7	88	60.0–68.7	99	54.7–60.0	70

compound, all four compounds were recovered with a yield ranging between 57% for apigenin to 99% for β-ionone.

In order to improve the recovery yields for all four natural compounds used here, purification of these compounds using CPC was carried out with an elution/extrusion mode. Briefly, in this method after pumping a given amount of a mobile phase throughout a column initially filled with a stationary phase, additional stationary phase is pumped through the column. This results in an extrusion of the stationary phase initially set up in the column, allowing recovery of all natural compounds that were partitioned predominantly to the stationary phase, hence recovering them.

Chromatogram corresponding to the separation of natural compounds using this mode is presented in Fig. 5. Recovery yield and purity for all four recovered compounds are reported in Table 10. In this procedure, elution with the top, apolar phase as mobile phase was carried out for 50 min. Interestingly, using this mode, α-tocopherol elutes first, before β-ionone, which is consistent with the fact that it exhibits the highest partition coefficient in the biphasic system: H/B/Ch:LevA (1:3).

Even though α-tocopherol and β-ionone are fully eluted after approximately 25 min., a 50 min. long elution time was used in order to improve later separation of apigenin from coumarin, two compounds that remain in the bottom phase, the stationary phase, but that still undergo some separation within the rotor. This long elution time was required since no separation of these compounds occurs during the extrusion step. During the extrusion step, coumarin was found to elute before apigenin, in agreement with the apigenin lower value for K_D in H/B/Ch:LevA (1:3), that makes it the compound remaining mostly at the bottom, stationary phase.

In order to avoid excessive pressure, possibly arising from the low, though non negligible, viscosity of the bottom phase containing the DES Ch:LevA, a reduced flow rate of 0.65 mL/min for the mobile phase during extrusion was required.

Similarly to the previous experiments using dual elution mode, two levels of purity for each compound were selected. Because of a long tailing of apigenin leading to a coelution with coumarin, 99% pure apigenin could not be obtained. On the opposite, and unlike results obtained with dual elution mode, above 84% of coumarin, β-ionone and α-tocopherol were recovered with a 99% purity. When aiming at recovering separately 94% pure natural compounds, results are improved compared to those obtained in

Table 10
Natural compounds recovery yields according to the desired purity in the CPC experiment performed using elution/extrusion procedure. The engaged mass of apigenin, coumarin, β-ionone and α-tocopherol were of 0.83 mg, 2.01 mg, 1.80 mg and 3.93 mg respectively. *: No purity higher than 94% reachable.

	Apigenin		**Coumarin**		***β*-Ionone**		***α*-Tocopherol**	
	Retention times (min)	Yield (%)	Retention times (min)	Yield (%)	Retention times (min)	Yield (%)	Retention times (min)	Yield (%)
Purity ≥ 99%	–	0*	80.0–102.0	90	16.0–28.7	87	8.7–14.0	84
Purity ≥ 94%	108.0–124.7	62	80.0–104.7	92	14.7–28.7	96	8.7–15.3	86

dual elution mode. As detailed in Table 10, apigenin is recovered with the lowest yield, namely 62%. Recovery of coumarin is slightly better while that of tocopherol is significantly higher than those obtained using dual elution mode. Only β-ionone is recovered with a slightly lower yield, namely 96%, compared to 99% obtained in dual elution mode.

A few works report application of organic biphasic system based on DES to the separation of natural compound using CPC in ascending or descending elution mode. Specifically, Roehrer et al. [17]. successfully separated a mixture of naringenin, β-ionone, retinol and α-tocopherol using CPC and a system composed of heptane, ethanol and Ch:LevA (1:2) in composition 30/40/30 wt%. The most polar compound studied in this previous work was naringenin, that exhibits a relatively high log (K_{OW}) value, namely 2.4.[48] The latter was separated from β-ionone, the second most polar compound out of the four mentioned above, exhibiting a log(K_{OW}) value of 3.8.[49] Coumarin was excluded from their investigation because it was supposedly too polar (log K_{OW} = 1.4). In this work, coumarin and apigenin are well separated. These are two polar molecules with log K_{OW} of 1.4 and 3.0 respectively [50,51]. Moreover, apigenin and coumarin exhibit partition coefficient values log(K_D) of −1,2 and −0.9 respectively whereas Roehrer et al. [17]. reported values of −2.5 and −1 for naringenin and coumarin respectively. Keeping in mind apigenin and naringenin are very similar molecules, values reported by Roehrer et al. [17]. differ more significantly than those reported here, but are also far away from the CPC preferred range. Therefore, replacing ethanol with 1-butanol and slightly modifying the HBA/HBD molar ratio in the DES to 1:3, appears to yield a better separation of the most polar compounds while maintaining the separation of apolar compounds. This is mainly due to the presence of 1-butanol replacing ethanol, the former being less polar than the latter. A significant amount of 1-butanol is therefore present in the upper phase, unlike what was observed previously for heptane/ethanol-based systems [34]. This, in turn, increases polarity of the upper phase, or at least yields phases with more similar polarity, compared to systems based on heptane, ethanol and Ch:LevA. This allows more polar compounds to partition towards the top phase more significantly than when ethanol is present. Compared to other previously reported systems based on ethanol, a biphasic system based on 1-butanol and a DES, as introduced in this study, appears to be more versatile in order to separate compounds exhibiting close and moderate polarity.

The system based on heptane, 1-butanol and ChCl:LevA DES can therefore be applied to separation and purification from plant extract of moderately polar to apolar compounds. In the system studied here, compounds close to the preferred range should exhibit log P ranging from 1.4 to 3.8. After the separation of these compounds, in case they are partitioned towards the apolar phase,their recovery from the solvent can be envisaged by evaporating heptane and 1-butanol, solvents with boiling point of 98 and 118 °C respectively [41]. However, for thos epartiitoned towards the DES-containg phase, evaporation is not envisaged. As this DES is non-toxic and choline chloride is allowed as an animal feed additive, such phase ocntianig natural compounds could be used directly, without further separation steps in applications such animal feed supplementation. Regarding other applications, for instance in cosmetics, for which choline chloride is forbidden, separation of natural compounds from DES are necessary. recently developed depending on the nature of the target compounds. Separation methods, recently developed depending on the nature of the natural compounds to remove include resins or solid phase extraction (SPE), liquid-liquid extraction, anti-solvent addition or supercritical carbon dioxide.[52]

4. Conclusions

A range of new organic biphasic systems based on hydrophobic solvents, heptane, 1-butanol or ethyl acetate and deep eutectic solvents based on choline chloride or thymol are reported in this work. Binodal curves show that the use of a solvent less polar than ethanol reduces the biphasic domain. In addition, replacing choline chloride with thymol further reduces the biphasic domain, that almost vanishes for the system H/EtAc/LevA:Thy. This work reports, for the first time, direct measurements of the experimentally phase composition for such organic biphasic systems using GC-FID and HPLC-DAD. Such analysis revealed the influence of choline chloride and thymol on the composition of the phases in equilibrium and helped understanding at the mechanisms responsible for changes in the volume ratios (R_V) observed experimentally.

The partition coefficients of six natural compounds in the biphasic systems under study were measured. Using 1-butanol or ethyl acetate generally leads to an increase in the K_D value, i.e., an increase in the partition of natural compounds towards the upper apolar phase. Furthermore, it appears that the influence of choline chloride on K_D values is more pronounced when a second solvent such as 1-butanol or ethyl acetate is used than with ethanol. In presence of thymol, immiscible phases appear to be less different in terms of compositions and polarity. Natural compounds therefore tend to partition similarly between the two phases.

Using these results it was possible to separate apigenin, coumarin, β-ionone and α-tocopherol with very good yields using CPC and a DES-based biphasic system H/B/Ch:LevA. The presence of 1-butanol replacing ethanol in the biphasic systems allows to separate compounds with relatively high polarity, increasing the versatility of the system and the number of potential applications.

The application of such DES-biphasic sytems to the purification of raw material extracts obtained from raw material is currently under investigation and will be the subject of a forthcoming article.

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.

CRediT authorship contribution statement

Jean-Baptiste Chagnoleau: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Inês LD Rocha:** Methodology, Investigation. **Ryan Khedher:** Investigation.

João AP Coutinho: Supervision, Writing – review & editing. **Thomas Michel:** Resources. **Xavier Fernandez:** Resources. **Nicolas Papaiconomou:** Supervision, Conceptualization, Validation, Writing – review & editing.

Data Availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.463812.

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