Biotechnology Education

Aqueous Biphasic Systems in the Separation of Food Colorants[S]

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Abstract

Aqueous biphasic systems (ABS) composed of polypropylene glycol and carbohydrates, two benign substances are proposed to separate two food colorants (E122 and E133). ABS are promising extractive platforms, particularly for biomolecules, due to their aqueous and mild nature (pH and temperature), reduced environmental impact and processing costs. Another major aspect considered, particularly useful in downstream processing, is the "*tuning*" ability for the extraction and purification of these systems by a proper choice of the ABS components. In this work, our intention is to show the concept of ABS as an alternative and volatile organic solvent-free tool to separate two different biomolecules in a simple way, so simple that teachers can effectively adopt it in their classes to explain the concept of bioseparation processes. Informative documents and general information about the preparation of binodal curves and their use in the partition of biomolecules is available in this work to be used by teachers in their classes. In this sense, the students use different carbohydrates to build ABS, then study the partition of two food color dyes (synthetic origin), thus evaluating their ability on the separation of both food colorants. Through these experiments, the students get acquainted with ABS, learn how to determine solubility curves and perform extraction procedures using colorant food additives, that can also be applied in the extraction of various (bio)molecules. © 2018 by The International Union of Biochemistry and Molecular Biology, 46:390–397, 2018.

Keywords: *Biotechnology education; bioseparation; aqueous biphasic systems; phase diagrams; partition; food colorants*

Introduction

The study of the separation of target compounds from complex mixtures using liquid–liquid extraction (LLE) techniques is a formative experiment in undergraduate Chemistry, Chemical Engineering, and Biotechnology laboratories. Presently, liquid–liquid extractions are widely used in the industry for the concentration, the extraction or the purification of a variety of compounds. In this sense, the target molecule is separated from its matrix based on its relative solubility in two different immiscible (or partially miscible) liquids. These systems are frequently formed by water (polar) and an organic solvent (nonpolar). Both liquids should be carefully chosen so as to maximize the partition of the target molecule from one solvent to another and minimize the coextraction of impurities. This technique is hence a useful tool to illustrate and apply relevant topics such as molecular polarity, intermolecular forces, thermodynamic equilibrium, acid–base reactions, and several laboratory works regarding LLE were recently proposed in Chemical Education Journals [1–4]. In those, as in typical conventional industrial and laboratorial systems, at least one liquid phase is formed by a volatile organic solvent (VOC); however useful, these volatile solvents may have deleterious effects in the environment. The education of students in the XXI Century should focus on the crucial relevance of environmental issues and societal

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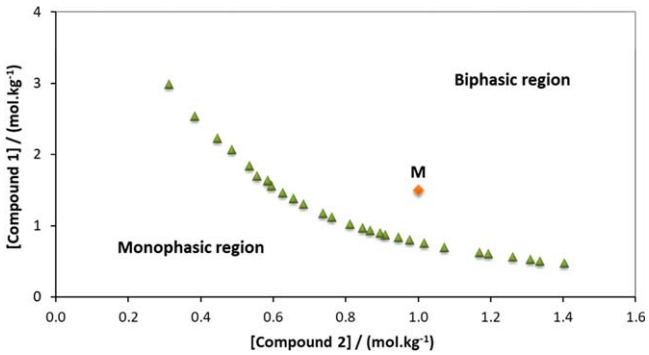

FIG 1

Example of a phase diagram of a system composed of two different compounds, in molality units, with the experimental binodal data (▲), a mixture point in the biphasic region (♦), and the mono- and biphasic regions identified. [Color figure can be viewed at wileyonlinelibrary.com]

concerns. In one of the published LLE experiments, environmental concerns are addressed by reducing the amounts of solvents employed [3]; although useful, this is a limited improvement in reducing environmental impacts. Another disadvantage of VOCs, particularly when the target molecule is a biomolecule, is the mutual (in)compatibility of those organic solvents and biological substrates. This is a most important issue at times where biotechnological processes assume paramount importance. To overcome these difficulties, Albertsson proposed, in 1986, the use of two partially immiscible aqueous phases for the partition of cells and cell particles [5]. Those aqueous biphasic systems (ABS) are formed when two distinct substances, both miscible with water, *e.g.*, polymer/polymer or polymer/salt are combined; above a critical concentration of the phase-forming components the spontaneous phase separation takes place and an aqueous two-phase system is formed. As both phases have a great amount of water in their composition, these systems are most compatible with biomolecules [6].

For students to be aware of both environmental concerns and the biocompatibility concept in technological separation procedures, they should be acquainted with safer alternatives to the conventional methodologies that use VOCs. Henceforth, a set of experiments using ABS formed by a synthetic but innocuous polymer, polypropylene glycol 400 g mol^{-1} (PPG 400), and a carbohydrate is proposed for undergraduate students majoring Biotechnology, Biochemistry, Chemical Engineering, or Chemistry. To evaluate the influence of the carbohydrate structure on its capability of promoting the phase separation, various carbohydrates were investigated, namely monosaccharides (mannose, fructose, and xylose) and disaccharides (sucrose and mannose). These sugar molecules bear a high number of hydroxyl groups that bond extensively with water, thus reducing "free" water molecules content, and promoting the separation (splitting) of the two aqueous phases. The students firstly determine the solubility curve (also designated as binodal curve) to obtain the ternary phase diagrams at room temperature—each group being assigned a different carbohydrate. Then, design of the respective ABS is proposed and these are further analyzed taking into account their application in separation processes (definition of binodal curve, mixture point, monophasic, and biphasic region). In Fig. 1 it is possible to observe a binodal curve, the M point, which corresponds to a mixture point in the biphasic region, and also the mono- and biphasic region are annotated. Next, an appraisal of the extractive potential of the studied ABS will follow by using a suitable composition (biphasic region) to separate (or purify) two synthetic food additives (E122 and E133).

Aqueous Biphasic Systems

Aqueous biphasic systems (ABS) are formed when two water-soluble compounds mixed in water and when above certain concentrations allow the spontaneous phase separation. This phenomenon was firstly observed by Beijerinck in 1896, when agar was mixed with soluble starch or gelatin [7]. Originally, these systems were based on aqueous mixtures of two incompatible polymers, such as polyethylene glycol (PEG), dextran, and/or maltodextrin [5, 6]. The combination of dextran and PEG represents one of the most studied systems; dextran, more dense and hydrophilic in the lower phase and PEG, more hydrophobic and less dense, in the upper phase. Since then, many immiscible aqueous systems were found using mainly hydrophilic polymers. In recent years, however, with the purpose of increasing the mass transfer rates and the selectivity of the fractionation of biomolecules, other types of ABS have been studied, in which different phase components are used. Ionic liquids (ILs), inorganic salts and carbohydrates are three examples of solutes used in ABS [8–11] that were applied in the separation or purification of a wide range of compounds, from proteins and enzymes to antibiotics and organic acids and many other bio- or synthetic molecules [8, 9].

Polymer-Carbohydrate Based ABS Extraction

For this laboratory experiment, ABS formed by aqueous solutions of the synthetic polymer poly(propylene glycol) 400 g mol^{-1} (PPG 400) and a carbohydrate were chosen since these are novel and completely benign compounds. PPG is a biocompatible polymer that has many properties in common with PEG: both are liquid at room temperature and their solubility in water decreases rapidly while increasing their molar mass. PPG is similar to PEG, but the replacement of a methyl group in the ethylene glycol repeating unit renders PPG more hydrophobic than PEG. Moreover, secondary hydroxyl groups in PPG are less reactive than primary hydroxyl groups in PEG. Both polymers are considered safe and have been approved for human injections and oral application but PPG is considered less toxic than PEG, so biotechnological matrices are now being

produced in PPG and are widely used by the chemical, food, and pharmaceutical industries [12].

Carbohydrates or saccharides (or sugars) are a large and diverse group of organic compounds that include sugars, cellulose, and starch. Carbohydrates are polyhydroxy aldehydes or ketones with high affinity for water since they present several —OH groups, with a dual hydrogen-bond donor/acceptor character. For that reason, carbohydrates can be involved in hydrogen bonding, and thus, present a strong ability to bind water. As a result, carbohydrates may be advantageous substitutes of polymers in the formation of ABS, since they embody more biocompatible routes for the extraction of biomolecules [11, 13].

Food Color Additives

Food color additives are any dye, pigment, or substance that imparts color when it is added to food or drinks. Food additives play an important role in today's complex food supply since their use has become more prominent in recent years due to the increased production of prepared, processed, and convenience foods. Brilliant Blue FCF (E133) is a blue color synthetic dye produced using aromatic hydrocarbons from petroleum. This food additive is found in diverse range of foods such as ice cream, canned peas, packet soups, bottled food colorants, icings, ice pops, blue raspberry flavored products, dairy products, and sweets. It is also used in drinks and tooth elixir. Brilliant Blue FCF is an approved food colorant and pharmacologically inactive substance for drug formulations in the EU and the United States. Azorubine, also known as carmoisine (E122), is a synthetic red color dye allowed as a food additive in the EU (*e.g.*, cheeses, dried fruit, and some alcoholic beverages) and is permitted for use as an excipient in pharmaceutics but it was never used in food in the US.

Experimental Procedures

Experimental Overview

The proposed laboratory work is intended to provide students with tools to design a safe and environmental benign extraction procedure at reduced cost. The experiments require two class periods: in the first period the students characterize an ABS (binodal curve) and in the second part they assess its potential as an extraction platform. The solubility (binodal) curve is determined and analyzed, the monophasic and biphasic regions are identified and the formation and the meaning of an ABS biphasic region is discussed. To build a phase diagram, the orthogonal representation using concentrations is to be preferred, instead of the conventional triangular phase diagram, due to its simplicity. In the second period, the students ascertain the potential of the ABS in the partition of food colorants as model substances. ABS were shown to be efficient extractive platforms for food additives [15–17]. The color of the

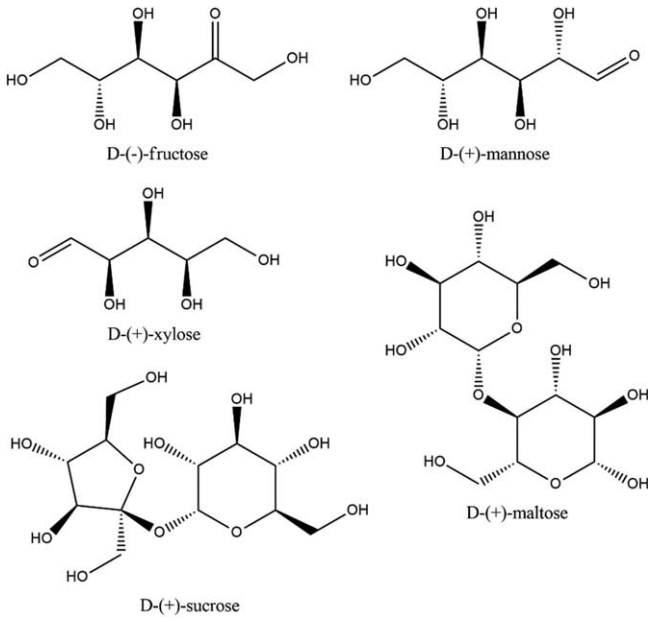

FIG 2 *Chemical structures of the carbohydrates studied in this work.*

target molecules facilitates the visualization of its migration towards each phase and helps to understand the partition of a solute between the two phases [14]. Moreover, the quantification of the dyes is easy to perform and interpret using UV–Vis spectroscopy; the calibration curves may be determined by the students or by the instructor (according to the duration of the lab class).

In the first class, the students determine the solubility curves of PPG 400 + carbohydrates (Experiment 1 in Supporting Information). An initial 30 min lecture is recommended to explain the rationale and the experimental details of ABS formation. The students are then organized into five (or more) pair-groups and a different carbohydrate is assigned to each group: D-(−)-fructose; D-(+)-mannose; D-(+)-xylose; D-(+)-maltose, and D-(+)-sucrose. Then, the experimental determination of the solubility curve (taking 120 min) will follow. Mass data should be registered and treated using a spread sheet.

The students use the information from the solubility (binodal) curve representation previously obtained to establish the ABS composition to be adopted for the partition of the dyes. A fixed composition is recommended for all systems, regardless the variability among the different solubility curves, so that the results for the different carbohydrates may be compared (*e.g.*, 40 wt % of PPG, 20 wt % of carbohydrate, and 40 wt % of food additive solution). In the next class, the students study the partitioning of the two dyes (during ∼120 min)—Experiment 2 in Supporting Information. The calibration curves needed to compute the concentration of the dyes in each phase may be experimentally

determined by the students or given by the instructor, according to the duration of the class and/or desired pedagogic goals. The complete set of final results should be made available to all students so that they can analyze and compare them.

Prelab

Students must learn how to use the spectrophotometer UV–Vis, the analytical balance and the micropipets. Knowledge on how to prepare and use a calibration curve is needed.

Materials

The carbohydrates used were: D-(−)-fructose (98.0 wt % pure from Panreac), D-(+)-mannose (99.5 wt % pure from Sigma-Aldrich), D-(+)-xylose (≥99.0 wt % pure from Sigma-Aldrich), D-(+)-maltose (≥98.0 wt % pure from Fisher Bioreagents), and D-(+)-sucrose (99.5 wt % pure from HiMedia). The polypropylene glycol with a molecular weight of 400 g mol^{-1} (PPG 400) was bought highly pure from Sigma-Aldrich. The chemical structures of the carbohydrates used are shown in Fig. 2. The food additives: azorubine (E122) and brilliant blue FCF (E133) were purchased from Vahiné with a purity of >98.0 wt %. Distilled and deionized water was used in all experiments. For more details on the equipment and materials used please check Supporting Information file.

Design of Binodal Curves of PPG 400 + Carbohydrates + H_2O

The ternary phase diagrams were determined at 298 (±1) K and at atmospheric pressure by the cloud point titration method. Stock solutions 40–60 wt % of the carbohydrates, depending on the carbohydrate solubility saturation in water (Supporting Information), are previously prepared to use in the determination of the phase diagrams. Repetitive drop-wise addition of the carbohydrate solution to pure PPG 400 was carried out until the detection of a cloudy solution, followed by the drop-wise addition of deionized water until the detection of a monophasic region (clear and limpid solution). In case of any doubts, the teacher should do one assay to demonstrate the appearance of turbidity. All these additions are carried out under continuous stirring, in a 20 mL glass vial or test tube. This procedure should be explained in detail and strictly followed by the class as defined in Supporting Information–Experiment 1.

Partition of Food Additives Through Polymer-Carbohydrate Based ABS

A calibration curve should be prepared for each food additive with concentration ranges of 0.2–1.5 mg mL^{-1} (E122) and 0.5–4.0 mg mL^{-1} (E133); the absorbance of the aqueous solutions being measured at 524 nm (E122) and 633 nm (E133).

The partition systems were prepared in Eppendorf tubes by weighing the appropriate amounts of carbohydrate (PPG 400) and the aqueous solution containing food additives (E122 and E133), in order to obtain a mixture point of fixed composition for all systems (40 wt % of PPG 400, 20 wt % of carbohydrate, and 40 wt % of food colorant aqueous solution). The teacher should ascertain that the students understand that the mixture point used was chosen so as to be located in the biphasic region for all systems. The detailed procedure is described in Supporting Information–Experiment 2.

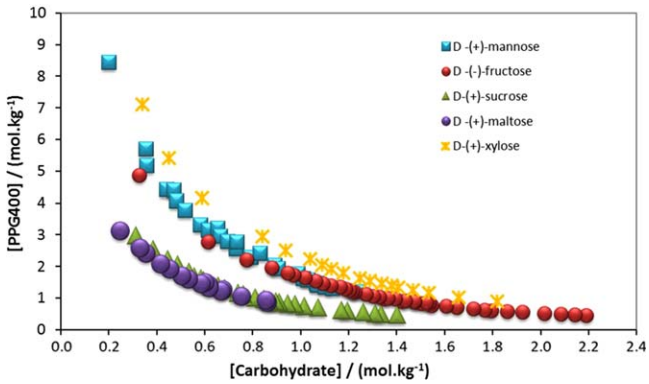

FIG 3

Binodal curves for the ternary systems composed of PPG 400 + carbohydrate + H_2O at 298 K: (●) D-(-)-fructose; (■) D-(+)-mannose; (●) D-(+)-maltose; (▲) D-(+)-sucrose and (✳) D-(+)-xylose. [Color figure can be viewed at wileyonlinelibrary.com]

When the thermodynamic equilibrium is attained the formation of two phases is visually detected, and the separation of top and bottom-phases may be accomplished. Using a Pasteur pipette, the top phase is carefully and quantitatively transferred to a microvial and the respective mass is measured. Next, the concentration of food color additive is quantified through UV-spectroscopy, using the calibration curves previously established for each food colorant. The procedure is repeated for the bottom phase. This protocol should be strictly followed by the class.

Hazards

PPG 400 is not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008 nor classified as dangerous according to Directive 67/548/EEC. Prolonged contact with PPG is essentially nonirritating to the skin. Undiluted PPG is minimally irritating to the eye and can produce slight transient conjunctivitis (the eye recovers after the exposure is removed). Carbohydrates and food additives are benign since these can enter the food supply. Students should wear laboratory coats, gloves, and safety goggles during this experiment in order to minimize possible safety risks.

Discussion

Development and Analysis of the Phase Diagrams

The binodal curves corresponding to the systems investigated in this work, combining PPG 400 and each of the five carbohydrates, are presented in Fig. 3.

TABLE I *Extraction parameters (EE%, K, and S) regarding the partition of both E122 and E133 in different polymer-carbohydrate-based ABS*

	E122		E133		
Carbohydrate	*EE%*	$K_{(E122)}$	*EE%*	$K_{(E133)}$	S
D-(+)-mannose	76.98 ± 4.18	2.68 ± 0.94	104.72 ± 4.43	>100	37.29
D-(–)-fructose	72.96 ± 2.20	2.31 ± 0.17	112.58 ± 3.65	>100	43.35
D-(+)-maltose	74.23 ± 0.60	2.43 ± 0.66	104.61 ± 1.75	>100	41.16
D-(+)-sucrose	82.72 ± 0.67	3.34 ± 0.29	100.73 ± 3.74	>100	29.98
D-(+)-xylose	70.02 ± 4.81	1.84 ± 0.48	103.35 ± 1.25	>100	54.31

It should be mentioned to the students that the closer the curve is to the axes, the less amount of solute is required to promote the separation of the two aqueous phases, the better the solute acts as a two-phase promoter. All binodal curves are represented in molality units to avoid disparities in the evaluation of the carbohydrate potential in inducing the liquid–liquid demixing that could result from their distinct molecular weights.

The experimental ternary phase diagrams should also be determined by students in mass units (Fig. S4 and Tables S1–S2 in Supporting Information). These curves indicate that the carbohydrates capability to induce ABS follows the decreasing order: D-(+)-maltose ≈ D-(+)-sucrose ≫ D-(−)-fructose > D-(+)-mannose > D-(+)-xylose. Maltose and sucrose are both disaccharides and have higher molar mass and more —OH groups than fructose or mannose, therefore these interact more extensively with water and show greater capacity to form ABS. Moreover, since both are disaccharides, similar biphasic regions are obtained. In terms of monosaccharides, the gathered data suggests that hexoses [D-(–)-fructose and D-(+)-mannose] are more effective in promoting the formation of two-phase systems than pentoses [D-(+)-xylose]. The higher number of hydroxyl groups per molecule results in a higher number of potential hydrogen bonds with water turning them into stronger "salting-out" agents, *i.e.*, agents with higher ability to form hydration complexes with water. Decreasing the number of water molecules available to interact with the other phase forming compound, thus "expels" the polymer to a distinct phase more easily. This tendency is actually in agreement with previous studies, which used ionic liquid-based ABS formed with carbohydrates [11]. Students should be advised to search into literature and to find other examples of phase diagrams where these tendencies are found.

Development and Analysis of the Food Colorants Partition

The partition of the food colorants was performed using a fixed composition for each studied ABS (40 wt % of PPG 400 + 20 wt % of carbohydrate + 40 wt % of food colorants aqueous solution). The results of partition coefficient [K – Eq. (1) in Supporting Information] and extraction efficiency [EE% – Eq. (2) in Supporting Information) for the partitioning of each dye are presented in Table I and equally illustrated in Fig. 4. Students should be guided to prepare a similar table and figure after their experiments.

Analyzing the partitioning phenomena, it was found that Brilliant Blue FCF (E133) almost completely partitions towards the bottom carbohydrate-rich phase. The concentration of this dye in the PPG-rich top phase is below the detection limit (Table I, Fig. 4), therefore, no equilibrium constants are provided, and by convention, the $K_{(E133)}$ is presented as >100. This particularity should be explained in detail to students and they should define the results for this specific food colorant following the same convention here described. Regarding the E122, it also partitions predominantly to the carbohydrate-rich phase but a significant amount is still present in the polymer-rich phase. The partition constants of this dye do not vary much with the carbohydrate; from $K_{(E122)} = 1.8$ when D-(+)-xylose was used and $K_{(E122)} = 3.3$ for sucrose, the correspondent extraction

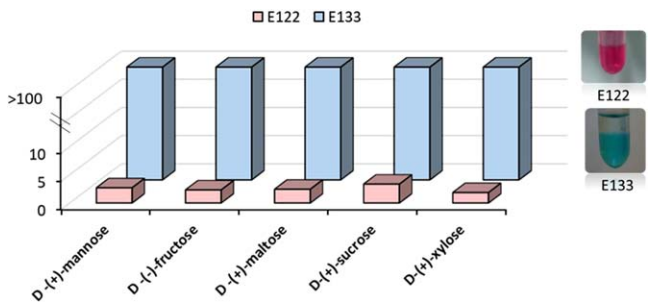

FIG 4 *Partition coefficients (K) for both food additives - E122 and E133-in the ABS composed of 40 wt % of PPG 400 + 20 wt % of carbohydrate + 40 wt % of food colorant aqueous solution. [Color figure can be viewed at wileyonlinelibrary.com]*

TABLE II *Summary of the performance of the students (%) by the end of the lab practical classes*

Skills and outcomes	*Able (%)*	*Not able (%)*
Experiment 1		
a. The student was able to experimentally determine the binodal curve.	88%	12%
b. The student understood the concept of binodal curve.	62%	38%
c. The student correctly identified the mono- and biphasic region of the phase diagram.	100%	0%
d. The student could draw the chemical structure of the carbohydrates in study.	94%	6%
e. The student understood the effect of the carbohydrate chemical structure on the ability to form ABS.	56%	44%
Experiment 2		
a. The student was able to identify the components/solutes of top and bottom aqueous-rich phases.	81%	19%
b. The student was able to correctly use a calibration curve.	75%	25%
c. The student correctly determined the extraction parameters (K, %EE, and S) and recognized their meaning.	81%	19%
d. The student correctly identified the partitioning profile of each food dye.	62%	38%
e. The student was able to predict the partition behavior of each dye in other complex matrixes.	44%	56%

efficiencies being 70% for D-(+)-xylose and 83% for D-(+)-sucrose. The ability to promote phase separation seems to be related to the partition results. The extended results are depicted in Table S3, Supporting Information, in which the concentrations of top and bottom phases and the mass balance values [Eq. (4) in Supporting Information] are available. These parameters should also be calculated and presented by the students for further discussion helping to point out the conclusions previously described.

This experiment allows students to calculate the selectivity value of the different ABS. Selectivity was estimated as the ratio between the partition constants of the two dyes, $S = K_{(E133)}/K_{(E122)}$ [Eq. (3) in Supporting Information], and the results are presented in Table I. In this particular case, since K values of E133 > 100, they were set equal to 100 for the sake of selectivity values calculation. Thus, the students were able to compute experimental S values for every system. From these S results, it may be concluded that the E133 manifests stronger preference for the carbohydrate rich-phase, the highest selectivity being provided by the D-(+)-xylose-based ABS ($S = 54.31$).

Student Learning

The experiment has been completed by 16 students in the 1st semester of the 3rd year of Chemical Engineering and Biotechnology degrees. They performed the calculations and presented their results using a spreadsheet. Additionally, they answered a short questionnaire. The global performance of the students is summarized in Table II. It may be observed that 88% of the students were able to correctly determine the binodal curves for one of the systems D-(–)-fructose and D-(+)-sucrose + PPG 400, in spite of having felt some practical difficulties in the determination of the first cloud point in Experiment 1. This practical detail is perhaps the most critical experimental step in the elaboration of the solubility curve, so instructors are recommended to give particular advice and support here. Another practical challenge for the students was the careful separation of top and bottom phases in Experiment 2, an operation that may be greatly responsible for the deviations in the results. Yet, in terms of extraction efficiencies and partition coefficients for the partitioning of both food dyes, the results were acceptable. The average values obtained for EE% E122, EE% E133, $K_{(E122)}$, and $K_{(E133)}$, considering both D-(–)-

fructose and D-(+)-sucrose + PPG 400 ABS, were 76.92%, 115.81%, 2.03 and 5.20, respectively (RSDs between 1.68 and 12.49% for extraction efficiencies and from 0.32 to 3.58 for partition coefficients). These results agree reasonably well with the authors' ones, which demonstrates that the proposed activities are feasible and adequate for the students' level. As data presented in Table II shows, the performance of the students when lower order learning skills were addressed (1a, 1c,1d, and 2a and 2c) was very good, but the performance level decreases when higher order learning skills (requiring analysis and evaluation) are at stake, as items 1e and 2d and 2e demonstrate. As expected, the students did not all entirely master the scientific reasoning that explains the observed distribution pattern, although half of them could explain and predict the partition behavior, which is quite satisfactory. Globally, the results show that this set of lab experiments is most successful in enabling the students to construct binodal curves and use them for the purification of target molecules, which are very positive outcomes.

Conclusion

ABS are excellent alternative techniques to promote the partition/separation and purification of different (bio)molecules, from the simplest (amino acids or phenolic compounds) to the most complex, namely proteins and enzymes. Actually, ABS are claimed as promising extractive platforms, particularly for biomolecules, due to their aqueous and mild nature (pH and temperature), reduced environmental impact and processing costs.

In the proposed lab work, the students prepared and used benign purification platforms of ABS that were also particularly well suited for the purification of biomolecules. Even if students are already familiar with liquid–liquid extraction from previous conventional experiments using volatile organic solvents, this laboratory work provides the opportunity to get acquainted with a "green" and cost-effective alternative. A particular strength of this experimental work is that it enhances both student laboratory skills (solubility curve determination and calibration curve) and the understanding of previously learned specific concepts (calibration curve, distribution equilibria, and structure-properties relationship) as well as new ones (the formation of two immiscible aqueous phases and the "salting-out" effect). The work also fosters students to grasp new concepts and apply them in the fields of biotechnology, biochemistry, and chemistry.

This two-class laboratory experimental work is proposed as an alternative to conventional LLE systems that use deleterious volatile organic solvents and are almost ubiquitous in Chemistry and Chemical Engineering curricula. This work was meant to provide a simple, low-cost, and benign LLE technique that teachers can easily adopt in their classes to introduce the concept of benign bioseparation processes. Also, it introduces novel ABS (whose inherent concepts may not be easy to grasp by lecturing alone) using a hands-on approach. Along with doing the experiments and building the solubility curves, the students become more engaged and at ease with both the fundamental and the practical concepts of phase diagrams. In particular, they will understand how to choose a point in the biphasic region to implement a totally benign LLE.

Another major aspect to be considered, particularly useful in downstream processing, is the "*tuning*" ability of these systems by the proper choice of the ABS components. In these experiments, the best selectivity for the food colorant molecules extraction was achieved with the ABS based in maltose.

Moreover, and since PPG and carbohydrates are two substances usually present in food additive formulations, no further polishing steps to remove the phase components are required. So, by performing these laboratory activities, the students will be able to infer about the influence of using different components, in the case, carbohydrates, as phase components in two main areas: the ability to form ABS and the extraction yield and selectivity of a target molecule. These are most useful and fundamental issues in the food industry and the downstream processing towards a more responsible and greener future.

Conflict of Interest

There are no conflicts of interests associated to this work.

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