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Recovery of crocins from saffron stigmas (*Crocus sativus*) in aqueous two-phase systems☆

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ABSTRACT

Crocins are carotenoid derivates that have recently attracted the interest of the scientific community due to their nutraceutical properties. Saffron (dry *Crocus sativus* stigmas) is one of the main known sources of crocins. In this study the potential use of aqueous two-phase system (ATPS) for the extraction of crocins from *C. sativus* stigmas was evaluated. The partitioning behavior of crocins in different types of ATPS (polymer–polymer, polymer–salt, alcohol–salt and ionic liquid–salt) was evaluated. Ethanol–potassium phosphate ATPS were selected based on their high top phase recovery yield and low cost of system constituents. The evaluation and optimization of system parameters rendered conditions (V_R = 3.2, ethanol 19.8% (w/w), potassium phosphate 16.5% (w/w), TLL of 25% (w/w), 0.1 M NaCl and 2% (w/w) of sample load) under which more than 75% of total crocins were recovered in the top (ethanol rich) phase, whereas the wasted stigmas accumulated in the bottom phase. Lastly, a comparison between an optimized solid–liquid extraction using ethanol:water as solvent and ATPS was conducted demonstrating that similar yields are achieved with both strategies (76.89 ± 1.8% and 79.27 ± 1.6%, respectively). However, ATPS rendered a higher extraction selectivity of 1.3 ± 0.04 mg of crocins for each mg of phenolic compound, whereas ethanolic extraction showed a selectivity of 0.87 ± 0.01. The results reported herein demonstrate the potential application of ATPS, particularly ethanol–potassium phosphate systems, for the recovery of crocins from *C. sativus* stigmas.

1. Introduction

Saffron is one of the most popular spices worldwide due to its flavoring and coloring properties. This spice is obtained from the dry stigmas of *Crocus sativus Linnaeus* (*Iridaceae*) which is widely cultivated in Iran, Spain, France, Italy, Switzerland and India [1,2]. Besides its applications in the food industry, saffron is regarded as a high-value medicinal plant in India and China, where it has been used for the treatment of various kinds of mental illnesses without reported negative effects [3–5].

The organoleptic properties of saffron spice are given by the presence of three carotenoid derivatives (crocins for color, picrocrocin for flavor, and safranal for aroma), mainly synthesized during flowering. These metabolites are produced by oxidative cleavage of zeaxanthin, followed by oxidative modifications and glycosilations [4,6]. The interest on the saffron carotenoid derivates, particularly crocins, is increasing due to their nutraceutical properties and wide range of application on the food, cosmetic and nutrimental supplement industry. Crocins are red-colored and water soluble carotenoids, which are a series of mono and di-glycosyl esters of crocetin (a polyene dicarboxilic acid consisting of seven conjugated double bonds and four side-chain methyl groups) [7]. *Trans*-crocin 4, digentibiosyl moiety, and *trans*-crocin 3, glucosyl and gentibiosyl moiety, have been described as the most abundant crocins in several saffron samples [8].

Different studies have demonstrated that crocins have several nutraceutical properties including antioxidant [9], hypolipidemic [10], neuroprotective [11], antidepressive [12], anticholesterolemic [13], antitumoral and anticarcinogenic [14–16] activity. With the increasing market of natural products with nutraceutical activity with emphasis in chemoprevention, the development of efficient and scalable downstream processes to obtain such products has become a priority. In the particular case of saffron, the best results for solid–liquid extraction of crocins are obtained with mixtures of ethanol:water and methanol:water [8,9,15]. Methods such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PSE), molecular imprinted polymer (MIP), liquid–liquid extraction (LLE) are emerging as novel techniques with high resolution and effectiveness for nutraceuticals extraction [17–20].

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MAE offers shorter extraction time, less solvent and in most cases higher extraction efficiencies than solid–liquid extraction. However, as in the case of solid–liquid extraction, subsequent operations for wasted solids removal are needed. Furthermore, the industrial application of this technique is still limited [21,22]. SFE represents one of the novel techniques with greater potential for selective extraction of nutraceuticals. This technique offers advantages for the recovery of carotenoids and non-polar compounds. However, the cost related to the investment, operation and maintenance of this technology has limited its application at large scale [23]. With MIP specific recognition sites are synthesized by radical polymerization with a template molecule. MIP may be loaded as sorbent for solid-phase extraction (SPE) or as stationary phase in HPLC, offering selectivity and high recovery yields [20]. Even though MIP is considered as a selective recovery step, a previous extraction stage is needed in order to extract the crocins from the biological material (solid–liquid extraction). LLE represents an attractive alternative since early processing steps can be combined into a single operation (such as extraction and primary recovery). Additionally, LLE can be easily implemented at industrial scale due to its simplicity and low cost. Moreover, LLE has demonstrated to be suitable for the extraction, recovery and purification of low molecular weight products [18,19].

In this context, the use of aqueous two-phase systems (ATPS), a type of LLE, has proved to be highly effective for the primary recovery and partial purification of biological products [24]. ATPS are formed when hydrophilic compounds, such as two polymers (polyethylene glycol, dextran, polypropylene glycol, etc.), a polymer and a salt (phosphates, sulfates, citrates, etc.), an alcohol (ethanol, propanol, isopropanol, etc.) and a salt, or an ionic liquid (IL) (1-butyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium acetate, 1-butyl-3-methylimidazolium tetrafluoroborate, etc.) and a salt are combined over certain critical concentration [24]. This combination results in the formation of two hydrophilic phases which primordial component is water. ATPS has been used for the recovery and partial purification of a wide range of bioproducts including proteins, genetic material, low-molecular weight compounds (such as secondary metabolites and phytochemicals), cells, organelles, virus and virus-like particles [23–25]. The fractionation of the compounds in ATPS is dictated by the affinity of each particular solute toward a specific phase. Affinity can be enhanced by the manipulation of ATPS parameters such as tie-line length (TLL; which is function of the constituents concentrations on each phase) and the volume ratio (V_R; defined as the ratio between the top and the bottom phase volumes), among others [25]. Some of the advantages of ATPS are: (a) biocompatibility, (b) low costs, (c) ease of scaling-up, (d) specificity, and (e) process integration capability [24–26]. Ionic liquids-ATPS have recently emerged as a possible strategy for recovery and purification of biomolecules. However, some ionic liquids present high toxicity and cost. Therefore, an adequate selection of the ionic liquid to be use for ATPS formation is necessary in order to avoid problems related to economic, biocompatibility and toxicity constrains [27–29]. The use of ATPS for the recovery and partial purification of crocins from *C. sativus* represents an interesting case of study. Crocins are typically extracted by solid–liquid methods (organic solvent extraction) and to our knowledge, no studies addressing the potential use of ATPS for the fractionation and recovery of crocins from saffron stigmas have been previously reported.

The objective of this study was to characterize the partition behavior of pure crocins on different types of ATPS (polymer–polymer, polymer–salt, alcohol–salt and ionic liquid–salt), where the effect of system parameters was investigated in route to process development. Additionally, system and process parameters were investigated to optimize the recovery of crocins from *C. sativus* stigmas on ethanol–potassium phosphate ATPS. Finally, recovery, process capability and selectivity achieved in ATPS were compared with traditional solid–liquid optimized extraction.

2. Materials and methods

2.1. Biological material

Commercial saffron (*C. sativus*) dried stigmas (Roland, USA) were obtained from a local market. Stigmas were stored in the dark and dry conditions at 4 °C until used.

2.2. Chemicals and standards

Polyethylene glycol (PEG) of nominal molecular mass of 400, 1000, 3350, 4000, 8000 and 10,000 g mol^{-1}, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM] [BF_4]), 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]), dextran (DEX) from *Leuconostoc spp.* of nominal molecular mass of 110,000 g mol^{-1} and Folin–Ciocalteu reagent 2 N (FCR) were purchased from Sigma Aldrich (St. Louis, MO, USA). A standard of crocins (constituted mainly by *trans* crocin 4, *trans* crocin 3, and two unidentified peaks at 440 nm) [2,8] was purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol (96%), sodium phosphate, sodium carbonate, dipotassium hydrogen orthophosphate and potassium dihydrogen orthophosphate were purchased from DEQ (Mexico). All chemicals were of analytical grade, and all the solutions were prepared using bi-distilled water. A stock solution of crocins (1 mg ml^{-1}) in water was prepared and stored at 4 °C under dark conditions until used. PEG (50%, w/w of PEG 1000, 3350, 4000, 8000 or 10,000 g mol^{-1}) and DEX (30%, w/w) stock solutions were prepared to be used on ATPS formation. Since PEG 400 g mol^{-1} is liquid at room temperature it was used at 100% (w/w). Potassium phosphate solution (40%, w/w) was prepared in a ratio 18:7 dipotassium hydrogen orthophosphate/potassium di-hydrogen orthophosphate. Sodium phosphate (NaH_2PO_4) solution was prepared (40%, w/w). Adjustment of pH was made by addition of orthophosphoric acid, potassium phosphate or sodium hydroxide as needed, after the pH adjustment the volume was completed using a volumetric flask.

2.3. Aqueous two-phase experiments

2.3.1. Influence of type of aqueous two-phase system and system parameters on crocins partition behavior

Selected PEG–potassium phosphate, PEG–DEX, ionic liquids (IL)–potassium/sodium phosphate and ethanol–potassium phosphate ATPS (29 systems in total, identified in Table 1) were constructed in order to determine the effect of system parameters on the fractionation of pure crocins, as well as define the most adequate system type for crocins recovery. ATPS were prepared for convenience on a fixed mass basis using a top-loading balance. Crocins and components of the systems were weighted in a Mettler-Toledo X564 analytical balance (Mettler-Toledo International, USA). Predetermined quantities of stock solutions were mixed with bi-distilled water and 0.2 g of crocins standard solution (1 mg ml^{-1}) to give a final total weight of 2.0 g. The stock solutions were mixed and phases dispersed by mixing for 10 min at 25 °C. Completely phase separation was achieved by low speed centrifugation at 3000 × *g* for 10 min at 25 °C using a Galaxy 10 centrifuge (VWR International, USA). Visual estimation of the volume of the top and bottom phase was done in graduated tubes. Phase volumes were used to estimate the volume ratio (V_R = volume of the top phase/volume of the bottom phase). Samples were carefully pipetted off and collected on clean tubes for further analysis.

Table 1
Systems selected for the evaluation of the partition behavior of crocins in ATPS.

System	PEG MW ($g\,mol^{-1}$)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Volume ratio (V_R)	Top phase recovery Y_{TOP} (%)
Polymer–salt (polyethylene glycol–potassium phosphate)						
1	400	14.0	18.0	25	1.05 ± 0.01	72.41 ± 0.51d
2		17.0	20.0	45	1.03 ± 0.02	90.79 ± 0.69c
3	1000	13.0	15.0	25	0.90 ± 0.01	89.65 ± 1.55c
4		19.2	17.1	45	1.11 ± 0.01	99.78 ± 1.33a
5	3350	11.9	11.8	25	0.92 ± 0.02	94.96 ± 1.39abc
6		18.8	15.0	45	1.00 ± 0.01	97.24 ± 0.86ab
7	8000	13.0	10.4	25	1.00 ± 0.01	93.00 ± 1.53bc
8		20.2	14.8	45	1.06 ± 0.03	92.62 ± 1.14bc

System	PEG MW ($g\,mol^{-1}$)	PEG (%, w/w)	Dextran (%, w/w)	TLL (%, w/w)	Volume ratio (V_R)	Top phase recovery Y_{TOP} (%)
Polymer–polymer (polyethylene glycol–dextran 110,000)						
9	4000	5.4	10.0	15	1.04 ± 0.01	47.49 ± 0.69c
10		5.4	12.1	20	0.90 ± 0.02	45.41 ± 0.32c
11		6.1	14.1	25	0.87 ± 0.01	56.30 ± 0.44b
12		7.6	15.5	30	1.00 ± 0.02	66.20 ± 1.66a
13	10,000	3.5	8.2	15	0.99 ± 0.05	38.24 ± 0.22d
14		4.0	11.1	20	0.87 ± 0.02	47.11 ± 6.95c
15		4.9	13.5	25	0.91 ± 0.02	47.27 ± 1.00c
16		6.8	15.0	30	1.00 ± 0.00	55.24 ± 0.98b

System	Ionic liquid (IL)	IL (%, w/w)	Phosphate (%, w/w)	IL in top phase, IL_{TOP} (%, w/w)	Volume ratio (V_R)	Top phase recovery Y_{TOP} (%)
Ionic liquid–salt						
17	[BMIM]-[BF_4]	20.0	12.1	NR	0.33 ± 0.0	90.30 ± 1.13a
18		32.6	21.0	65	0.64 ± 0.01	86.47 ± 2.45a
19		34.2	17.0	63	0.68 ± 0.0	89.42 ± 0.29a
20		35.2	19.4	64	0.68 ± 0.0	89.63 ± 2.54a
21	[EMIM]-[Ac]	13.0	30.8	45.5	0.93 ± 0.03	81.59 ± 0.31b
22		13.8	29.83	47.8	1.15 ± 0.02	83.89 ± 1.45b
23		15.8	30.47	59.3	1.05 ± 0.01	79.35 ± 1.49b
24		17.0	25.0	46.7	2.44 ± 0.0	94.22 ± 3.17a
25		20.0	25.0	62.3	2.12 ± 0.02	97.34 ± 1.46a
26		30.0	15.2	68.8	7.25 ± 0.0	74.02 ± 0.61c

System	EtOH (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Volume ratio (V_R)	Top phase recovery Y_{TOP} (%)
Alcohol–salt					
27[a]	14.0	23.0	25	1.03 ± 0.01	91.14 ± 0.40b
28[b]	14.5	24.7	37.5	0.92 ± 0.01	97.98 ± 1.39a
29[c]	17.0	26.1	50	1.02 ± 0.01	96.92 ± 1.26a

Systems composed with salt were prepared with potassium phosphate (pH 7.0), except for [BMIM][BF_4] which were prepared with sodium phosphate (pH 4.0). TLL and compositions of polymer–salt/polymer were estimated using the binodal curves presented by Zaslavsky [60]. TLL and compositions of [BMIM][BF4]–salt were estimated using the binodal curves presented by Li et al. [28]. Nr: not reported. The recovery of crocin from the phase is expressed relative to the initial amount of crocin in the solution loaded to the ATPS. Levels not connected by the same letter are significantly different. Statistical analysis was realized by type of ATPS.

[a] For V_R 3.2: EtOH 19.8% (w/w); PO4 16.5% (w/w).
[b] For V_R 3.2: EtOH 22.0% (w/w); PO4 16.0% (w/w).
[c] For V_R 3.2: EtOH 26.0% (w/w); PO4 16.0% (w/w).

Crocins concentration in each phase was determined by measuring the absorbance at 440 nm. A blank system (where water was used as sample) was prepared for each treatment and used as analytical blank for the corresponding phase (top or bottom). A calibration curve for crocins quantification was constructed in water (5–300 $\mu g\,ml^{-1}$) with a correlation coefficient (R^2) of 0.9997. Recovery yield at the top and bottom phase was estimated as the amount of crocins present in the phase (volume of the phase × crocins concentration) and expressed relative to the original amount loaded into the system. Partition coefficient (K_p) was calculated as the crocin concentration ratio between the phases (concentration of crocins in the top phase/concentration of crocins in the bottom phase).

2.3.2. Recovery of crocins from C. sativus in ethanol–potassium phosphate aqueous two-phase systems

Partition behavior studies showed that the use of ethanol–potassium phosphate ATPS represent an interesting option for the fractionation of crocins due to their particular polarity properties and economics. Therefore, ethanol–potassium phosphate ATPS were selected for crocins recovery on complex systems (where dried stigmas of *C. sativus* were used as sample). ATPS were prepared with a fixed mass basis. Predetermined quantities (see Table 1) of stock solutions were mixed with bi-distilled water to give a total weight of 50.0 g. Sample load was kept constant as a 2.0% (w/w) dried stigmas from *C. sativus*. The components were mixed thoroughly using a magnetic stirrer in order to allow the complete contact of all system constituents and subsequently were allowed to settle for 2 h for phase separation. Phase volumes were used to estimate V_R. Phases were carefully extracted (pipetted off) for further analysis. Total crocins concentration in commercial saffron was quantified using the average extinction coefficient for crocins of $\varepsilon_{440\,nm} = 103{,}000\,dm^3\,mol^{-1}\,cm^{-1}$ in water and a molecular mass of $977\,g\,mol^{-1}$. Recovery yield at the top and bottom phase was estimated as the amount of crocins present in the phase (volume of the phase × crocins concentration) and expressed relative to the original amount loaded into the system. An aliquot of the top phase was taken, diluted with water and

Table 2
Influence of process parameters for crocins recovery using solid–liquid extraction.

Treatment	EtOH (% v/v)	Temperature (°C)	Sample load (% w/v)	Crocins recovery (%)*
1	0.0	25	5.0	52.01 ± 1.9c
2	25.0	25	5.0	58.60 ± 4.5bc
3	50.0	25	5.0	76.89 ± 1.8a
4	75.0	25	5.0	67.97 ± 0.3abc
5	100.0	25	5.0	17.75 ± 1.5d
6	50.0	50	5.0	70.68 ± 3.4ab
7	50.0	25	2.5	75.20 ± 4.5a
8	50.0	25	10.0	68.47 ± 2.6ab

Extractions were made as describe in Section 2. Levels not connected by the same letter are significantly different. Statistical analysis was made using JMP software version 5.0.
* Crocins recovery is expressed relative to the total amount of crocins loaded to the ethanolic treatment.

filtrated using a 0.45 μm syringe filter for crocins quantification. Samples were protected from light and stored at −20 °C until further analysis.

2.3.3. Determination of ionic liquid [EMIM][Ac] in top phase

Since no binodal curve was found on literature for [EMIM][Ac] ATPS such systems were characterized in terms of ionic liquid (IL) concentration at the top phase. The [EMIM][Ac] concentration in the top phase of the ionic liquid based ATPS was estimated by UV absorbance at 210 nm. A calibration curve for [EMIM][Ac] quantification was constructed in water (0.1–0.9%, w/w) with a correlation coefficient (R^2) of 0.999. IL-based ATPS without sample were constructed as previously described (Table 1, Systems 21–26). Samples from the top phase were carefully pipetted off and collected on clean tubes for further analysis. IL concentration estimated at 210 nm in the top phase of ATPS was established for further comparison between the systems in order to understand possible effects of this factor on crocins partitioning.

2.4. Establishment of optimal process conditions for the ethanolic extraction of crocins from C. sativus

Ethanol concentration, extraction temperature, sample load and time were varied in order to determine their effect on crocins extraction. The extraction processes were carried out in an incubator with agitation under controlled temperature at 200 rpm and protected from light for up to 60 min. Table 2 shows the factorial used for crocins extraction optimization. An aliquot of the extraction was taken, diluted with water and filtrated with a 0.45 μm syringe filter for crocins quantification. Sample was protected from light and stored at −20 °C until further analysis. The content of crocins per gram of saffron stigmas (31.48 ± 3.88 mg g^{-1} of dried saffron stigma) was determined by serial extractions of the wasted solids at optimal extraction conditions (ethanol aqueous solution at 50% (v/v), temperature 25 °C and sample load of 5%, w/v).

2.5. Analytical procedures

Crocins, system constituents and other reagents were weighted in a Mettler-Toledo X564 analytical balance (Mettler-Toledo International, USA). Absorbance measurements were made in an Epoch microplate spectrophotometer (BioTek Intruments, Inc., USA) using a flat bottom 96-well quartz microplate (Hellma, Germany).

2.5.1. Analysis of crocins by HPLC–DAD

HPLC analysis was performed in order to determine the purity achieved using ATPS compared with solid–liquid extraction. Crocins were analyzed using reversed-phase HPLC-DAD technique based on the method described by Tarantilis et al. [30] with some modifications. The HPLC system consisted on an Agilent-1200 (Palo Alto, CA) series instrument equipped with a UV–Vis photodiode array (DAD) detector, quaternary pump, vacuum degasser and an auto sampler. An Agilent Zorbax XDB-C18 Solvent Saver Plus (3.5 μm) stainless-steel column (100 mm × 3 mm I.D.) was used. The mobile phase was a linear gradient from 40% to 100% methanol in water in 17 min, followed by 5 min at 100% methanol. The solvent flow-rate was kept constant at 0.3 ml min^{-1} and the sample injection volume was 5 μl. Detection was performed simultaneously at 190–600 nm. The relative purity analysis was performed using three different wavelengths for crocins (440 nm) and the principal contaminants (250 and 308 nm) absorbance [30]. Relative purity was expressed as the relationship between the area of the major crocin peak (i.e. crocetin di-β-D-gentibiosyl ester) and the total area for each wavelength [8].

2.5.2. Total phenolic compounds analysis

A final analysis to determine extraction selectivity for crocins upon phenolics was assessed. The total phenolic concentration in the extracts was determined by using the Folin–Ciocalteu method with gallic acid as the standard [31]. Briefly, 100 μl methanolic solutions of standard (50–250 ppm) or diluted extract (10-fold in bi-distilled water) was added to 500 μl of Folin–Ciocalteu reagent 2 N (diluted 9-fold in bi-distilled water). This mixture was allowed to stand for 5 min before the addition of 400 μl of 7.5% (w/w) sodium carbonate (Na_2CO_3). After incubation for 1 h at room temperature (22–24 °C), the absorbance was measured at 760 nm in an Epoch microplate spectrophotometer (BioTek Intruments, Inc., USA) using a flat bottom 96-well microplate. All determinations were carried out in triplicate using proper blank solutions (aqueous ethanol or top phase of ATPS). The results were expressed as mg gallic acid equivalents (GAE) g^{-1} of dry stigmas (DS). Equation obtained for the standard curve was Abs = 0.0059 × GAE + 0.0019 (R^2 = 0.9988).

2.6. Statistical procedures

Results reported are the average of at least three independent experiments, and the standard error was calculated as the standard deviation divided by the square root of the number of replicas. The total crocins content was analyzed using analysis of variance (ANOVA) with JMP™ Statistical Discovery™ Software Version 5.0 (SAS Institute Inc., NC, USA). Significant differences among means ($p < 0.05$) were determined by all pairs Tukey HSD.

3. Results and discussion

3.1. Influence of type of aqueous two-phase system and system parameters on pure crocins partition behavior

The partition experiments in ATPS using pure crocins as sample revealed that these compounds exhibited a top-phase preference. The top-phase preference of the crocins resulted (for most of the

systems studied) in partition coefficients (K_p) higher than 100, particularly on polymer–salt, ionic liquid–salt and alcohol–salt systems. Due to these extreme K_p values it was decided to use the recovery of crocins (expressed relative to the initial amount of crocins loaded to the ATPS) from the top phase as the response variable to evaluate the effect of system parameters on the behavior of the product in the ATPS.

3.1.1. Partition behavior of crocins in PEG–potassium phosphate systems

PEG–potassium phosphate ATPS has been the most studied for the fractionation of biological products, particularly for proteins, virus-like particles, and genetic material (genomic or plasmidic) [24]. The partition behavior of crocins on selected PEG–potassium phosphate ATPS is shown in Table 1. As it can be seen crocins exhibit a top phase preference in PEG–potassium phosphate systems (Systems 1–8). An interaction effect between low-molecular weight polymers and increasing TLL upon crocins recovery was observed. Increasing the TLL promoted an increase in the top phase recovery of crocins from $72.41 \pm 0.51\%$ to $90.79 \pm 0.69\%$ and $89.65 \pm 1.55\%$ to $99.78 \pm 1.33\%$ for PEG 400 and 1000 g mol^{-1}, respectively. This partition behavior may be explained in terms of phase free volume and hydrophobicity [32]. As TLL increases the polymer concentration increases in the top phase, and as a consequence, hydrophobicity of such phase also increases. Since crocins are amphipathic molecules (sugar constituents gives them a polar character while its polyene core is mostly hydrophobic) an increment on top phase hydrophobicity and excluded volume seem to favor their partition toward such phase.

From all the PEG–potassium phosphate ATPS studied, Systems 4 (PEG 1000 TLL 45%, w/w) and 6 (PEG 3350 TLL 45%, w/w) exhibit the best recovery of crocins from top phase, $99.78 \pm 1.33\%$ and $97.24 \pm 0.86\%$, respectively (see Table 1). The increase in PEG molecular weight (PEGMW) and TLL has been associated with an increment on the hydrophobicity in the top phase [32]. ATPS with low PEGMW (400 g mol^{-1}) exhibited the lower crocins recovery, particularly at TLL (25%, w/w). Therefore, partition behavior of crocins can be related to hydrophobic interactions between PEG chain and polyene core. However, a further increase on PEGMW and TLL causes a slight decrease in recovery yield (Table 1, Systems 7 and 8). This may be explained in terms of increase of excluded volume on the top phase of the system. Cisneros et al. reported the partitioning behavior of lutein from *Chlorella protothecoides* in PEG–potassium phosphate systems [32]. The results showed a top phase preference, where at least 70% of the carotenoid can be recovered from this particular phase when high PEGMW were used. Crocins and lutein have a polyene as central core. These structures can favor hydrophobic interactions with the polymer chains, which can cause an affinity partitioning of the molecules to the top PEG-rich phase.

3.1.2. Partition behavior of crocins in PEG–dextran systems

In regard to the fractionation of crocins on PEG–dextran ATPS as PEGMW increases the partition behavior of crocins into the top phase system decrease. The top phase recovery yields were less than 70% for all cases, and there was a notable accumulation of the target product at the interface of the systems (Table 1, Systems 9–16). It seems that a high TLL (i.e. 30%, w/w) favored the top phase recovery of crocins independently of the PEG molecular weight used (PEG 4000 and 10,000 g mol^{-1}). ATPS constructed using PEG 4000 showed a significant increment in the top phase recovery (from $45.41 \pm 0.32\%$ to $66.2 \pm 1.66\%$) when TLL was increased from 20.0 to 30.0% (w/w).

For polymer-based ATPS it has been reported that low molecular weight compounds usually partition in an even way between the phases [33,34]. Nevertheless, this behavior can be affected by an interaction between the target molecule and the phase forming compound [35]. In this particular case, crocins partitioning exhibits a top phase preference when a high TLL is applied. This behavior has been observed with some hydrophobic low molar mass antibiotics (vancomycin and pristinamycins) [34,36], and it was postulated that the hydrophobic interaction between the molecule and the ethylene group of PEG can be responsible for the compound partitioning to the PEG-rich phase [36]. As TLL increases, PEG concentration in the top phase and dextran concentration in the bottom phase increase, and as a consequence, the hydrophobic interactions may be favored [37].

3.1.3. Partition behavior of crocins in ionic liquid–salt systems

Ionic liquids (ILs) have attracted the interest on the bioprocess research area due to their versatility. For this study two ionic liquids with different hydrophobicity ([EMIM][Ac] < [BMIM][BF_4]) were used to analyze the partition behavior of crocins on IL–salt ATPS [29]. [BMIM][BF_4] is one of the most used ionic liquids for the extraction of biological compounds. For the systems constructed using [BMIM][BF_4] (Table 1, Systems 17–20) crocins exhibited a top phase preference.

[EMIM][Ac] has been proposed as a safer ionic liquid due to the absence of a halogen anion [38,39]. However, [EMIM][Ac]–potassium phosphate ATPS have not been characterized in terms of TLL. Therefore, the partition behavior on these systems was analyzed in terms of the top phase concentration of the ionic liquid (determined by absorbance at 210 nm) instead of TLL. Crocins exhibited, in [EMIM][Ac] and [BMIM][BF_4] systems, an evident top phase preference (Table 1, Systems 21–26), where at least 70% of the crocins were recovered at such phase. Ionic liquid concentration in the top phase (45.5–59.3%, w/w) showed no effect in the partition behavior of crocins (Table 1, Systems 21–23).

From Table 1 it can be seen that using a V_R higher than 1.0 affected the crocins partition. Systems with similar IL concentration in top phase (i.e. ~46%, w/w) showed that an increase in the V_R could enhanced crocins partition into the [EMIM][Ac]-rich phase (Table 1, Systems 21 and 24). Hydrophobic and electrostatic interactions have been proposed as the principal factors in the partitioning behavior of biomolecules in IL-ATPS [33,40,41]. Systems 25 and 26 showed a peculiar behavior, although a similar top phase concentration of ionic liquid was determined for both systems (~65%, w/w). System 26 with the higher V_R showed a decrease in crocins recovery at the top phase. Such behavior can be related with the migration of inorganic salts upon the top phase reducing the available water molecules for crocins solubilization.

Ionic liquids in the presence of water are dissociated in ions. Therefore, electrostatic interactions between the IL-cation and negative charge molecules or IL-anion and positive charge molecules have been proposed as the main force that drives molecules partitioning [33,42]. However, crocins have not electrochemical charge due to the lack of ionizable functional groups within the working pH range. Consequently, a second driving force must be involved on crocins partition behavior. Keith et al. [43] reported that an increase in the concentration of IL is associated with a higher hydrophobicity in the top phase of the system. Hydrophobic interactions have been associated with the alkyl chain length of the cation [40]. This driving force may be associated with the partitioning behavior of crocins toward the top IL-rich phase.

3.1.4. Partition behavior of crocins in ethanol–potassium phosphate systems

Polymer–polymer/salt ATPS have been well documented for the extraction of several biomolecules. Ionic liquids ATPS has recently emerged as a "green" option for the recovery and extraction of several biological compounds [29]. However, the main drawbacks of these kinds of systems are the difficulty of recycling the

components, the high cost of some phase forming compounds and the efficiency of separating high molecular weight system constituents (particularly polymers) from low molecular weight products [44]. The characterization of alcohol–salts ATPS have been described in literature since the early 90s [45]. However, the number of studies focusing on their potential application for the fractionation, recovery and purification of biological compounds is limited [44,46,47]. The advantages of these ATPS include low viscosity, high polarity, ease of ethanol recovery, lower cost system constituents and lower environmental impact [48]. Characterization of alcohol–salt based ATPS has been limited due to the low compatibility with macromolecules such as proteins and enzymes that can be inactivated or denatured in the organic solvent phase [49]. Due to the particular physicochemical properties of crocins (i.e. low molecular mass, stability and efficient extraction in organic media), the application of alcohol–salt ATPS for their extraction and recovery represents an interesting alternative to present extraction strategies.

In the case of ethanol–potassium phosphate ATPS crocins partition behavior is clearly favored toward the top (alcohol–rich) phase of the systems (Table 1, Systems 27–29). An increase in TLL (from 25%, w/w to 37.5%, w/w) increased the top phase preference of crocins. Wang et al. [47] reported the effect of system parameters in alcohol–salt ATPS on the partition behavior of tetracycline hydrochloride. The authors concluded that the concentrations of salt and alcohol produced a significant effect on the solubility of target molecules, since the "salting-out" effect caused by the high salt concentration can lead to an exclusion of low molecular weight compounds. This exclusion may favor partitioning toward the top phase for molecules soluble in alcohol. System 29 (TLL 50.0%, w/w) shows no effect upon recovery of crocins in the top phase when compared with System 28 (TLL 37.5%, w/w). This behavior may be related with the little effect of TLL upon salt concentration in the bottom phase for these particular systems (i.e. Systems 28 and 29).

Based on the results presented herein it can be concluded that PEG–potassium phosphate, IL–phosphate and ethanol–potassium phosphate ATPS represent a viable option for crocins recovery. PEG–phosphate ATPS rendered the highest recovery yields. However an ultrafiltration process is needed to separate the target molecule (i.e. low molecular weight compound) from the high molecular weight top phase constituent. This process may represent some technical disadvantages since PEG may accumulate in the membrane surface forming a gel layer reducing flux considerably at a fixed operation pressure [44,50]. In the other hand, alcohol–salt systems offer several advantages over traditional ATPS, such as low cost of forming compounds, easy separation of target molecule from system constituents and ease of ethanol recovery for further extractions. A rough economic comparison, in which the cost of ATPS constituents were taken into account, was realized between PEG 1000-phosphate and ethanol–phosphate ATPS for crocins recovery. It was estimated that traditional polymer–salt ATPS where approximately 2.5 times more expensive than alcohol–salt systems on a crocins mass recovered basis. Therefore, ethanol–potassium phosphate ATPS were selected for consecutive experimental stages with complex systems (i.e. dry stigmas of *C. sativus*).

3.2. Recovery of crocins from C. sativus stigmas in ethanol–potassium phosphate aqueous two-phase systems

Purified crocins exhibit an evident top phase preference in ethanol–phosphate systems. However these systems did not take into account the influence of contaminants from saffron stigmas upon the partition behavior of crocins on ATPS. In this section the extraction of crocins from stigmas of *C. sativus* was optimized by the manipulation of system parameters, such as TLL and V_R, and process parameters (such as sample load and addition of neutral salts) to maximize crocins recovery on the top (ethanol-rich) phase.

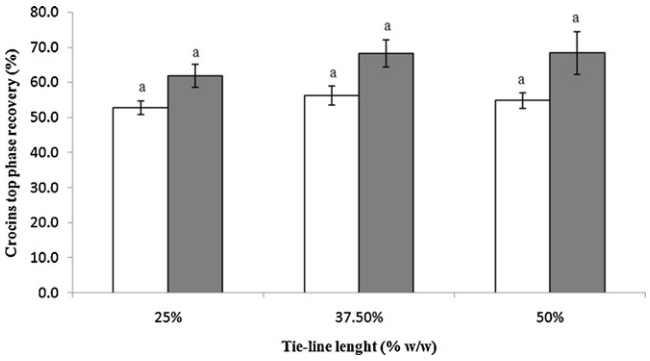

Fig. 1. Influence of volume ratio and tie-line length on crocins top phase recovery in ethanol–phosphate aqueous two phase systems. The recovery of crocins from the top phase (Y_{TOP}) in V_R 1.0 (□) and V_R 3.2 (■) ATPS is expressed relative to the total amount of crocins loaded to the ATPS. For all systems, pH and saffron loaded to the system were kept constant at 7.0 and 2% (w/w), respectively. Treatments not connected by the same letter are significantly different. The ATPS were constructed as described in Section 2. Results reported are the average of at least three independent experiments.

Initially, the effect of increasing TLL upon crocins partition behavior was evaluated with a sample load of dry stigmas of 2%, w/w. Changes in the TLL affect the free volume available for a solute, and as a consequence the partition behavior of the compound is affected [32]. The impact of increasing TLL upon crocins recovery is illustrated in Fig. 1. The results showed that increasing the TLL has little or no effect upon the recovery of crocins into the top ethanol rich phase. Independently from the TLL used (from 25% to 50%, w/w) ethanol–potassium phosphate ATPS with $V_R = 1.0$ recovered at least 50% of the crocins present in the saffron stigmas ($31.48 \pm 3.88\,mg\,g^{-1}$ of saffron) on the top phase while wasted solids accumulate in the interface. A decrease in ATPS performance for the recovery of crocins is observed when compared with model systems (pure crocins; Table 1, Systems 27–29). This behavior may be related to a saturation effect of the top phase, as a result of crocins and contaminants (not present in crocins standard) fractionation into such phase. It was determined that the standard had three times more crocins per gram than the crude extract. However, the mass of contaminants in crude extract is much higher than that the crocins standard, which may support a possible saturation effect of the top-phase.

The effect of changing V_R (from 1.0 to 3.2) upon the recovery of crocins from stigmas of *C. sativus* was evaluated in selected systems (Systems 27–29 in Table 1). It has been reported that an increment in V_R usually causes an increment in recovery for low molecular weight products on the top phase. This behavior has been associated with the relative affinity of the product and contaminants to the top phase and the available free volume [25]. Fig. 1 compares the top phase recovery yields of crocins after V_R modification from 1.0 to 3.2 (from blank systems). As the V_R of the system increases, the free volume available in the top phase for the partition of solutes also increases. As a result the recovery yield achieved also increases. For each TLL the same effect was observed. In the particular case of TLL 50% (w/w) (Table 1, System 29) an apparent increase in the top phase recovery of crocins was observed from 54.80 ± 2.2 to $68.31 \pm 6.1\%$. Statistical analysis indicates that no significance difference is observed when the V_R of the ATPS is increased ($p = 0.11$). However, ATPS with high V_R (3.2) offer an important process advantage for crocins recovery since while crocins partition selectively toward the top phase, wasted biomass (stigmas) partitions to the bottom phase facilitating its separation

Table 3
Effect of NaCl concentration (M) upon top phase recovery yield of crocins in ethanol–phosphate, TLL 25% (w/w) system.

System	EtOH (%, w/w)	Phosphate (%, w/w)	NaCl (M)	V_R	Top phase recovery, Y_{TOP} (%)
27	19.8	16.5	0	3.29 ± 0.1	61.83 ± 3.3b
			0.1	3.05 ± 0.1	79.27 ± 1.6a
			0.3	2.79 ± 0.1	69.58 ± 4.1ab
			0.5	2.75 ± 0.2	73.30 ± 5.3ab

Sample load was kept at 2% (w/w). Levels not connected by the same letter are significantly different. Statistical analysis was made using JMP software version 5.0.

and preventing contamination problems when the top phase is removed for further processing. System 28 (comprising TLL 37.5% (w/w) and V_R = 3.2) exhibited the best recovery of crocins from the top phase (68.18 ± 3.9%) in a single extraction. However, System 27 (comprising a TLL 25% (w/w) and a V_R = 3.2) offers the advantage of a high top phase recovery of crocins (61.83 ± 3.3%) using less phase constituents, particularly ethanol. Since no statistical significant difference was observed between the recovery obtained with System 27 and 28 (p = 0.55, when different TLLs are compared with V_R = 3.2), System 27 was selected as the most favorable to maximize the partitioning of the product of interest and biomass to opposite phases.

In an attempt to increase crocins recovery at the top phase of ethanol–potassium phosphate ATPS, the effect of addition of a neutral salt (NaCl) was studied. For polymer–salt ATPS it has been reported that as the concentration of NaCl increases, hydrophobic interactions between low molecular weight compounds and the polymer chains are enhanced [36,51]. It has also been reported that the addition of neutral salt in typical ATPS (i.e. polymer–polymer or polymer–salt) often produces an electrical potential difference between the two phases caused by the unequal distribution of the ions; therefore the partitioning behavior of charged molecules is strongly affected by this electrostatic potential difference [52,53]. As far as the authors know, the effect of neutral salt addition (i.e. NaCl) into ATPS conformed by ethanol–potassium phosphate has not been studied.

The effect of NaCl concentration upon crocins recovery in System 27 (comprising a TLL 25% (w/w), V_R = 3.2 and sample load of 2%, w/w) is shown in Table 3. The increase of NaCl concentration from 0 to 0.5 M is associated with a decrease in the V_R from 3.29 to 2.75. This behavior may be associated with the unequal partitioning of NaCl between the phases [51]. In this particular case, NaCl migrates preferentially to the bottom phase (Table 3). The use of a NaCl concentration of 0.1 M favored the fractionation of crocins toward the top phase, increasing the recovery from 61.83 ± 3.3 to 79.27 ± 1.6%. This behavior can be related to the salting out effect. Wang et al. [47] reported that an increment of salt concentration in alcohol–salt ATPS promotes the exclusion of hydrophobic low molecular weight compounds (i.e. tetracycline) into the top alcohol-rich phase. It has also been reported that salt-induced phase separation of water-miscible organic solvents in aqueous solution, these systems has been used successfully for the recovery of polar analytes into the organic phase (i.e. acetone, methanol, ethanol, and acetonitrile) [54,55]. In this last case, the addition of NaCl may be related with an increase of ethanol at the top phase (due to the salting-out effect), increasing crocins solubility at such phase. Further increase in NaCl concentration (0.3 and 0.5 M) did not result in an increment in the concentration of crocins in the top phase when compared with a system without neutral salt. Addition of NaCl had shown to change phase composition in PEG–potassium phosphate systems [56], and to decrease the free volume change in the top phase [53,57]. Therefore, the marginal effect of NaCl upon crocins partitioning may be attributed to the decrement in the free volume in the top phase, which may lead to a saturation effect. Based on these results the ethanol–potassium phosphate ATPS comprising TLL 25% (w/w), V_R 3.2 and 0.1 M NaCl was selected as the most favorable for crocins recovery at the top phase, while the wasted solids accumulated in the bottom phase.

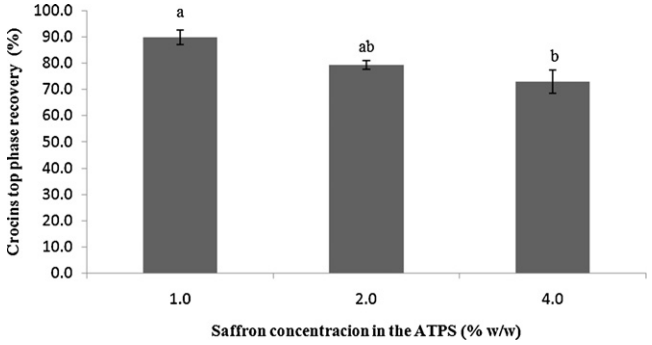

Fig. 2. Effect of sample load (%, w/w) on crocins top phase recovery yield in ethanol–phosphate aqueous two phase system. The recovery of crocins from the top phase (Y_{TOP}) in ATPS is expressed relative to the total amount of crocins loaded to the ATPS. For all systems, pH was kept constant at 7.0. ATPS System 27 was constructed as described in Section 2 using a V_R 3.2 with 0.1 M NaCl. Results reported are the average of at least three independent experiments.

Finally, in order to assess process robustness, the effect of sample loading (dry *C. sativus* stigmas, 1.0, 2.0 and 4.0%, w/w) on the performance of ATPS was evaluated. The ATPS selected on the previous experimental stage, System 27 (V_R = 3.2) with 0.1 M NaCl, was used to carry out the sample loading effect studies. Fig. 2 illustrates the effect of sample loading on the recovery of crocins at the top phase of the selected system. It is clear that top phase recovery of

Table 4
Comparison of sample load for crocins recovery between ethanolic extraction and ethanol–potassium phosphate ATPS.

Extraction	Sample load (%, w/v)	Crocins recovery (%)	Process capability[c] (mg crocins g^{-1} EtOH)
Ethanolic[a]	2.5	75.20 ± 4.5ab	1.43 ± 0.04
	5.0	76.89 ± 1.8ab	2.52 ± 0.05
	10.0	68.47 ± 2.6b	4.63 ± 0.28
ATPS[b]	1.5	89.8 ± 2.6a	1.52 ± 0.09
	3.0	79.27 ± 1.6ab	3.10 ± 0.07
	6.0	72.82 ± 4.4b	5.52 ± 0.21

[a] Ethanolic extractions were made under optimal conditions (ethanol 50%, v/v, temperature 25 °C and agitation).
[b] Ethanol–phosphate ATPS extractions were made under optimal conditions (System 27, TLL 25%, V_R = 3.2 with 0.1 M NaCl).
[c] The recovery is expressed as crocins rendered per gram of ethanol needed for the extraction process. Crocins recovery is expressed relative to the total amount of crocins loaded to the treatment. Levels not connected by the same letter are significantly different. Statistical analysis was made using JMP software version 5.0.

crocins decrease as sample loading is increased. Such behavior can be explained on the basis of solute deposition at the interface due to a saturation effect of the top phase with both, crocins and contaminants. Sample load of 1.0% (w/w) exhibits a top phase crocins recovery of 89.8 ± 2.6% in a single extraction, whereas a sample load of 4.0% (w/w) rendered a total recovery of 72.82 ± 4.4%. It is clear that an attempt to intensify recovery process by increasing sample load results in a negative performance, decreasing the recovery of crocins. Sample load of 1.0% and 2.0% (w/w) may rendered high top phase recovery (89.8 ± 2.6% and 79.27 ± 1.6%, respectively), where no significantly difference was observed between the treatments. However, sample loading of 2.0% (w/w) offer potential benefits over 1.0% (w/w) since an unnecessary dilution of the sample is avoided.

From the studies of the influence of system and process parameters upon the extraction and primary recovery of crocins from ATPS, process conditions comprising V_R = 3.2, ethanol 19.8% (w/w), phosphate 16.5% (w/w), TLL of 25% (w/w) and 0.1 M NaCl were selected as optimal. Such extraction conditions resulted in a top phase recovery of 79.27 ± 1.6% using a sample load of 2.0% (w/w). The results reported here highlight the potential application of ATPS for the recovery of crocins.

3.3. Establishment of optimal process conditions for the ethanolic extraction of crocins from C. sativus

The optimal conditions for crocins extraction using typical organic solvent extraction (solid–liquid extraction) were determined as a benchmark for crocins recovery. Also, consecutive solid–liquid extraction stages under optimal conditions were employed in order to determine the amount of crocins on dry *C. sativus* stigmas. As shown in Table 2, ethanol concentration (%, v/v) demonstrated to be the main factor influencing the recovery of crocins from dry *C. sativus* stigmas. Extraction temperature was expected to be a critical factor as shown by Yang et al. for the extraction of crocins from *Gardenia jasminoides* fruits [58]. In the case of saffron stigmas, no effect was observed when temperature was increased. This behavior may be related with saturation of the extraction phase since crocins concentration between both materials is different, it was reported that *G. jasminoides* fruits have 8.47 mg g^{-1} of dried powder, and in this study it was found that saffron stigmas have a concentration of 31.48 ± 3.88 mg g^{-1} of dried saffron stigma [58]. Optimal conditions comprise the following parameters: ethanol:water ratio (1:1), temperature 25 °C, sample load of 5% (w/v) and shaking (50–200 rpm). Under such conditions a recovery yield of 76.89 ± 1.82% can be achieved on a single extraction stage.

3.4. Process comparison between solid–liquid extraction and ethanol–phosphate ATPS for crocins recovery

In order to compare processes capability and robustness between ethanolic solid–liquid extraction and ethanol–potassium phosphate ATPS sample load effect must be expressed in the same basis. Sample loading effect would be compared as the mass of saffron stigmas per volume of extraction media (%, w/v). For ATPS the extraction volume corresponds to the top (ethanol rich) phase, since it is the volume that needs to be recovered for further processing. Therefore, sample loading evaluated for ATPS robustness 1.0, 2.0 and 4.0% (w/w) are equivalent for 1.5, 3.0 and 6.0% (w/v), respectively. Table 4 shows the effect of sample loading in the optimized extraction processes. ATPS with a sample load of 1.5% (w/v) shows the higher crocins recovery in the top phase. However, this sample loading renders a highly diluted sample. Ethanolic and ATPS render similar crocins extraction when a sample load of 2.5–10.0% and 3.0–6.0% (w/v) is used, respectively. Under optimal conditions with the higher sample load tested in this study, ethanolic extraction achieved a crocins recovery of 68.47 ± 2.6% (i.e. 10.0%, w/v sample load), whereas ethanol–phosphate ATPS rendered 72.82 ± 4.4% (i.e. 6.0%, w/v sample load).

Process capability was also analyzed in terms of crocins rendered per gram of ethanol needed for the extraction process. As a general result, when sample load (i.e. %, w/v) is evaluated ethanol–potassium phosphate ATPS renders a higher recovery of crocins per gram of ethanol used for the extraction (Table 4). Under optimal conditions with the higher sample load for ATPS (i.e. 6.0%, w/v) the process may extract 4.62 ± 0.28 mg crocins g^{-1} EtOH; whereas for the ethanolic extraction under optimal conditions with a similar sample load a yield of 3.47 mg crocins g^{-1} EtOH was estimated. This demonstrates the feasibility of using ATPS process for the extraction as an alternative for the traditional extraction methods.

As far as the authors know, an optimized solid–liquid extraction of crocins from saffron stigmas has not been developed. However, some research groups have worked in the optimization of recovery and purification of crocins using other strategies. Mohajeri et al. developed a molecular imprinted polymer (MIP) which had affinity for the gentiobiose present in the principal crocin molecule. With the application of the MIP as sorbent in SPE, an 84% recovery of crocin was achieved [20]. However, a previous step is required for the extraction of crocins from raw materials. Zhang et al. developed a two-step chromatography process, with a novel stationary phase, for the isolation of crocins from saffron extract [59]. Such process achieved high-purity for the three principal crocins [crocetin (di-β-D-gentobisyl) ester, crocetin (mono-β-D-gentobisyl-mono-β-D-glycoside) ester and crocetin (β-D-gentobisyl) ester]. However, the process requires 22 h for pre-separation and a second chromatography step for the complete separation of the pigments. Lastly, a crystallization method for the extraction and purification of crocin from saffron stigmas was developed by Hadizadeh et al. using ethanol as crystallization medium. Even though high-purity was achieved by the process, industrial application may be limited since it requires high-volume of organic solvents and 44 days for process completion [2]. Therefore, based on the results presented herein, the use of ethanol–potassium phosphate ATPS may represent a viable alternative for the extraction and recovery of crocins from *C. sativus* stigmas as a previous stage of chromatography or SPE.

3.4.1. Extraction selectivity comparison under optimal extraction conditions

A final analysis using HPLC was performed to determine the purity achieved using ATPS compared with solid–liquid extraction. Relative purity was defined as the ratio between the area of the major crocin peak (i.e. crocetin di-β-D-gentibiosyl ester; identified by mass spectrometry; data not shown) and the total area for each wavelength. It was observed, at 440 nm, that the extraction of the principal crocin shown a similar behavior among the different treatments, which represents 42.95 ± 0.3% and 45.27 ± 2.7% for ethanolic extraction and ethanol–potassium phosphate ATPS, respectively. In respect to the contaminants behavior (picrocrocin, safranal, phenolic acids) [30], measured at 250 nm, it can be concluded that there is a significant effect upon principal crocin relative purity when the extractions are analyzed at 250 nm (p = 0.0014). In the HPLC profile at 250 nm, major crocin represents 3.25 ± 0.1% and 2.15 ± 0.1% for ethanol–phosphate ATPS and ethanolic extraction, respectively. Based on these results, ATPS show a higher relative purity in this particular wavelength, which is related to picrocrocin and flavonoids recovery. A further study was realized in order to evaluate ethanol–potassium phosphate ATPS extraction selectivity for crocins extraction over phenolic compounds (expressed as mg of gallic acid equivalents, GAE). The results showed that ethanolic solid–liquid extraction rendered a yield of 27.86 ± 0.6 mg GAE g^{-1}

of saffron (ethanol:water ratio (1:1), temperature 25 °C, sample load of 5%, w/v and 200 rpm), while ATPS rendered 19.18 ± 0.4 mg GAE g^{-1} of saffron (System 27, $V_R = 3.2$, 0.1 M NaCl with a sample load of 3%, w/v). Based on these results it can be supported that ethanol–potassium phosphate ATPS rendered a higher extraction selectivity of 1.30 ± 0.04 mg of crocins mg^{-1} GAE than ethanolic extraction, which showed a selectivity of 0.87 ± 0.01 mg of crocins mg^{-1} GAE ($p = 0.0003$). Extraction selectivity analysis by total phenolic component supported that ATPS extraction has a higher relative purity of crocins over phenolic component. It is clear that such results demonstrate the potential application of ATPS processes in the recovery of crocins from *C. sativus*.

4. Conclusions

The present study reports, for the first time, the recovery of crocins from saffron stigmas using ATPS. From the different types of ATPS studied, ethanol–potassium phosphate demonstrated the greater potential to be used for this particular application. Characterization of the partitioning behavior of target product by studying the effect of system parameters resulted in the establishment of an optimized extraction stage. The operating conditions established for ethanol–potassium phosphate ATPS extraction rendered conditions ($V_R = 3.2$, ethanol 19.8% (w/w), potassium phosphate 16.5% (w/w), TLL 25% (w/w) and 0.1 M NaCl) under which crocins are recovered in the top phase. ATPS shows higher selectivity and relative purity for crocins extraction over phenolic components than solid–liquid extraction. The findings report here demonstrates an efficient and ease of scaling up ethanol–potassium phosphate ATPS process for the selective extraction of crocins from *C. sativus*, with application in the food and cosmetic industry, as an alternative for solid–liquid extraction.

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References

[1] V. Sujata, G.A. Ravishankar, L.V. Venkataraman, J. Chromatogr. 624 (1992) 497.
[2] F. Hadizadeh, S.A. Mohajeri, M. Seifi, Pakistan J. Biol. Sci. 13 (2010) 691.
[3] M. Schmidt, G. Betti, A. Hensel, Wien. Med. Wochenschr. 157 (2007) 315.
[4] F.S. Bouvier, C. Suire, J. Mutterer, B. Camara, Plant Cell 15 (2002) 47.
[5] F.I. Abdullaev, Biofactors 4 (1993) 83.
[6] M.H. Namin, H. Ebrahimzadeh, B. Ghareyazie, T. Radjabian, S. Gharavi, N. Tafreshi, Afr. J. Biotechnol. 8 (2009) 5378.
[7] S. Pfister, P. Meyer, A. Steck, H. Pfander, J. Agric. Food Chem. 44 (1996) 2612.
[8] H. Caballero-Ortega, R. Pereda-Miranda, F.I. Abdullaev, Food Chem. 100 (2007) 1126.
[9] Y. Chen, H. Zhang, X. Tian, C. Zhao, L. Cai, Y. Liu, L. Jia, H.-X. Yin, C. Chen, Food Chem. 109 (2008) 484.
[10] L. In-Ah, L. Jin, B. Nam-In, K. Dong-Hyun, Biol. Pharmaceut. Bull. 28 (2005) 2106.
[11] T. Ochiai, S. Ohno, S. Soeda, H. Tanaka, Y. Shoyama, H. Shimeno, Neurosci. Lett. 362 (2004) 61.
[12] Y. Wang, T. Han, Y. Zhu, C.-J. Zheng, Q.-L. Ming, K. Rahman, L.-P. Qin, J. Nat. Med. 64 (2010) 24.
[13] S.-Y. He, Z.-Y. Qian, F.-T. Tang, N. Wen, G.-L. Xu, L. Sheng, Life Sci. 77 (2005) 907.
[14] S.C. Nair, B. Pannikar, K.R. Panikkar, Cancer Lett. 57 (1991) 109.
[15] J. Escribano, G.-L. Alonso, M. Coca-Prados, J.-A. Fernández, Cancer Lett. 100 (1996) 23.
[16] F.I. Abdullaev, J.J. Espinosa-Aguirre, Cancer Detect. Prev. 28 (2004) 426.
[17] R. Tsao, Z.Y. Deng, J. Chromatogr. B 812 (2004) 85.
[18] J.G. Huddleston, H.D. Willauer, R.P. Swatloski, A.E. Visser, R.D. Rogers, Chem. Commun. (1998) 1765.
[19] P.G. Mazzola, A.M. Lopes, F.A. Hasmann, A.F. Jozala, T.C. Penna, P.O. Magalhaes, C.O. Rangel-Yagui, A. Pessoa, J. Chem. Technol. Biotech. 83 (2008) 143.
[20] S.A. Mohajeri, H. Hosseinzadeh, F. Keyhanfar, J. Aghamohammadian, J. Sep. Sci. 33 (2010) 2302.
[21] V. Mandal, Y. Mohan, S. Hemalatha, Pharmacogn. Rev. 1 (2007) 7.
[22] L. Wang, C.L. Weller, Trends Food Sci. Technol. 17 (2006) 300.
[23] C.X. Li, J. Han, Y. Wang, Y.S. Yan, X.H. Xu, J.M. Pan, Anal. Chim. Acta 653 (2009) 178.
[24] J. Benavides, O. Aguilar, B.H. Lapizco-Encinas, M. Rito-Palomares, Chem. Eng. Technol. 31 (2008) 838.
[25] J. Benavides, M. Rito-Palomares, J. Chem. Technol. Biotech. 83 (2008) 133.
[26] P.A.J. Rosa, I.F. Ferreira, A.M. Azevedo, M.R. Aires-Barros, J. Chromatogr. A 1217 (2010) 2296.
[27] C.F. Poole, S.K. Poole, J. Chromatogr. A 1217 (2010) 2268.
[28] C. Li, J. Han, Y. Wang, Y. Yan, J. Pan, X. Xu, Z. Zhang, J. Chem. Eng. Data 55 (2010) 1087.
[29] S. Opperman, F. Stein, U. Kragl, Appl. Microbiol. Biotech. 89 (2011) 493.
[30] P.A. Tarantilis, G. Tsoupras, M. Polissiou, J. Chromatogr. A 699 (1995) 107.
[31] V.L. Singleton, J.A. Rossi, Am. J. Enol. Viticult. 16 (1965) 144.
[32] M. Cisneros, J. Benavides, C.H. Brenes, M. Rito-Palomares, J. Chromatogr. B 807 (2004) 105.
[33] S. Dreyer, P. Salim, U. Kragl, Biochem. Eng. J. 46 (2009) 176.
[34] V. Paquet, M. Myint, C. Roque, P. Soucaille, Biotechnol. Bioeng. 44 (1994) 445.
[35] H. Walter, R. Tung, E.J. Krob, Exp. Cell Res. 102 (1976) 14.
[36] C.-K. Lee, S.I. Sandle, Biotechnol. Bioeng. 35 (1990) 408.
[37] M. Rito-Palomares, A. Lyddiat, Chem. Eng. J. 87 (2002) 313.
[38] P. Wasserscheid, R. van Hal, A. Bösmann, Green Chem. 4 (2002) 400.
[39] S.D. Arco, R.T. Laxamana, O.D. Giron, J.M. Obliosca, Philipp. J. Sci. 138 (2009) 133.
[40] Y. Pei, J. Wang, K. Wu, X. Xuan, X. Lu, Sep. Purif. Technol. 64 (2009) 288.
[41] C. He, S. Li, H. Liu, K. Li, F. Liu, J. Chromatogr. A 1082 (2005) 143.
[42] A. Soto, A. Arce, M.K. Khoshkbarchi, Sep. Purif. Technol. 44 (2005) 242.
[43] E.G. Keith, A.B. Grant, R.D. Rogers, J. Am. Chem. Soc. 125 (2003) 6632.
[44] T. Tianwei, H. Qinang, L. Qiang, Biotechnol. Lett. 24 (2002) 1417.
[45] A. Greve, M.-R. Kula, Fluid Phase Equilib. 62 (1991) 53.
[46] C.W. Ooi, B.T. Tey, S.L. Hii, S. Mazlina, M. Kamal, J.C.W. Lan, A. Ariff, T.C. Ling, Process Biochem. 44 (2009) 1083.
[47] Y. Wang, J. Han, X. Xu, S. Hu, Y. Yan, Sep. Purif. Technol. 75 (2010) 352.
[48] W. Zhi, Q. Deng, J. Chromatogr. A 1116 (2006) 149.
[49] M. van de Weert, J. Hoechstetter, W.E. Hennink, D.J.A. Crommelin, J. Control. Release 68 (2000) 351.
[50] C. Bhattacharjee, P.K. Bhattachary, J. Membr. Sci. 72 (1992) 137.
[51] J. Huddleston, A. Veide, K. Köhler, J. Flanagan, S.-O. Enfors, A. Lyddiatt, Trends Biotechnol. 9 (1991) 381.
[52] T.T. Franco, R. Hatti-Kaul, in: R. Hatti-Kaul (Ed.), Methods in Biotechnology, Aqueous Two Phase Systems, Methods and Protocols, Human Press, Totowa, New Jersey, 2000, p. 47.
[53] S. Chethana, C.A. Nayak, K.S.M.S. Raghavarao, J. Food Eng. 81 (2007) 679.
[54] G.B. Frankforter, L. Cohen, J. Am. Chem. Soc. 36 (1914) 1103.
[55] R.E. Majors, LCGC North America, Chromatography Online, 2009. http://chromatographyonline.findanalytichem.com/lcgc/article/articleDetail.jsp?id=613590&sk=&date=&pageID=5.
[56] J.P. Chen, J. Ferment. Bioeng. 73 (1991) 140.
[57] M.A. Eitman, J.L. Gainer, Biochim. Biophys. Acta 992 (1989) 125.
[58] B. Yang, X. Liu, Y. Gao, Innov. Food Sci. Emerg. 10 (2009) 610.
[59] H. Zhang, Y. Zeng, F. Yan, F. Chen, X. Zhang, M. Liu, W. Liu, Chromatographia 59 (2004) 691.
[60] B. Zaslavsky, Aqueous Two-phase System Partitioning: Physical Chemistry and Bioanalytical Applications, New York, 1995.