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Approach for simultaneous cannabidiol isolation and pesticide removal from hemp extracts with liquid-liquid chromatography

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ABSTRACT

In this work, a computer-aided approach for selecting biphasic solvent systems for simultaneous cannabidiol (CBD) isolation and pesticide removal from hemp extracts with liquid-liquid chromatography (LLC) was investigated. By taking advantage of a fully predictive thermodynamic model (COSMO-RS), a considerable number of ternary solvent systems were screened and potential systems were identified with practically no experimental effort; the partition coefficient of the target component CBD was used as screening parameter. After the experimental validation, three solvent systems were chosen: *solvent system I*: *n*-heptane/methanol/water 4/3/1 v/v/v, *solvent system II*: *n*-heptane/acetone/water 2/5/1 v/v/v and *solvent system III*: *n*-heptane/acetonitrile/water 5/3/2 v/v/v. The 59 pesticides regulated by the state of Oregon in cannabis products were included in the next step with the aim to propose a classification system of the most critical pesticides for obtaining pesticide-free CBD. Based on CBD/pesticide separation factors ($\alpha_{CBD/PEST}$), four critical levels were defined. Depending on the solvent system, it was shown that 50–70 % of the Oregon-listed pesticides were found to be non-critical ($\alpha_{CBD/PEST} > 4$), while only 13–22 % were retrieved as highly and medium critical ($\alpha_{CBD/PEST} < 2$). Using the acquired knowledge, a guideline for the selection of the best solvent system candidate for the simultaneous LLC separation of CBD and removal of Oregon-listed pesticides from hemp extracts was proposed. Following a series of LLC experiments with a pesticide-spiked hemp extract, it was shown that the pesticide classification lists can be used to select the most promising solvent system to achieve the removal of most of the contaminating pesticides.

1. Introduction

Cannabis sativa L. (Cannabaceae, cannabis) has been used for more than 6000 years as a source of food, fiber, oil and medicine, as well as for recreational or religious purposes (Bonini et al., 2018; Schluttenhofer and Yuan, 2017). Cannabis has a very complex chemical composition, with around 540 reported specialized metabolites, such as (phyto)cannabinoids, terpenoids, flavonoids and alkaloids. The most active of these are by far the cannabinoids, a class represented by more than 100 known terpenophenolic compounds that accumulate mainly in the resin secreted from the trichomes of female plants (Bonini et al., 2018). Based on its use, cannabis can be technically divided into two distinct groups: marijuana (medicinal and recreational) and (industrial) hemp. Traditionally, marijuana is almost exclusively grown in greenhouses or other controlled environment facilities, being primarily bred for its main psychoactive cannabinoid, Δ^9-tetrahydrocannabinol (THC). On the other hand, hemp serves more as an agricultural commodity, being valued for its fibers and seeds and, more recently, for its non-intoxicating medicinal compounds, notably cannabidiol (CBD) (Sandler et al., 2019; Schluttenhofer and Yuan, 2017). From a legal point of view, in most European and North American countries, cannabis is classified as hemp if the crop contains less than 0.2 − 0.3% THC (Schluttenhofer and Yuan, 2017). Due to their low THC content, hemp and CBD products have recently gained an increased popularity, as their attributed medical benefits are achieved without the "high" effects of marijuana (VanDolah et al., 2019). Consequently, a wide panel of products are nowadays marketed as "full-spectrum" formulas, dietary supplements or CBD-enriched products (King, 2019; VanDolah et al., 2019).

The presence of pesticides in hemp crops is a very challenging issue nowadays, not only for cultivators, but also for regulators, consumers or public health researchers (Subritzky et al., 2017). Many studies have revealed there is no clear relationship between pesticide use and augmentation of cannabis yields, being repeatedly claimed that their role in

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weed and pest control might be actually unnecessary (Sandler et al., 2019). Beside their concealed use during cannabis growth, other reasons for pesticide contamination might be related to the spray drift from adjacent crops or assimilation from the contaminated soil; numerous pesticides are environmentally mobile, being carried away by groundwater and rain, whilst cannabis is recognized as a robust and fast-growing plant able to absorb pollutants with a great efficiency (Chandra et al., 2017; Seltenrich, 2019). Thence, pesticide contamination of cannabis materials has been repeatedly brought into attention (Sandler et al., 2019). A survey of 389 cannabis products from the state of Oregon found 24 residual pesticides, with piperonyl butoxide as the most commonly retrieved contaminant. Furthermore, 12 pesticides were found in up to 50 % of the cannabis samples collected from central Californian dispensaries (Chandra et al., 2017). Out of 26 investigated cannabis samples, 84.6 % were confirmed to be positive for pesticides from various classes, such as insecticides, miticides, fungicides or growth regulators (Russo, 2016). In addition, a multi-screening study testing for 71 residual pesticides in various cannabis products from Italy (such as hemp oils and flours) revealed that amitraz, chlorpyrifos and trifluralin were above the acceptable residual limits set at 0.010 mg/kg (Fusari et al., 2013). Nevertheless, occupational or non-occupational exposure to residual pesticides has become an important issue due to potential adverse health effects. For example, organophosphate pesticides, such as chlorpyrifos, malathion, parathion methyl, ethoprophos or coumaphos, have been found to be highly toxic, inducing neurobehavioral and cognitive disorders, teratogenicity, immunotoxicity or endocrinal and metabolic disturbances (Triassi et al., 2019). Daminozide and paclobutrazol are two plant growth regulators that have been banned in the USA and numerous European countries, due to their carcinogenetic properties; however, both have been found as contaminants in cannabis products (Atapattu and Johnson, 2020). In this light, more and more regulatory agencies are addressing the residual pesticides issue in cannabis products. For example, in the USA, pesticide regulations are specific to each state, mostly due to the fact that cannabis cultivation is still not yet federally legal. Among the states, California has the most severe requirements, monitoring 66 pesticides, followed by Oregon with 59 pesticides. In comparison to these, Canada has set wider and stricter controls of pesticides in cannabis products, demanding testing for 96 pesticides, with limits of quantification (LOQs) usually lower than those set in the USA (Atapattu and Johnson, 2020; Craven et al., 2019). From a processing point of view, cannabis-derived extracts are frequently obtained using CO_2-supercritical fluid extraction (SFE-CO_2) followed by winterization (to filter out waxes and some color compounds), decarboxylation (to transform acidic cannabinoids into their neutral forms), distillation and, optionally, a chromatographic-based method for the isolation of specific cannabinoid compounds (King, 2019). For instance, CBD purification is mostly achieved by recrystallization, conventional chromatography with solid stationary phase or liquid-liquid chromatography (LLC) (Hazekamp et al., 2004; King, 2019).

LLC, better known as centrifugal partition chromatography (CPC) and countercurrent chromatography (CCC), is a preparative separation technique where the two phases of a biphasic solvent system are used as the mobile and stationary phase. One of the two phases is kept stationary inside the column with the help of a centrifugal field, while the other one is pumped through the column (Berthod, 2002; Foucault, 1994). In this sense, LLC enables high column loading, while expensive solid stationary phases and time intensive column packing procedures are not required. In addition, the almost limitless variety of possible biphasic solvent systems (Skalicka-Woźniak and Garrard, 2015) and easy preparation of tailor-made solvent systems make LLC a very flexible, scalable, as well as selective separation technique (Friesen et al., 2015; Morley and Minceva, 2019; Roehrer and Minceva, 2019). Taking advantage of these benefits, Hazekamp et al. (2004) showed that CBD (and other cannabinoids, namely THC, cannabinol and cannabigerol) can be separated by CPC using *n*-hexane/acetone/acetonitrile 5/2/3 (v/v/v) as a biphasic solvent system, with the acetonitrile-rich phase as the stationary phase (ascending mode, ASC).

To this end, a considerable attention has been paid either on the large-scale purification of CBD (and other cannabinoids) by LLC (Hazekamp et al., 2004; Popp et al., 2019) or the ultra-trace detection of residual pesticides (Atapattu and Johnson, 2020; Craven et al., 2019) in cannabis products, but not to the removal of the contaminating pesticides. Therefore, the aim of this study was to establish a systematic computer-aided approach for the selection of biphasic solvent systems for the simultaneous purification of CBD from hemp extracts and removal of specific-contaminating pesticides. In this sense, a predictive thermodynamic model was used for solvent system screening, guiding the user toward the design of tailor-made solvent systems for the isolation of pesticide-free CBD from a particular hemp extract using LLC.

2. Material and methods

2.1. Chemicals

Acetone (≥99.0 %), ethyl acetate (≥99.0 %), *n*-heptane (≥99.0 %), tetrahydrofuran (THF, ≥99.0 %) and methanol (≥99.0 %) were purchased from Merck KGaA (Darmstadt, Germany), whereas acetonitrile (≥99.0 %) was achieved from J.T. Baker (Deventers, the Netherlands). The deionized water was obtained from an in-house network system, whilst the Milli-Q water was acquired from a Milli-Q Direct Water Purification System from Merck Millipore (Darmstadt, Germany). Cannabidiol (CBD) solution (1 mg/mL in methanol), ethoprophos (≥95.0 %), fenoxycarb (≥98.0 %), kresoxim methyl (≥95.0 %), piperonyl butoxide (≥98.0 %), pyrethrum extract (40.7 % pyrethrins, with 3.1 % cinerin I, 2.4 % cinerin II, 0.8 % jasmolin I, 1.2 % jasmolin II, 20.4 % pyrethrin I, 12.5 % pyrethrin II) and trifloxystrobin (≥98.0 %) were purchased from Merck KGaA (Darmstadt, Germany), while propiconazole (≥98.4 %) was bought from LGC Labor GmbH (Augsburg, Germany). The 59 pesticides from Oregon list were obtained from Restek (Bad Homburg, Germany), as six separate mixes (Cat. # 32586-32591), with each pesticide in a concentration of 600 µg/mL in acetonitrile. The decarboxylated hemp extract (≥7.5 % CBD; < 0.2 % THC) was provided by BAFA Neu GmbH (Malsch, Germany); a voucher specimen (HF655_2dc) is deposited in the Assistant Professorship of Biothermodynamics, Technical University of Munich (Germany).

2.2. Preparation of biphasic solvent systems

The biphasic liquid solvent systems were prepared by mixing the respective portions of each solvent at room temperature. The mixtures were then vigorously shaken and equilibrated at room temperature for at least two hours. Afterwards the two phases were split into separate vials or containers.

2.3. Determination of the partition coefficients

In LLC (CCC and CPC), the separation is based on the different distribution of the solutes between the two liquid phases, which can be described by the partition coefficient P_i of a solute i. The partition coefficient P_i is defined as the ratio of the concentration of a solute i in the stationary phase (c_i^{SP}) to its concentration in the mobile phase (c_i^{MP}) at thermodynamic equilibrium (Eq. 1).

$$P_i = \frac{c_i^{SP}}{c_i^{MP}} \tag{1}$$

The separation factor α (Eq. 2) between two compounds i and j is defined as the ratio of their partition coefficients, where $P_j > P_i$:

$$\alpha_{ij} = \frac{P_j}{P_i} \tag{2}$$

In this study, the partition coefficients of the different solutes in various biphasic systems where determined in different ways described in the following section.

2.3.1. Computational determination with COSMO-RS

The partition coefficient of a solute i was predicted with the Conductor-Like Screening Model for Realistic Solvation (COSMO-RS) according to the approach from Hopmann et al. (2012) and Frey et al. (2014). The conformers of all solutes were generated in COSMOconf (Version 4.0, COSMO-logic, Germany) with the Balloon algorithm, implemented in the software. The conformers were considered as a Boltzmann-weighted mixture of conformers for the calculations and the maximum number of conformers was set to 20 within an energy window of 25 kcal/mol from the lowest energy structure. The partition coefficients were then calculated with the software COSMOtherm (Version C30 Release 16.01, COSMOlogic, Germany) with the triple-zeta valence polarization (TZVP) basis set parametrization.

For the calculation of the partition coefficient of a solute i in a biphasic solvent system, the composition of the solute-free phases has to be known. In this study, the liquid-liquid equilibrium (LLE) data was taken from literature or experimentally determined by GC-TCD (Section 2.5.1). The LLE conditions for a solute i in UP and LP are defined by Eq. (3).

$$x_i^{UP}\gamma_i^{UP} = x_i^{LP}\gamma_i^{LP} \tag{3}$$

with the mole fraction x_i and the activity coefficient γ_i of solute i in the two phases, respectively. The partition coefficient K of a solute i in the linear range of the partitioning equilibria can then be calculated based on the mole fractions in the phases at thermodynamic equilibrium:

$$K_i = \frac{x_i^{UP}}{x_i^{LP}} = \frac{\gamma_i^{\infty,LP}}{\gamma_i^{\infty,UP}} \tag{4}$$

where γ_i^{∞} is the limiting activity coefficient of solute i. It must be noted, that the distribution of a solute i in the linear range of partitioning equilibria is constant. The partition coefficient K_i can be converted to P_i considering the molar volumes ν of the phases:

$$P_i = \frac{c_i^{UP}}{c_i^{LP}} = \frac{x_i^{UP}\nu^{LP}}{x_i^{LP}\nu^{UP}} = K_i\frac{\nu^{LP}}{\nu^{UP}} \tag{5}$$

The molar volumes of the phases were calculated from the composition of the phases and the molar volume of the pure solvents. Neglecting the excess volume of mixing, the molar volumes of the phases were approximated as the weighted sum of the molar volumes of the pure solvents ν_{0j}:

$$\nu = \sum x_j\nu_{0j} \tag{6}$$

2.3.2. Experimental determination with shake-flask experiments

Two experimental protocols were followed in the shake-flask experiments, depending on the purpose. For the initial screening of the partition coefficient of CBD in different solvent systems, 5 mg of hemp extract were added to upper (10 mL) and lower (10 mL) phases of pre-equilibrated solvent systems and placed into a 20 mL vial. Then, each phase was analyzed by HPLC-DAD (Section 2.5.2). Once the three solvent systems were selected, the six pesticide mixes and CBD standards were combined together and a stock solution, in which CBD and each pesticide had a concentration of 50 ppm, was obtained by a proper dilution in acetonitrile. 20 µL of the stock solution were added to 20 mL of each of the three selected solvent systems, affording a nominal concentration of 50 ppb of each solute. Afterwards, 1 mL of upper phase and 1 mL of lower phase were taken, evaporated to dryness, re-dissolved in 1 mL methanol and analyzed by LC–MS/MS (Section 2.5.3) to determine the concentration of each analyte in the phases. The partition coefficient (descending, DSC, mode) of the solute i was then calculated according to Eq. 1.

2.4. Liquid-liquid chromatography experiments

LLC experiments were carried out on a countercurrent chromatography column, model HPCCC-Mini Centrifuge (0.8 mm i.d.) from Dynamic Extractions (Wales, UK), with a column volume of 18.2 mL. Two isocratic Gilson 306 pumps (Gilson, USA), equipped with an 806 Manometric Module (Gilson, USA), were used for delivering the mobile and stationary phases. The elution profiles were monitored with a DAD 171 diode array detector (Gilson, USA) at a wavelength of 220 nm. The CCC experiments were performed at room temperature. All LLC separations were carried out as pulse injections in DSC mode, where the lower phase is used as mobile phase. At the beginning of each experiment, the column was filled with stationary phase. Afterwards, the rotational speed was set to 1900 rpm and the mobile phase was pumped through the column at 1 mL min^{-1} until no more stationary phase eluted from the column. The samples were dissolved in the corresponding mobile phase (5 mg min^{-1} hemp extract spiked with 50 ppm of each pesticide), filtered and then injected *via* a 1 mL sample loop with a manual injection valve, with the mobile phase continuously pumped at 1 mL min^{-1}. All CCC runs were manually fractionated after injection in fraction intervals of 1 min. The collected fractions were analyzed by HPLC-DAD. Based on the analysis of the fractions, a reconstructed offline LLC-chromatogram was generated for each separation run.

2.5. Analytical methods

2.5.1. GC-TCD analysis

GC-TCD analysis was performed using a Nexis GC 2030 coupled with a thermal conductivity detector (TCD) from Shimadzu (Tokyo, Japan). A Restek Rxi-624Sil MS (30 m × 0.25 mm, 1.4 µm) capillary column was used, with helium as carrier gas at a linear velocity flow of 40 cm s^{-1}. The temperature of the injection port was set to 250 °C, at a split-ratio of 50 during injection. After an isothermal step of 1 min at 35 °C, a linear temperature gradient of 31 °C min^{-1} to 190 °C was applied. The TCD temperature was set at 260 °C. The biphasic solvent systems were prepared in 20 mL vials by mixing the corresponding portions of the solvents at ambient temperature and pressure. The mixture was then vigorously shaken and equilibrated at 25 °C for 2 h. Then, 100 µL of upper and lower phases were separately diluted with 900 µL THF and their compositions were analyzed by GC-TCD, using six-point calibration curves established for each of the analyzed solvents.

2.5.2. HPLC-DAD analysis

HPLC-DAD analysis was performed using a Prominence HPLC system from Shimadzu (Tokyo, Japan) equipped with a LC-20AB mobile phase delivery module, DGU-20A_3 degasser, SIL-20A auto-sampler, and a SPD-M20A diode array detector. The separations were achieved on a Poroshell 120 EC-C18 (3.0 × 150 mm, 2.7 µm) column guarded with a Poroshell 120 UHPLC Guard EC-C18 (3.0 mm) pre-column. Milli-Q water (A) and acetonitrile (B) were used as mobile phase, at a flow-rate of 0.25 mL min^{-1}, with the following gradient: 0 min–60 % B, 20 min–80 % B, 21 min–95 % B, 25 min–95 % B, 30 min–60 % B, 35 min–60 % B. The injection volume was 3 µL and the detection wavelength was set at 220 nm. The concentrations of CBD, ethoprophos, fenoxycarb, kresoxim methyl, piperonyl butoxide, pyrethrins (cinerins I and II, pyrethrins I and II) and trifloxystrobin were calculated by preparing six-point calibration curves of the corresponding standards, over individual concentration ranges (Table S5). LabSolutions 5.82 (Shimadzu) was used for both data acquisition and data analysis.

2.5.3. LC–MS/MS analysis

LC–MS/MS analysis was performed using an Agilent1200 HPLC system (Santa Clara, CA, USA) equipped with a G1312A pump, G1316A column oven, a CTC PAL autosampler and coupled with a QTRAP5500

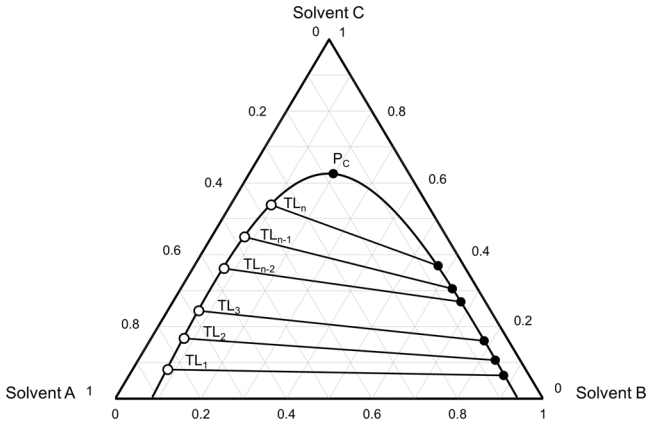

Fig. 1. Example of a ternary phase diagram (mol/mol) composed of solvent A, B and C; ○ composition of phase 1 in mole fraction; ● composition of phase 2 in mole fraction; TL, tie line; P_C plait point.

mass spectrometer detector from AB Sciex Instruments (Darmstadt, Germany). The separation was carried out on a Phenomenex Kinetex5 u XB-C18 (100 × 2.1 mm, 5 µm) column at 40 °C, with a mobile phase composed of 5 mM ammonium formate (A) and methanol with 5 mM ammonium formate (95/5, v/v) (B); the following gradient was applied: 0 – 0.5 min–10 % B, 2 min–30 % B, 9 min–60 % B, 11 min–80 % B, 12 – 17 min–100 % B, 18 – 22 min–10 % B, at a flow-rate of 0.3 mL min^{-1}; the injection volume was 5 µL. The MS parameters were as follows: positive ionization mode, curtain gas 40 psi, CAD gas -2, ion source voltage: 5500 V, source temperature: 400 °C, gas1: 55 psi, gas2: 65 psi, entrance potential 10 V. Most multiple reaction monitoring mode (MRM) transitions were obtained from literature data (Wong et al., 2010). Only pralletrhin, phosmet, CBD and MGK-264 were freshly tuned. The concentration of CBD and each pesticide was obtained from five-point calibration curves, over the concentration domain ranged from 5 to 100 ppb. Analyst 1.7.0 was used for data acquisition and MultiQuant 3.0.3 for data analysis (both AB Sciex).

3. Results and discussion

In this work, a computer-aided approach for selecting biphasic solvent system candidates for preparative LLC isolation of CBD from hemp extracts, with simultaneous removal of contaminating pesticides that might be present in the starting material is proposed. First, a list of biphasic solvent systems from a predefined pool of solvents was created. After that, a fully predictive thermodynamic model (COSMO-RS) was used to screen for potential systems (Section 3.1). The objective of the screening was to identify biphasic solvent systems in which the partition coefficient of the target component CBD is within a predefined range. Three of the most promising solvent systems were next evaluated in terms of their ability to separate the target compound CBD from a list of pesticides as impurities. In this work, pesticides whose limits in cannabis products are regulated by the state of Oregon were considered. Consequently, a list of critical pesticides was determined for each solvent system based on separation factors $\alpha_{CBD/PEST}$ values predicted by COSMO-RS and validated by shake-flask experiments (Section 3.2). For the proof-of-concept, a hemp extract spiked with seven pesticides was subjected to LLC separations with each of the three selected solvent systems (Section 3.3).

3.1. Screening for biphasic solvent systems for LLC isolation of CBD from hemp extracts

A computer-aided approach based on the fully predictive thermodynamic model COSMO-RS was applied, following the procedures proposed by Hopmann et al. (2012) and Brace and Engelberth (2017). Even though the solvents used during LLC separations are removed at the end of the process (*i.e.* vacuum evaporation) and their residual levels in the final products are regulated by different organizations (*i.e.* Food and Drug Administration, European Food Safety Authority), their toxicity, safety profile and environmental impact have to be considered during the process design. The pool of solvents used to screen for biphasic solvent systems for LLC isolation of CBD from hemp extracts was selected according to several guidelines used in pharmaceutical industry (Alder et al., 2016; Byrne et al., 2016; Prat et al., 2014). For instance, benzene and diethyl ether as well as chlorinated solvents were excluded, with the following categories of solvents included: hydrocarbons (*n*-heptane, *n*-hexane), alcohols (methanol, ethanol, *i*-propanol, *n*-butanol), ketones [acetone, methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK)], acetonitrile, methyl *tert*-butyl ether (MTBE) and water. Next, from this list, possible ternary solvent systems were defined by choosing two poorly miscible solvents and a third bridge solvent that is (partly) miscible in these two solvents. Despite the fact that quaternary solvent systems are frequently used in LLC separations of natural products (Skalicka-Woźniak and Garrard, 2015), they were excluded due to their difficult and cost-intensive recycling, especially at industrial scale (Hopmann et al., 2012).

The liquid-liquid equilibrium (LLE) data of considered solvent systems were taken from literature or experimentally determined in our lab. The partition coefficient of CBD ($P_{CBD}^{COSMO-RS}$), determined as described in Section 2.3.1, was used as a screening parameter; the target was to find solvent systems in which $P_{CBD}^{COSMO-RS}$ was in the inclusion range of 0.2–5 (“theoretical sweet spot”). This range exceeds the conventional limits of the “experimental sweet spot” (0.4–2.5) (Friesen et al., 2015), but this extension was considered in order to account for partition coefficients that could be over- or under-predicted with COSMO-RS. Within the biphasic region, $P_{CBD}^{COSMO-RS}$ was first calculated for the lowest and highest available tie lines (TL_1 and TL_n in the

Table 1
Percentage distribution of Oregon-listed pesticides among four critical levels, as evaluated according to COSMO-RS predicted and experimentally determined CBD/pesticide separation factors ($\alpha_{CBD/PEST}$) for the three selected biphasic solvent systems.

Critical level	*Solvent system I*		*Solvent system II*		*Solvent system III*	
	COSMO-RS	*EXP*	*COSMO-RS*	*EXP*	*COSMO-RS*	*EXP*
Highly critical pesticides	5/65	5/65	14/65	9/65	5/65	4/65
Red-zoned ($\alpha_{CBD/PEST} < 1.5$)	(8%)	(8%)	(22 %)	(14 %)	(8%)	(6%)
Medium critical pesticides	3/65	3/65	8/65	5/65	4/65	5/65
Orange-zoned ($1.5 < \alpha_{CBD/PEST} < 2.0$)	(5%)	(5%)	(12 %)	(8%)	(6%)	(8%)
Low critical pesticides	17/65	9/65	10/65	17/65	10/65	8/65
Yellow-zoned ($2.0 < \alpha_{CBD/PEST} < 4.0$)	(26 %)	(14 %)	(15 %)	(26 %)	(15 %)	(12 %)
Non-critical pesticides	40/65	48/65	33/65	34/65	46/65	48/65
Green-zoned ($4.0 < \alpha_{CBD/PEST}$)	(61 %)	(74 %)	(51 %)	(52 %)	(71 %)	(74 %)

Solvent system I: *n*-heptane/methanol/water 4/3/1 v/v/v; *Solvent system II*: *n*-heptane/acetone/water 2/5/1 v/v/v; *Solvent system III*: *n*-heptane/acetonitrile/water 5/3/2 v/v/v; *α* separation factor.

Table 2
Classification of Oregon-listed pesticides for the three selected solvent systems according to their COSMO-RS predicted ($P_i^{COSMO-RS}$) and experimentally determined (P_i^{EXP}) partition coefficients.

No	Compound	Solvent system I		Solvent system II		Solvent system III	
		$P_i^{COSMO-RS}$	P_i^{EXP}	$P_i^{COSMO-RS}$	P_i^{EXP}	$P_i^{COSMO-RS}$	P_i^{EXP}
0	CBD	0.44	1.04	1.33	1.67	5.93	1.12
1a	Abamectin, abamectin B1a	0.01	0.01	0.50	0.27	0.89	0.01
1b	Abamectin, abamectin B1b	0.01	0.00	0.20	0.12	0.33	0.01
2	Acephate	0.00	0.00	0.05	0.00	0.00	0.00
3	Acequinocyl	19.11	38.45	4.74	49.97	444.06	40.89
5	Acetamiprid	0.00	0.00	0.02	0.03	0.00	0.00
5	Aldicarb	0.01	0.01	0.15	0.13	0.02	0.01
6	Azoxystrobin	0.00	0.00	0.05	0.14	0.00	0.00
7	Bifenazate	0.04	0.03	0.29	0.35	0.10	0.00
8	Bifenthrin	5.54	68.52	2.01	27.01	49.62	77.39
9	Boscalid	0.06	0.04	0.22	0.40	0.06	0.04
10	Carbaryl	0.01	0.00	0.14	0.25	0.01	0.00
11	Carbofuran	0.01	0.00	0.17	0.26	0.02	0.00
12	Chlorantraniliprole	0.00	0.00	0.10	0.00	0.00	0.00
13	Chlorfenapyr	0.30	nd	0.50	nd	0.64	nd
14	Chlorpyrifos	2.67	18.96	1.40	3.55	9.60	9.18
15	Clofentezine	0.11	3.00	0.30	1.09	0.12	0.96
16	Cyfluthrin	0.77	10.39	0.72	3.08	2.40	0.28
17	Cypermethrin	1.36	20.20	0.95	4.10	4.83	0.21
18	Daminozide	0.00	0.08	0.03	0.12	0.00	6.51
19	Diazinon	1.71	2.36	1.28	1.71	9.16	1.98
20	Dichlorvos	0.05	0.00	0.27	0.00	0.06	0.00
21	Dimethoate	0.01	0.00	0.08	0.06	0.00	0.00
22	Ethoprophos	0.39	0.58	0.83	1.06	2.07	0.69
23	Etofenprox	6.19	73.28	2.06	7.58	52.88	27.41
24	Etoxazole	1.71	4.93	1.15	4.14	11.47	8.32
25	Fenoxycarb	0.07	0.33	0.31	0.51	0.15	0.11
26	Fenpyroximate	0.45	5.40	0.71	1.94	2.28	2.31
27	Fipronil	0.01	0.02	0.16	0.55	0.01	0.02
28	Flonicamid	0.00	0.00	0.01	0.00	0.00	0.00
29	Fludioxonil	0.00	0.00	0.07	0.31	0.00	0.00
30	Hexythiazox	0.62	5.68	0.86	3.18	3.25	3.87
31	Imazalil	0.04	0.14	0.14	0.38	0.02	0.07
32	Imidacloprid	0.00	0.00	0.04	0.00	0.00	0.00
33	Kresoxim methyl	0.97	1.50	0.82	1.00	2.28	0.32
34	Malathion	0.29	0.61	0.56	0.70	0.52	0.18
35	Metalaxyl	0.01	0.02	0.18	0.32	0.02	0.00
36	Methiocarb	0.02	0.09	0.24	0.57	0.05	0.09
37	Methomyl	0.00	0.00	0.08	0.07	0.00	0.00
38	Methyl parathion	0.17	0.35	0.33	0.70	0.12	0.22
39	MGK -264	0.81	3.33	1.22	2.33	6.35	2.27
40	Myclobutanil	0.00	0.02	0.07	0.35	0.00	0.01
41	Naled	0.17	0.01	0.41	0.01	0.20	0.00
42	Oxamyl	0.00	0.00	0.05	0.02	0.00	0.00
43	Paclobutrazol	0.02	0.00	0.15	0.33	0.03	0.00
44	Permethrins	7.78	63.49	2.12	0.78	52.01	0.09
45	Phosmet	0.09	2.12	0.26	0.41	0.06	0.08
46	Piperonylbutoxide	0.23	6.54	0.60	3.28	1.63	2.94
47	Prallethrin	0.17	2.42	0.54	1.73	0.64	1.10
48	Propiconazole	0.03	0.26	0.19	0.71	0.05	0.18
49	Propoxur	0.01	0.01	0.18	0.22	0.02	0.01
50a	Pyrethrins, cinerin I	1.21	9.04	1.50	4.05	13.72	10.47
50b	Pyrethrins, cinerin II	0.20	1.47	0.70	1.48	1.00	0.68
50c	Pyrethrins, jasmolin I	1.75	22.88	1.84	8.31	26.47	14.75
50d	Pyrethrins, jasmolin II	0.36	2.02	0.94	1.75	2.62	1.45
50e	Pyrethrins, pyrethrin I	1.03	12.76	1.27	4.81	8.82	7.01
50f	Pyrethrins, pyrethrin II	0.18	1.32	0.63	1.43	0.80	0.52
51	Pyridaben	1.39	10.02	1.21	4.86	13.40	7.18
52a	Spinosad, spinosyn A	0.05	0.69	0.56	3.62	2.25	0.58
52b	Spinosad, spinosyn D	0.12	1.00	0.85	3.98	10.71	0.89
53	Spiromesifen	1.43	5.33	1.61	3.76	19.52	4.44
54	Spirotetramat	0.01	0.03	0.17	0.17	0.02	0.00
55	Spiroxamine	9.93	6.46	3.68	80.27	339.50	81.86
56	Tebuconazole	0.02	0.06	0.17	0.45	0.05	0.04
57	Thiacloprid	0.00	0.00	0.02	0.02	0.00	0.00
58	Thiamethoxam	0.00	0.01	0.02	0.05	0.00	0.01
59	Trifloxystrobin	1.57	2.06	1.03	1.14	5.56	0.54

Solvent system I: *n*-heptane/methanol/water 4/3/1 v/v/v; *Solvent system II*: *n*-heptane/acetone/water 2/5/1 v/v/v; *Solvent system III*: *n*-heptane/acetonitrile/water 5/3/2 v/v/v.
Cells in *red* ("highly critical pesticides"): $\alpha_{CBD/PEST} < 1.5$; cells in *orange* ("medium critical pesticides"): $1.5 < \alpha_{CBD/PEST} < 2.0$; cells in *yellow* ("low critical pesticides"): $2.0 < \alpha_{CBD/PEST} < 4.0$; cells in *green* ("non-critical pesticides"): $4.0 < \alpha_{CBD/PEST}$; nd, not determined.

exemplary phase diagram in Fig. 1), accounting for the highest and lowest values of the partition coefficient, respectively. In case $P_{CBD}^{COSMO-RS}$ values for both tie lines were found to be > 100, those solvent systems were subsequently excluded. For all the other systems, more solvent system compositions situated on different tie lines (*i.e.* TL_2, TL_3 … TL_{n-1} in Fig. 1) were screened, until solvent system compositions giving $P_{CBD}^{COSMO-RS}$ within the inclusion range were eventually found. The list of all screened solvent systems is given in Table S1. Based on this, systems composed of *n*-heptane/*n*-hexane and water (as the two poorly miscible solvents) and acetone, acetonitrile, ethanol, methanol, or *i*-propanol as the bridge solvent were concluded to be most suitable. *n*-Heptane- or *n*-hexane-free aqueous solvent systems (*i.e.* MIBK/acetonitrile/water, MIBK/acetone/water, MTBE/ethanol/water, *n*-butanol/methanol/water) as well as systems composed of *n*-heptane/*n*-butanol/water and *n*-hexane/*n*-butanol/water were found to be too polar (very high $P_{CBD}^{COSMO-RS}$ values), no matter their composition. Lastly, several non-aqueous systems (*i.e.* *n*-heptane/methanol/acetonitrile; *n*-heptane/ethanol/acetonitrile; *n*-hexane/methanol/acetonitrile; *n*-hexane/ethanol/acetonitrile) also showed promising leads, as assessed by their $P_{CBD}^{COSMO-RS}$ values (Table S1).

Next, the above identified systems were experimentally evaluated by performing shake-flask experiments as described in Section 2.3.2. The objective was to find system compositions for which P_{CBD}^{EXP} values were situated within the inclusion range ("experimental sweet spot") of 0.4–2.5 (*i.e.* preferably close to 1) for each of the experimentally evaluated systems. The solvent system compositions tested in the shake-flask experiments were obtained from the available LLE data. The system compositions were transferred from mole fractions into volume portions. For practical reasons (*i.e.* easier preparation of the biphasic solvent system for separation purposes), the calculated volume portions were rounded to whole numbers. However, for this step, two solvents (*n*-hexane and ethanol) were excluded. Since *n*-hexane is posing "major issues" (Alder et al., 2016) or is regarded as "undesirable" in pharmaceutical and food processing (Prat et al., 2014), its substitution with *n*-heptane was proposed for this step, while ethanol was excluded due to cost-related factors. For several solvent systems (*i.e.* *n*-heptane/methanol/water, *n*-heptane/acetonitrile/water and *n*-heptane/acetone/water), a few P_{CBD}^{EXP} values were situated within the inclusion range (Table S2). Other solvent systems (*i.e.* *n*-heptane/*i*-propanol/water, *n*-

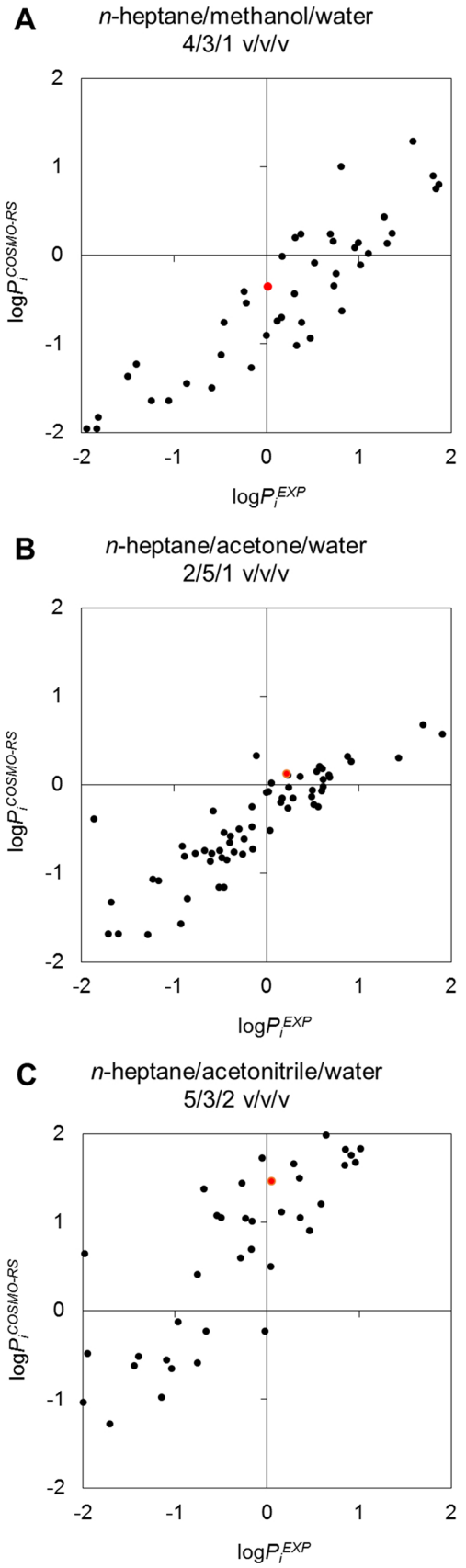

Fig. 2. $\log P_i^{COSMO-RS}$ *vs.* $\log P_i^{EXP}$ in (A) *Solvent system I*, (B) *Solvent system II* and (C) *Solvent system III*. Red point represents CBD, whilst the black points represent the pesticides; only pesticides with $-2.0 < \log P_i < 2.0$ were plotted.

heptane/methanol/acetonitrile) were excluded due to either P_{CBD}^{EXP} values situated outside the inclusion range or phase stability concerns related to the fact that those compositions were in the vicinity of the one-phase region in the ternary phase diagram (Table S2). Finally, three solvent system compositions were chosen for the next step:

- *Solvent system I*: *n*-heptane/methanol/water 4/3/1 v/v/v ($P_{CBD}^{EXP} = 1.04$);
- *Solvent system II*: *n*-heptane/acetone/water 2/5/1 v/v/v ($P_{CBD}^{EXP} = 1.67$);
- *Solvent system III*: *n*-heptane/acetonitrile/water 5/3/2 v/v/v ($P_{CBD}^{EXP} = 1.12$).

For all three systems, the equilibrium composition of the two phases at 25 °C (Table S3) was determined in our lab using the methods described in Section 2.5.1. These LLE data were next used for the COSMO-RS prediction of the partition coefficients of CBD and pesticides (Section 3.2).

3.2. Screening for biphasic solvent systems for LLC removal of Oregon-listed pesticides from hemp extracts

As mentioned, cannabis samples are quite often reported to be contaminated with traces of various pesticides, which might require additional processing for their removal from the final product. Therefore, a suitable biphasic solvent system would be one that can be used, not only for CBD isolation, but also for the simultaneous removal of contaminating pesticides. The ability of each of the three selected solvent systems to separate CBD (the target compound) from a list of pesticides (the impurities) was further evaluated. In this work, the test pesticides were represented by those regulated by the state of Oregon (https://www.oregon.gov/oha/ph/preventionwellness/marijuana/documents/oha-8964-technical-report-marijuana-contaminant-testing.pdf). According to this technical report, their action levels (meaning the concentrations of residual pesticides above which regulatory or remedial actions are demanded) are limited to 0.2 − 1 ppm. The list is constituted of 59 pesticides, which practically sums up 66 distinct chemical entities, as a few names include more than one congener (*i.e.* abamectin represented by abamectin B1a and abamectin B1b; spinosad represented by spinosyn A and spinosyn D; pyrethrins represented by cinerins I and II, jasmolins I and II and pyrethrins I and II).

To assess if a pesticide is raising issues regarding the LLC separation of CBD from a potentially contaminated hemp matrix, four *critical levels* were defined, based on the separation factor α, as follows:

- *highly critical (red-zoned) pesticides:* $\alpha_{CBD/PEST} < 1.5$;
- *medium critical (orange-zoned) pesticides*: $1.5 < \alpha_{CBD/PEST} < 2.0$;
- *low critical (yellow-zoned) pesticides*: $2.0 < \alpha_{CBD/PEST} < 4.0$;
- *non-critical (green-zoned) pesticides*: $4.0 < \alpha_{CBD/PEST}$.

In order to reduce the experimental effort, COSMO-RS was used to predict the partition coefficients of the pesticides ($P_{PEST}^{COSMO-RS}$), as well as the separation factors $\alpha_{CBD/PEST}$, calculated as the ratio of $P_{PEST}^{COSMO-RS}$ and $P_{CBD}^{COSMO-RS}$. 13 %, 34 % and 14 % of the pesticides were predicted as highly and medium critical pesticides in *solvent systems I*, *II* and *III*, respectively (Table 1). Practically, COSMO-RS indicated that *solvent systems I* and *III* would have the ability to remove a higher percentage of pesticides in comparison to *solvent system II*.

To validate these observations, the partition coefficients of CBD and Oregon-listed pesticides were experimentally determined for the *solvent systems I–III*. Experimental $\alpha_{CBD/PEST}$ values, defined as ratio of P_{PEST}^{EXP} and P_{CBD}^{EXP}, showed that the same percentages of red and orange-zoned pesticides as those predicted by COSMO-RS were found for *solvent systems I* (13 %) and *III* (14 %). According to the experimental values, 22 % of the pesticides were grouped as highly and medium critical for *solvent system II*. However, Table 1 does not depict which pesticides are

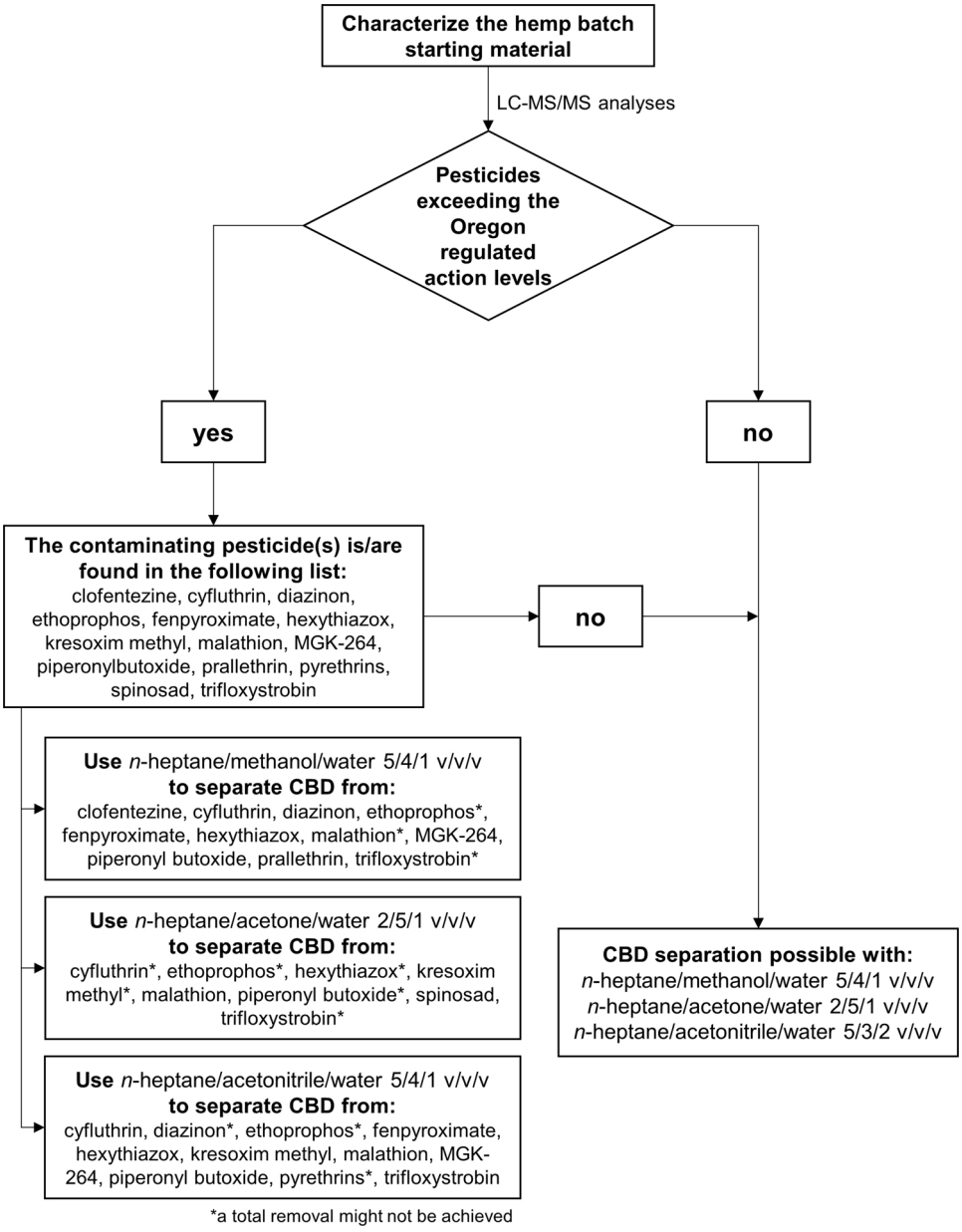

Fig. 3. Flow-sheet for selection of the biphasic solvent system for the simultaneous separation of CBD and removal of Oregon-listed pesticides from hemp extracts using liquid-liquid chromatography.

nominally critical and if the same pesticides found critical based on COSMO-RS predictions match the pesticide characterization based on experimentally obtained partition coefficients. Even though, COSMO-RS did not predict the exact values, it showed a considerably good prediction of the separation factors $\alpha_{CBD/PEST}$ (Tables 2 and S4). Some exceptions are represented by spinosyn A (predicted by COSMO-RS in the green zone in *solvent system I* and experimentally retrieved as highly critical) or clofentezine (shown as non-critical by COSMO-RS in *solvent systems II* and *III* and experimentally found in the red zone). As a general rule, a pesticide reported by COSMO-RS in the green zone (meaning 50–70 % of the Oregon-listed pesticides) was experimentally found in the same critical level.

To show the general tendency between the COSMO-RS predicted and experimentally determined partition coefficient values, $logP_{PEST}^{COSMO-RS}$ *vs.* $logP_{PEST}^{EXP}$ graphs were plotted for all three solvent systems (Fig. 2); it is worth mentioning that, since P_i values < 0.01 and > 100 cannot be considered experimentally accurate, only pesticides with $-2.0 < logP_i < 2.0$ were included in Fig. 1. Different solvent system-dependent tendencies can be observed: in *solvent systems I* and *II*, $P_{PEST}^{COSMO-RS}$ values were generally under-predicted as compared to P_{PEST}^{EXP} values, while an opposite situation (over-prediction) was observed in *solvent system III*. However, COSMO-RS provided the best prediction for *solvent system II*.

Finally, the above results are summarized in a solvent system selection guideline (Fig. 3) with the aim to help the user select the best solvent system for the purification of CBD from hemp extracts potentially contaminated with Oregon-listed pesticides. Before proceeding to the actual LLC separation, the user should firstly characterize the hemp batch in terms of the contaminating pesticides (usually performed in certified laboratories by LC–MS/MS analyses). If the batch is complying with the Oregon regulations (meaning no pesticide is found above the action levels), then any of the three solvent systems can be used for the purification of CBD, depending on which is preferred or more accessible to the user (in terms of cost-related aspects or local regulations). If one or more pesticides are found exceeding the limits, then the user has to check if that/those pesticides is/are retrieved in the following list:

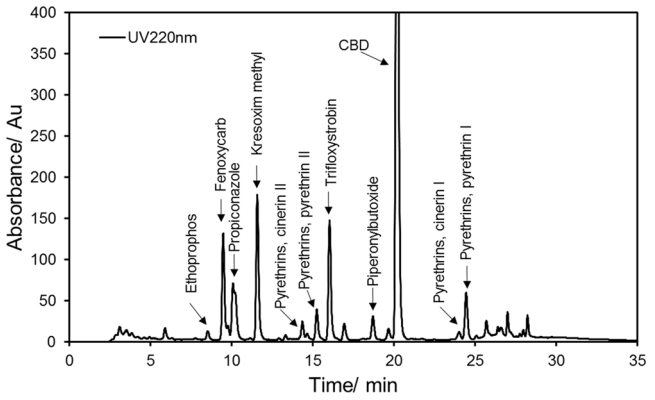

Fig. 4. HPLC-DAD chromatogram of hemp extract spiked with selected pesticides. Column: Agilent Poroshell 120 EC-C18 column (150 × 3 mm, 2.7 µm); mobile phase: water (A), acetonitrile (B); gradient: 0 min 60 % B, 20 min–80 % B, 21 min 95 % B, 25 min -95 % B, 30 min 60 % B, 35 min 60 % B; flow-rate: 0.25 mL/min; injection volume: 3 µL; UV: 220 nm; sample: hemp extract (5 mg/mL) spiked with 7 pesticides (50 ppm each).

clofentezine, cyfluthrin, diazinon, ethoprophos, fenpyroximate, hexythiazox, kresoxim methyl, malathion, MGK-264, piperonylbutoxide, prallethrin, pyrethrins, spinosad, trifloxystrobin. This list adds up the pesticides that were tagged as highly and medium critical in all three solvent systems. If the contaminating Oregon pesticides were not on the defined list, it means they are removable, no matter which of the three solvent systems might be used. If the contaminating pesticides were present on the list, then the most suitable solvent system is suggested. However, some precautions should be taken, as some of the pesticides (those marked with * in Fig. 3) may not be totally removable. This can result in a situation in which an additional purification step might be needed (*i.e.* second LLC separation step with a different solvent system). Having in hand only the results of the pesticide analysis of the hemp batch extracts, the knowledge acquired in the current study could help users, not only to select the adequate solvent system, but also decide which hemp batches have to be excluded from production or which, on the contrary, can still be kept and used for CBD isolation.

3.3. LLC experiments for CBD isolation from a pesticide-spiked hemp extract

To demonstrate the applicability and validity of the approach shown above for the selection of solvent systems for the preparative LLC separation of CBD from potentially contaminated hemp extracts, seven representative pesticides from the red, orange and yellow zones (namely, ethoprophos, fenoxycarb, kresoxim methyl, piperonyl butoxide, propiconazole, pyrethrins and trifloxystrobin) were selected. These were added to a decarboxylated hemp extract (Fig. 4) that was further subjected to LLC separations with each of the three solvent systems. The separations were performed on a lab-scale CCC column, in DSC mode at 1 mL min^{-1} and 1900 rpm. The feed mixture was injected in the column (V_{inj} = 1 mL) in a concentration of 5 mg mL^{-1} hemp extract (containing 250 – 350 ppm CBD) spiked with 50 ppm of each pesticide. In all separations, a stationary phase retention (S_F) of 0.6 was achieved. Even though the pesticide concentrations for spiking (50 ppm) might exceed those existing in real hemp batches, they were selected taking into consideration the higher LODs and LOQs (Table S5) of the UV detector of the HPLC-DAD system used to perform the off-line analyses of the collected fractions. The reconstructed off-line chromatograms presenting the individual fraction concentrations *vs.* elution time are depicted in Fig. 5. The peak shapes in Fig. 5 for CBD and each pesticide were obtained by fitting the experimental data points (×) into Gaussian equations with Origin2020 software. The on-line chromatograms recorded with the DAD detector of the CCC set-up (UV signal at

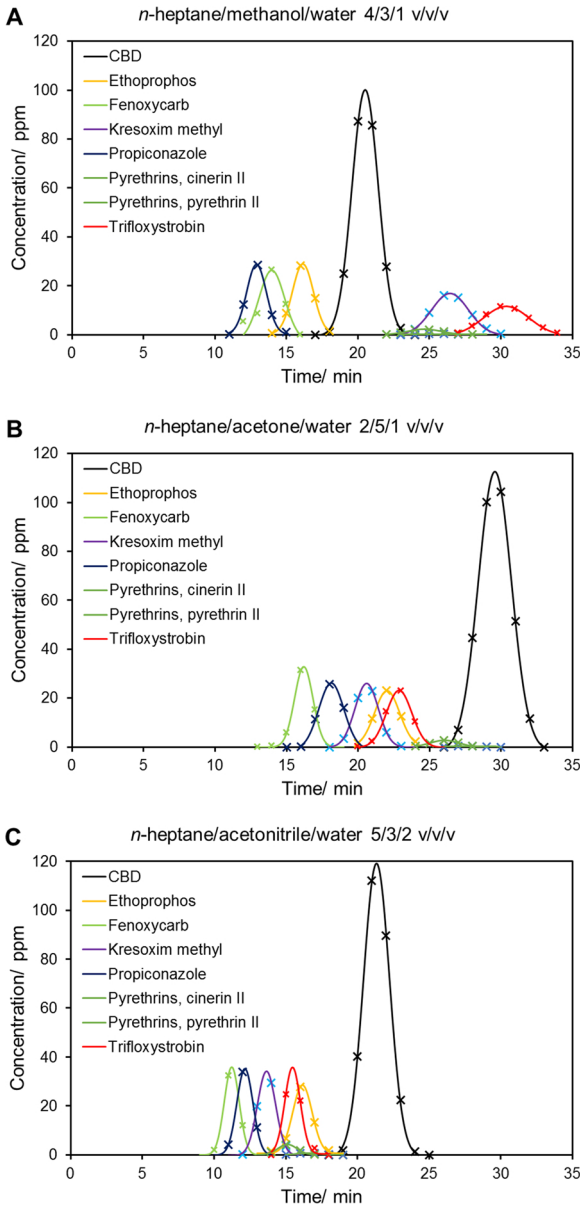

Fig. 5. Off-line reconstructed chromatograms of LLC separations of pesticides-spiked hemp extracts with (A) *Solvent system I*, (B) *Solvent system II* and (C) *Solvent system III*; DSC mode; 1900 rpm, 1 mL min^{-1}, V_{inj} = 1 mL; C_{inj} = 5 mg mL^{-1} hemp extract (containing 250-350 ppm of CBD) spiked with 50 ppm of each pesticide dissolved in mobile phase; V_{fr} = 1 mL; S_F = 60 %; points marked in "×" represent the concentrations obtained from off-line HPLC-DAD analysis of collected fractions; solid curve lines are obtained by fitting the experimental data points into Gaussian equations with Origin2020 software.

220 nm) is presented in Fig. S1 (Appendix A).

As expected from P_{CBD}^{EXP} values from Table 2, the yellow-zoned (fenoxycarb, propiconazole,) and green-zoned (piperonylbutoxide, cinerin I, pyrethrin I) pesticides as well as trifloxystrobin (orange zone, $\alpha_{CBD/PEST}$ = 2.0) were completely removed during the LLC separation with *solvent system I* (Figs. 5 and S1). Nevertheless, ethoprophos (orange zone, $\alpha_{CBD/PEST}$ = 1.8) and kresoxim methyl, cinerin I and pyrethrin II (red zone) were partly co-eluting with CBD peak. In the case of *solvent system II*, the pesticides from yellow (propiconazole, cinerin I, pyrethrin I) and orange (ethoprophos, kresoxim methyl, piperonylbutoxide) zones were eliminated, with the exception of the highly critical pesticides cinerin II and pyrethrin II that strongly overlapped with the CBD peak; trifloxystrobin ($\alpha_{CBD/PEST}$ = 1.5) only partly co-eluted. Since

none of the selected pesticides were in the red zone in *solvent system III*, the separation with this system showed the best outcomes, most of the pesticides being totally eliminated, while ethoprophos and cinerin II (orange zone) slightly overlapped with the CBD peak (Figs. 5 and S1).

4. Conclusions

In this work, a computer-aided approach for selecting biphasic solvent systems for simultaneous CBD isolation and pesticide removal from hemp extracts with LLC was proposed. By taking advantage of a fully predictive thermodynamic model, namely COSMO-RS, this work initially screened, with practically no experimental effort, a big number of ternary biphasic solvent systems in order to find the best candidates for the LLC purification of CBD from hemp extracts. After the experimental validation of the pre-selected systems, three solvent systems were chosen: *solvent system I*: *n*-heptane/methanol/water 4/3/1 v/v/v, *solvent system II*: *n*-heptane/acetone/water 2/5/1 v/v/v and *solvent system III*: *n*-heptane/acetonitrile/water 5/3/2 v/v/v. The 59 pesticides regulated by the state of Oregon in cannabis products were included in the next step with the aim to propose a classification system for the most critical pesticides to obtain pesticide-free CBD. Based on CBD/pesticide separation factors, (calculated using both COSMO-RS predicted and experimentally determined partition coefficients values), four critical levels were proposed, as follows: highly critical (red-zoned) pesticides, medium critical (orange-zoned) pesticides, low critical (yellow-zoned) pesticides and non-critical (green-zoned) pesticides. Since the three solvent systems showed different selectivity, a guideline for the proper selection of the best solvent system candidate for the simultaneous LLC separation of CBD and removal of Oregon-listed pesticides from particular hemp batches was proposed. For the proof-of-concept, a hemp extract spiked with several representative pesticides was subjected to LLC experiments with each of the three selected solvent systems. It was shown that the pesticide classification lists can be used to select the most promising solvent system for separation of the majority of the contaminating pesticides. The proposed approach is applicable to any LLC unit independent on its size. Nonetheless, after the selection of the solvent system, the operating conditions should be optimized for each particular hemp extract and LLC unit to achieve the maximum productivity for a specified CBD purity and/or recovery requirements.

Moreover, we would like to stress on the role of the thermodynamic models in providing valuable information that could significantly reduce the experimental efforts. Once the chemical structure of a contaminating pesticide is known, it can be used as input data for COSMO-RS, offering thus the possibility to extend the approach presented in this work to other groups of pesticides. Based on the predicted separation factors, the user can already have an idea if particular pesticides would interfere with the LLC separation of CBD. Furthermore, the proposed approach for the selection of solvent systems could be used in separating other cannabinoids (*i.e.* THC or cannabigerol) from different cannabis starting materials contaminated with pesticides.

CRediT authorship contribution statement

Simon Vlad Luca: Conceptualization, Methodology, Validation, Investigation, Project administration, Writing - original draft. **Simon Roehrer:** Conceptualization, Methodology, Validation, Funding acquisition, Writing - original draft. **Karin Kleigrewe:** Methodology, Validation, Investigation, Data curation. **Mirjana Minceva:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.112726.

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