Research Article

Received: 20 October 2017 Revised: 22 November 2017 Accepted article published: 28 November 2017 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.5524

Thermodynamic affinity-based considerations for the rational selection of biphasic systems for microbial flavor and fragrance production

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Abstract

BACKGROUND: Flavor and fragrance (F&F) compounds are increasingly produced by biotechnological instead of chemical means, as this allows them to be labeled *natural* additives. However, since most F&F compounds exhibit cytotoxicity towards common microbiological production hosts, *in situ* product removal strategies using two-phase partitioning bioreactors are desirable, making the rational selection of effective non-aqueous phases a crucial step. Here, thermodynamic first-principles methods and the experimental determination of partition coefficients were used to differentiate between sequestering phases with high and low thermodynamic affinity towards 17 important F&F compounds.

RESULTS: The approach was highly successful, enabling identification of outstanding extractants for several F&F compounds. Moreover, it was shown that certain solvent classes (e.g. long-chained alcohols and a variety of esters) function as efficient sequestering phases across all classes of target F&F compounds, whereas other solvent types (such as alkanes), and the liquid polymer silicone oil exhibit poor partitioning behavior. Finally, the tested absorptive solid polymers generally did not constitute effective sequestering phases for the target compounds, due to high fractions of hard/crystalline segments, however, this suggests that designing polymers with a higher proportion of soft segment could lead to enhanced sorptive capacity.

CONCLUSIONS: This study is the first in the current literature to systematically analyze sequestering phase choices for important F&F compounds and can serve as a guide for researchers working on biphasic microbial F&F production.

Supporting information can be found in the online version of this article.

Keywords: flavors and fragrances; two-phase partitioning bioreactors; biphasic systems; first-principles methods; *in situ* product removal

INTRODUCTION

Flavors and fragrances (F&Fs) fundamentally determine the appearance and appeal of present-day processed food and beverages, as well as household and cosmetic products. Their commercial significance is reflected in the F&F industry's world market value of approximately US$ 27 billion (IHS Markit: Specialty Chemicals Update Program - Flavors and Fragrances, 2014). In recent years, a strong market pull for more natural products has led to a successive replacement of chemically produced F&Fs by biotechnologically produced compounds,[1] as these can be labeled *natural* by US and European law as long as precursors isolated from nature are involved (US law: Code of Federal Regulations, Title 21, Food and drugs, part 101, sect 102.22(a)–(i); European law: Council Directive of 22 June 1988, 88/388/EEC, Official Journal of the European Communities, No L 184/61, 1988). Biotechnological production of F&Fs can be based either on the biotransformation of a natural precursor or on the *de novo*, fermentative synthesis from simple molecules such as sugars or amino acids.[2] Both routes can make use of naturally occurring microorganisms or genetically modified microorganisms (GMOs), however, *de novo* routes in GMOs seem to have a more promising future than biotransformations. This is largely due to the fact that no expensive or poorly-available precursors are required, as such routes utilize simple feedstocks such as monosaccharides, amino acids or renewable raw materials, and because continuously improved metabolic engineering strategies for popular microbiological hosts such as *Saccharomyces cerevisiae (S. cerevisiae)* facilitate the design of efficient bioprocesses.[3,4]

In spite of the possibility of engineering highly specialized production strains, the majority of common F&F compounds exhibits severe cytotoxicity towards the host cells, often ascribed to the molecules' tendency to compromise cellular membrane integrity and intracellular physiological processes as DNA, RNA and protein synthesis.[5–7] One strategy to overcome this limitation is the implementation of *in situ* product removal (ISPR) approaches which allow immediate and continuous removal of the inhibitory product from the vicinity of the biocatalyst.[8] In this context, two-phase partitioning bioreactors (TPPBs) with either liquid (e.g.

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Table 1. Overview of the selected representative F&F compounds with their chemical classification, their characteristic flavor or scent, literature references for *de novo* synthesis strategies, logP values and, if applicable, references for reported TPPBs (two-phase partitioning bioreactors) approaches

Compound	Class	Flavor/scent	Reported *de novo* synthesis	logP	Reported TPPB approaches
Benzyl alcohol	Alcohol, aromatic	Floral	26	1.1	9[b]
3-Methylbutanol	Alcohol, aliphatic	Fruity, roasted	30	1.2	-
2-Phenylethanol	Alcohol, aromatic	Floral (rose)	31	1.4	11[b],32[b]
Benzaldehyde	Aldehyde, aromatic	Almond-like	26[a]	1.5	9[b],33[b]
Isobutyraldehyde	Aldehyde, aliphatic	Malty	27	0.7	-
Vanillin	Aldehyde, aromatic	Vanilla	25	1.2	34[b],35[c]
Acetoin	Ketone, aliphatic	Buttery, creamy	24	−0.4	-
2-Heptanone	Ketone, aliphatic	Banana-like	10,36	2.0	10[b],36[b]
Raspberry ketone	Ketone, aromatic	Raspberry	37	1.3	-
Butyl butyrate	Ester, aliphatic	Fruity	38	2.8	38[b]
Isobutyl acetate	Ester, aliphatic	Fruity	39	1.8	39[b]
2-Phenylethyl acetate	Ester, aromatic	Sweet, honey	39	2.3	-
Geraniol	Monoterpenoid	Floral	40	3.2	41[b],42[b]
Linalool	Monoterpenoid	Floral	40	3.0	-
(S)-Limonene	Monoterpene	Herbal	43	4.4	43[b],44[b]
(R)-γ-Decalactone	Lactone	Peach-like	45	2.7	45[c]
6-Pentyl-α-pyrone	Lactone	Sweet, coconut-like	13	1.8	13[b],46[c],47[b]

[a] If additional strain engineering is applied to prevent the reduction to benzyl alcohol.
[b] Solute uptake into non-aqueous phase based on absorption.
[c] Solute uptake into non-aqueous phase based on adsorption.

decane, hexadecane, silicone oil, PPG 1200) or solid (e.g. Kraton, Hytrel, Desmopan) non-aqueous sequestering phases have been applied for the microbial production of F&Fs.[9–14] One challenge in the utilization of this processing strategy is the selection of a suitable sequestering phase that is compatible with both the process requirements and the microorganism being used. Instead of simply using non-aqueous phases that are 'on hand' in the laboratory and testing them for their efficacy, a more scientific and rational selection strategy is highly preferable. Different selection approaches have been applied for a variety of ISPR/TPPB bioprocesses in the past, either based on the assessment of the sequestering phase's safety,[15] or based on the evaluation of thermodynamic affinity between the target compound and the sequestering phase.[5,16,17] In this latter instance, thermodynamic first-principles methods such as UNIFAC[18,19] and Hansen or Hildebrand Solubility Parameters[20,21] have been used to predict phase equilibria in multiphasic systems.

Nevertheless, a comprehensive study and analysis of the rational selection of sequestering phases in biphasic systems for microbial F&F production is still missing in the literature. The present work aimed to fill this gap by, on the one hand, retrospectively examining previously-published two-phase partitioning systems for the performance of the selected sequestering phases for representative F&F compounds and, on the other hand, by applying thermodynamic first-principles methods and experimentally determining partition coefficients for selected systems. Finally, both the retrospective and the results obtained were combined to draw conclusions and recommendations for the selection of sequestering phases for microbial biphasic F&F production.

MATERIALS AND METHODS

Chemicals

The polymers Hytrel 8171 and Elvax 40 W were obtained from DuPont Canada. Silicone oil (icosamethylnonasiloxane with a viscosity of 10 cSt (25 °C) and an average molecular weight of 700 g mol^{-1}) was obtained from Scientific Polymer Products Inc. (Ontario). All other polymers and sequestering phases were purchased in technical grade from Sigma Aldrich (Oakville, Canada), whereas all F&F compounds were purchased from the same company at the highest purity available. A list of the used F&F compounds is shown in Table 1, with the rationale for their selection described later in the text.

Determination of partition coefficients

Partition coefficients (PCs) of F&F compounds, e.g. the target compounds, in different biphasic systems were determined in triplicate. All preparations were placed in glass scintillation vials with a liquid capacity of 24 mL and foil lined caps. In all cases, the aqueous phase was Type 1 ultrapure water. For liquid–liquid biphasic systems, the target F&F compounds were dissolved in the non-aqueous phases and these mixtures were contacted with water (20% (v/v) non-aqueous phase mixed with target compound), yielding overall target compound concentrations between 0.4 and 2 g L^{-1}. For solid–liquid biphasic systems, the polymers were washed in methanol and subsequently in Type 1 ultrapure water for 3 h each and air-dried overnight before use. The polymers (5% (w/w)) and Type 1 ultrapure water were placed in scintillation vials, followed by the addition of the target compounds with an aqueous concentration of 2 g L^{-1}. All vials were incubated at 30 °C and 180 rpm, either for at least 3 h for liquid–liquid mixtures or for at least 24 h for systems involving polymers. Subsequently, liquid–liquid preparations were centrifuged at 2800 rcf for 10 min, whereas solid–liquid preparations were processed directly. All aqueous phases were filtered (0.2 µm), if necessary diluted, and aqueous concentrations of the target compounds were quantified either by GC or HPLC. Mass balances were performed to determine the concentrations of the target compounds in the non-aqueous phases and finally PCs were

determined by calculating the ratio of the target compound's mass fractions in the non-aqueous and aqueous phase.

Quantification of F&F compounds

Benzyl alcohol, 2-phenylethanol, raspberry ketone, vanillin, benzaldehyde, 2-phenylethyl acetate, (S)-limonene, geraniol, linalool and 6-pentyl-α-pyrone were quantified by reversed-phase HPLC (ProStar, Varian), using a Viva C18 column (particle size 5 µm; 150 × 4.6 mm; Restek) kept at 60 °C, and UV detection. The flow rate was 1 mL min^{-1}, the injection volume was 10 µL. A binary gradient system consisting of water (solvent A) and 100% acetonitrile (solvent B) was applied. For benzyl alcohol, 2-phenylethanol, raspberry ketone and benzaldehyde, the following gradient sequence was used: 0–1 min 30% B, 1–3 min 30–100% B, 3–3.5 min 100% B, 3.5–4 min 100–30% B, then 1 min equilibration time. For vanillin, the following gradient sequence was used: 0–2.5 min 30% B, 2.5–3.5 min 30–100% B, 3.5–4 min 100–30% B, then 1 min equilibration time. 2-Phenylethyl acetate was eluted isocratically (50% acetonitrile) for 6 min, with 1 min equilibration time. For (S)-limonene, geraniol, linalool and 6-pentyl-α-pyrone, the following gradient sequence was used: 0–1 min 30% B, 1–3 min 30–100% B, 3–5 min 100% B, 5–7 min 100–30% B, then 2 min equilibration time. External standards were used for calculation of the target compound concentrations in the samples. All wavelengths used for detection and the elution times for the different compounds can be found in Table S1 (Supplementary Online Material).

3-Methylbutanol, 2-heptanone, acetoin, isobutyraldehyde, isobutyl acetate, butyl butyrate and (R)-γ-decalactone were quantified by GC (450-GC, Varian) equipped with a Rtx-502.2 column (particle size: 1.4 µm; 30 m × 0.25 mm; Restek) and a flame ionization detector. The carrier gas helium was used at a flowrate of 2.2 mL min^{-1}. Nitrogen, hydrogen and air flowrates were 25, 30 and 300 mL min^{-1}, respectively. The injection volume was 0.5 µL. Except for the analysis of (R)-γ-decalactone, the injector was kept at 215 °C, the detector at 250 °C and the oven temperature was ramped up from 35 to 215 °C at a rate of 40 °C min^{-1}, with an overall run time of 5.5 min and an equilibration time of 2 min. For the quantification of (R)-γ-decalactone, the injector was kept at 250 °C, the detector at 300 °C and the oven temperature was ramped up from 35 to 250 °C at a rate of 40 °C min^{-1}, with an overall run time of 6.5 min and an equilibration time of 2 min. External standards were used for calculation of the target compound concentrations in the samples. The elution times for the different compounds are listed in Table S1 (Supplementary Online Material).

Thermodynamic first-principles methods

Hansen solubility parameters (HSPs)

HSPs and *Ra* distances were calculated with the software HSPiP (5th edition, version 5.0.05, https://www.hansen-solubility.com/).[17] HSPs are predicated on the subdivision of binary, mutual solubility into three contributions: atomic dispersion interactions (δ_D), molecular dipole interaction (δ_P) and hydrogen bonding (δ_H). The total solubility parameter δ_{tot} can be determined by extracting the root of the sum of the squares of the individual parameters:

$$\delta_{tot} = \sqrt{\delta_D^2 + \delta_P^2 + \delta_H^2}$$

Moreover, the single contributions can be used to calculate the HSP 'distance' *Ra* between two materials:

$$Ra^2 = 4 * \left(\delta_{D2} - \delta_{D1}\right)^2 + \left(\delta_{P2} - \delta_{P1}\right)^2 + \left(\delta_{H2} - \delta_{H1}\right)^2$$

A low *Ra* distance implies high affinity between solute and solvent or polymer, arising from similar solubility parameters.[20] The software's 'Master Dataset' consisting of 1237 liquid sequestering phases and a dataset consisting of 530 polymers were used for the selection of suitable biphasic systems by selecting solute-sequestering pairs with small *Ra* distances.

Extractant screening program (ESP) based on UNIFAC

As a second thermodynamic method, ESP was used.[5] This program is based on UNIFAC (*UNI*QUAC Functional-group Activity Coefficients), a 'group-contribution method … for the prediction of activity coefficients in nonelectrolyte liquid mixtures'.[18] ESP can predict PCs for ternary systems that include the effect of the presence of water; this is in contrast to the use of HSPiP which considers only binary (solute–extractant) interactions. The qualitative ranking of different solvents can provide reliable data which can be used for the selection of suitable biphasic systems.[5] In the current study, approx. 1500 different solvents were screened in ternary systems consisting of 74% (w/w) water, 20% (w/w) solvent and 6% (w/w) target compound. The systems' temperature was set to 30 °C as this is a common temperature for bioprocesses.

RESULTS AND DISCUSSION

Selection of representative F&F compounds for subsequent thermodynamic affinity studies

In order to create a study as comprehensive as possible, special focus was placed on the selection of target compounds that can adequately represent the vast variety of different classes and structures of F&F molecules by utilizing a range of different selection criteria. First, only compounds with previously-reported *de novo* synthesis routes either in naturally occurring or in genetically modified microorganisms were chosen, reflecting the trend towards the replacement of biotransformations of externally added precursors with more sustainable and economical *de novo* pathways.[4,22] Second, different structural classes of F&F molecules were selected. As a starting point, Schrader's (2007) classification of F&Fs was used,[22] and six main classes of compounds were selected, namely alcohols, aldehydes, ketones, esters, monoterpenes/-terpenoids and lactones. Carboxylic acids, such as L-glutamic acid or citric acid, were excluded from the study as their potential dissociation into ions prevents the application of thermodynamic first-principles methods of either Hansen or UNIFAC. Other classes, such as aromatic heterocycles or compounds containing sulfur, were excluded due to the small number of associated F&F compounds.

Subsequently, four additional subcriteria were applied to select representative target compounds within each of the six main classes. First, only molecules with logP values (logP: logarithmic partition coefficient of a target compound in a biphasic system consisting of octanol and water)[23] lower than 3.4 were chosen in order to provide a rationale for the use of a biphasic system. The smaller the logP for a certain molecule, the higher is its hydrophilicity and thus the tendency to accumulate in the aqueous phase, where the biocatalyst is located, and to interfere with the cells' physiological processes.[5] The critical logP value, which prevents further metabolic activity or growth, is strongly dependent on the microorganism being used. Different strains of *Escherichia coli* (*E. coli*), a common host for the production of various platform chemicals, including F&F compounds,[24–27] have a critical logP of approximately 3.4.[28] For *S. cerevisiae*, the critical logP lies around 4.[29]

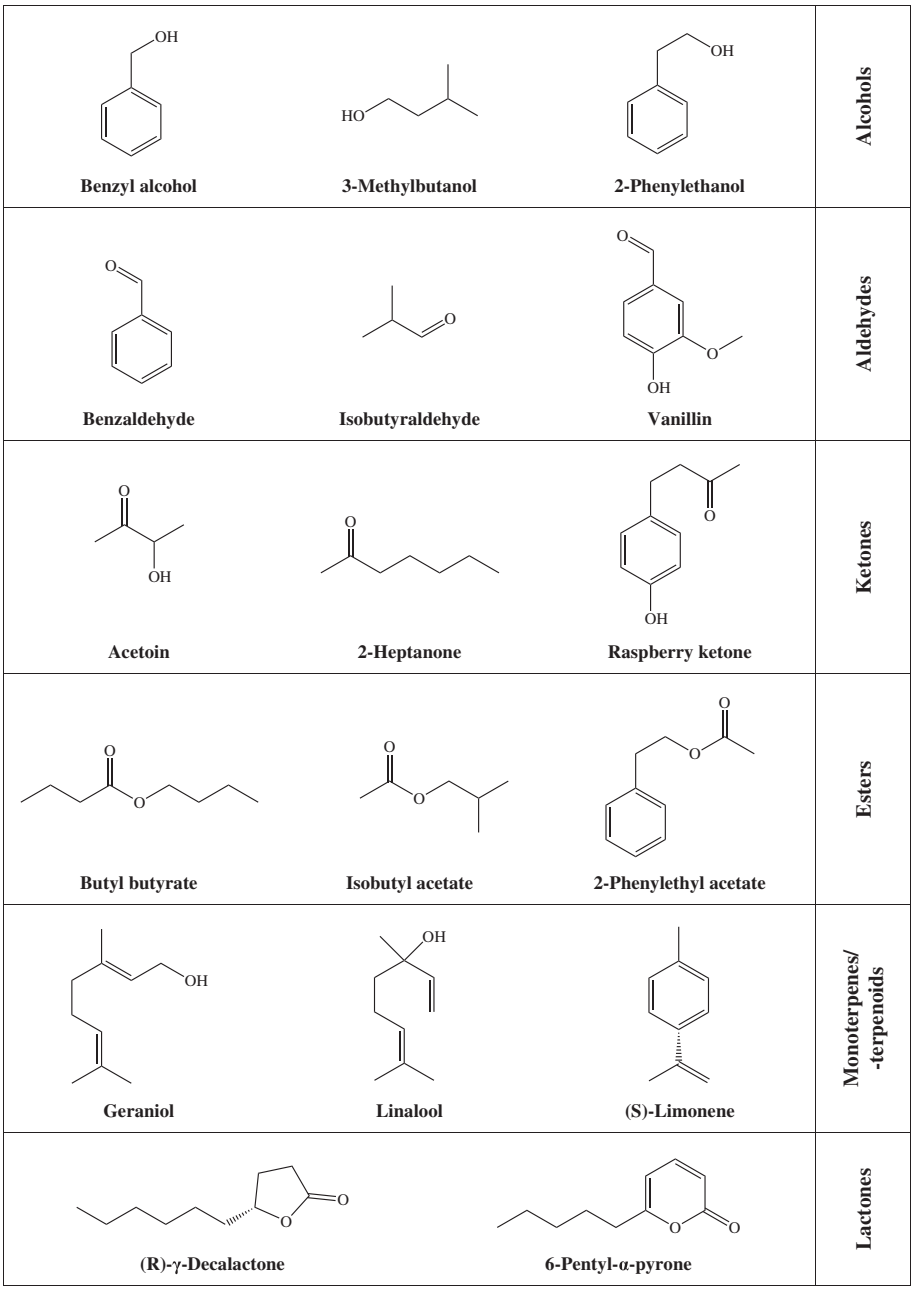

Figure 1. Chemical structures and associated classes of the selected representative F&F compounds.

To ensure that the two microorganisms most frequently used for F&F production are inhibited by the selected F&F compounds, and therefore to justify the use of ISPR, their respective logP values were to be lower than 3.4. The second subcriterion was to pick compounds with potential industrial relevance, either based on their market volume or on their unique flavor or scent. Third, within one main class of compounds the structural diversity was to be as high as possible, and thus both aliphatic and aromatic molecules were chosen within a class. Fourth, the selected compounds included ones which have been, and have not yet been, produced using ISPR approaches to allow for a comparison of retrospective and future F&F bioproductions.

All these considerations resulted in the selection of 17 representative F&F target compounds, two within the lactone group and three within all the other main classes, shown in Table 1, with chemical structures shown in Fig. 1. Except for (S)-limonene, all compounds meet the criterion of exhibiting a logP lower than 3.4. (S)-Limonene was included in the selection nonetheless, as on the one hand both enantiomers of this monoterpene ((R)-enantiomer: orange scent, (S)-enantiomer: herbal scent) depict the most commonly used fragrance compounds in household and cosmetic products,[48] and on the other hand this monoterpene, despite its high logP value of 4.4 and thus distinctively low water solubility (4.6 mg L^{-1} at 25 °C), exhibits severe toxicity towards *S. cerevisiae*.[44]

Comparison between predicted and experimental partitioning behavior of F&F compounds

ESP and HSPiP were used as thermodynamic first-principles methods to identify sequestering phases with both high (predicted to be a good extractant) and low (predicted to be a poor extractant)

thermodynamic affinity for the 17 representative target compounds. High thermodynamic affinity between two compounds implies their miscibility at the molecular level; the applied thermodynamic methods predict solubility by either using solubility parameters (HSPs) or activity coefficients (ESP) to calculate the Gibbs free energy of a system.[19,20,49] The HSPiP software sorts sequestering phases by their *Ra* distance to the target compound, whereas ESP ranks them according to their predicted PCs.

Only solvents with logP values higher than 4, boiling points higher than 250 °C and melting points lower than 20 °C were considered. The choice of these parameters ensured the solvents' biocompatibility with the common production strains *E. coli* and *S. cerevisiae*,[5] reasonably low volatility and hence low associated safety hazards (compounds with boiling points lower than 250 °C classify as *volatile*, as stated by the World Health Organization), and liquid state at room temperature. Solid polymers were screened only with HSPiP and not with ESP, as the latter program was not designed to predict PCs for systems involving solid polymers.

For each target compound, between two and four sequestering phases with predicted high thermodynamic affinity and one or two with predicted low affinity were selected, with one of the overall three to five sequestering phases being a (solid or liquid) polymer. The variation in the number of sequestering phases with predicted high thermodynamic affinity pre-selected for different target compounds can be ascribed to the intention of choosing sequestering phases from a variety of different chemical classes (e.g. alkanes, alkenes, alcohols, esters) represented in the top results of HSPiP and ESP. Owing to different original datasets as well as different algorithms, the HSPiP and ESP ranking results did not generally match up in terms of predicted extractants. Therefore, the top results from each method were picked, although solvents from the phthalate class, which were frequently highly ranked, were excluded due to severe health concerns associated with their application and a current shift in industry towards their stepwise replacement.[50] If TPPB approaches for a target compound had already been reported in the literature (see Table 1), the applied sequestering phases were also included as one of the overall sequestering phases, as an example of a 'retrospective' examination of somewhat random extractant selection. For all systems without PCs already reported in the literature, the PCs were determined experimentally.

In Figs 2 and 3 the PCs for all 17 F&F compounds in the different pre-selected biphasic systems are presented. In order to make the concept of the graphical representation more accessible, the partitioning of two selected target compounds will be described in more detail in the following. The partitioning behavior of the compound benzyl alcohol, which is presented in the top left in Fig. 2, was determined experimentally in four different biphasic systems. Moreover, its uptake by the polymer Hytrel 8171 is already described in the literature,[9] which is why the respective bar on the very right side of the graph is marked with a hash (retrospective) and an asterisk (polymer). Both ESP and HSPiP predicted hexadecane to be a poor extractant for this target compound and the very low experimental PC in the system with this sequestering phase, which is presented on the very left of the graph (dotted bar), confirms these predictions. The systems with the four sequestering phases predicted to be good, 10-undecen-1-ol, dibutyl maleate, oleyl alcohol and Hytrel 8171, exhibit significantly higher PCs. The second example, the partitioning behavior of the lactone 6-pentyl-α-pyrone in five different systems, is depicted in Fig. 3 at the bottom right. The PCs in the two systems with the sequestering phases predicted to be poor (hexadecane and the polymer PEG 1450) were not determined experimentally in this work, but were taken from the literature,[13,47] therefore, the respective dotted bars are again marked with hashes. Again, the sequestering phases predicted to be good, dibutyl maleate, diethyl sebacate and the polymer Hytrel 8171, show significantly higher PC values than the two systems previously described in the literature.

When looking at the entirety of the data presented in Figs 2 and 3, three major trends can be deduced. First, polymers which were predicted by HSPiP to be good sequestering phases, such as DuPont's Hytrels, generally tend to underperform in experimental absorption determinations. In some cases, e.g. Hytrel 8171 for the target compound 2-heptanone, polyethylene for butyl butyrate and polystyrene for 2-phenylethyl acetate, the determined PCs even lie below the PCs of biphasic systems with sequestering phases predicted to be poor. The often-low PCs in systems involving polymers are due to the fact that in using HSPiP only the predicted thermodynamic affinity between the 'active', absorbing fraction of a polymer and the target compound, and not the polymer as a whole, is considered. Solute uptake is affected by the chain mobility of a polymer and is strongly dependent on the proportion of crystalline/hard segments and amorphous/soft segments; a higher fraction of amorphous/soft segments results in higher overall PCs.[17,51] The material properties (e.g. hardness) of solid polymers often relate to the presence of crystalline regions in homopolymers, or the inclusion of hard segments in co-polymers, but these detract from the overall ability of polymers to absorb solutes. As commercially available polymers are often designed to have significant rigidity (e.g. high impact strength) and are not specially designed for TPPB applications (solute absorption), the fraction of the amorphous/soft segments in a polymer, if specified by the manufacturers at all, tends to be rather low. This does provide significant opportunity, however, for the optimization of biphasic systems involving polymers by selecting specific polymer grades, or designing customized materials with a higher proportion of segments actively participating in the absorption of target compounds. Overall, unexpectedly low PCs in biphasic systems involving polymers shown in Figs 2 and 3 are likely a result of polymers containing a high proportion of rigid segments that do not contribute to the target compounds' absorption. In contrast, as shown in Fig. 2, the three PCs for solid polymer biphasic systems derived from the literature (Hytrel 8171 for benzyl alcohol and benzaldehyde[9] and Hytrel 8206 for 2-phenylethanol[32]) are all significantly higher than the other sequestering phases tested for the respective target compounds. This suggests that the amorphous, absorptive components of these polymers are particularly effective at sequestering these particular target molecules.

A second trend emerging in the data presented is the fact that certain chemical classes of liquid extractants were shown to be effective sequestering phases for target compounds across all chemical F&F classes, while liquids from other classes were very poor. The two long-chain alcohols oleyl alcohol and 10-undecen-1-ol, and a variety of esters, most frequently dibutyl maleate and diethyl sebacate, were repeatedly top-ranked in ESP and HSPiP across all chemical classes of target compounds, and experimental determination of PCs confirmed their suitability as 'universal' sequestering phases for the absorption of F&F compounds. Thermodynamic affinity between two substances is based on intermolecular interactions,[20] and due to their molecular structure (long chains, presence of a double bond in most instances, alcohol and ester functionalities) the effective solvents belonging to the classes of alcohols and esters can interact with the target compounds through nonpolar Van der Waals forces,

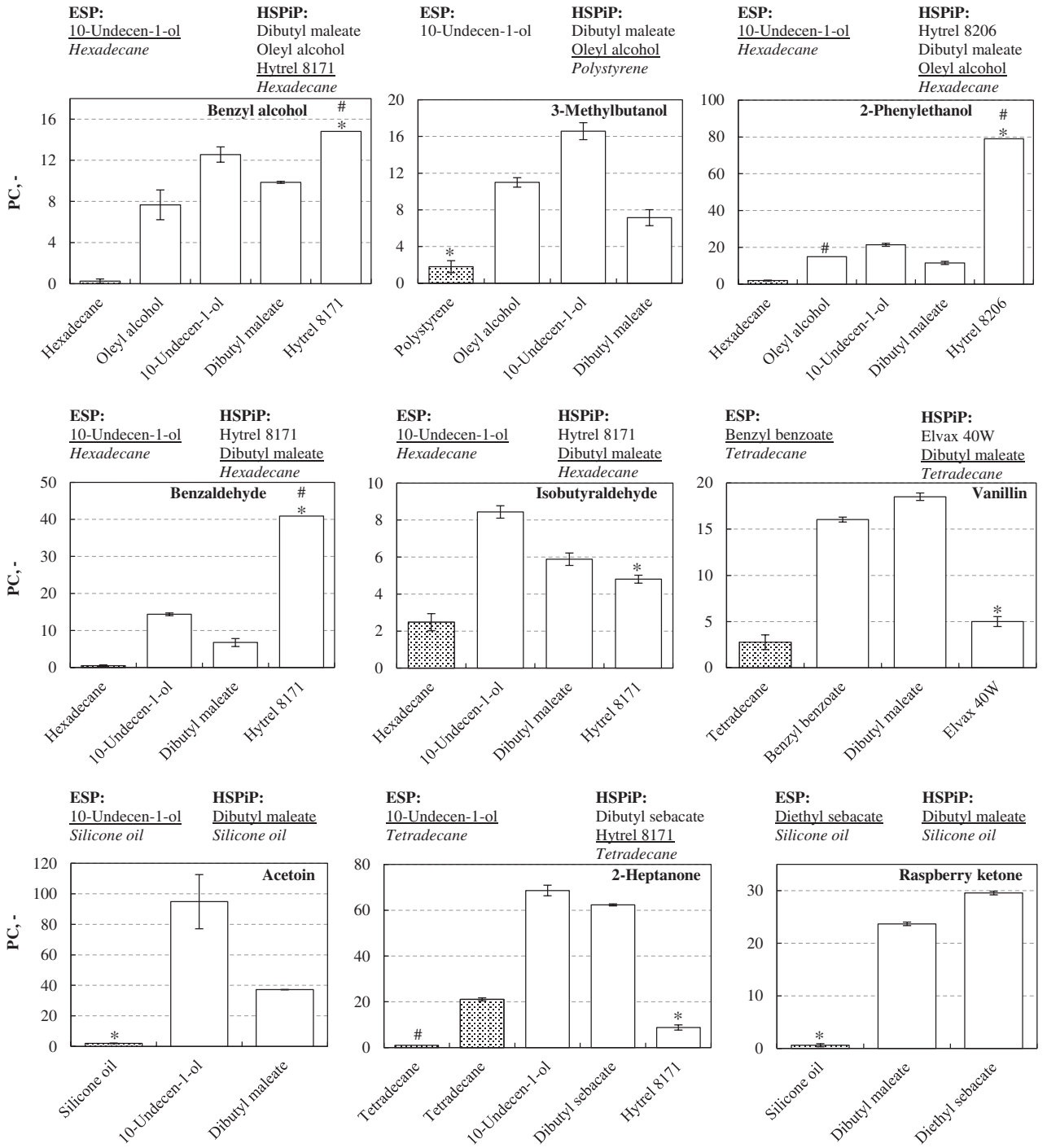

Figure 2. Partition coefficients with standard deviations (n = 3) for F&F compounds belonging to the chemical classes of alcohols, aldehydes and ketones in different biphasic systems with water as the aqueous phase. Above each chart, the sequestering phase rankings provided by ESP and HSPiP are listed, with sequestering phases printed in Roman being predicted as effective ones and with sequestering phases printed in *italic* being predicted as poor choices. Effective and unsuitable sequestering phases are separated by a horizontal line. White bars represent partition coefficients in biphasic systems with sequestering phases predicted to be effective, whereas dotted bars are used for systems with sequestering phases predicted to be poor. Asterisks (*) highlight systems which involved a polymer as the sequestering phase, hashes (#) indicate that the respective partition coefficient values are reported in the literature and were therefore not determined experimentally in this work.

polar dipole–dipole interactions and hydrogen bonding. This also becomes apparent when comparing the HSPs of olelyl alcohol, 10-undecen-1-ol, dibutyl maleate and diethyl sebacate with the HSPs of the alkane hexadecane and the liquid polymer silicone oil, which were predicted to be poor sequestering phases for target compounds across all chemical classes (see Table 2). Whereas the suitable extractants exhibit high δ_P and δ_H parameters, facilitating strong intermolecular interactions with the target compounds, hexadecane and silicone oil manifest only a modest δ_D parameter, but very low δ_P and δ_H values. Moreover, the δ_{tot} values of the effective sequestering phases are significantly closer to the δ_{tot} of the target compounds than those of

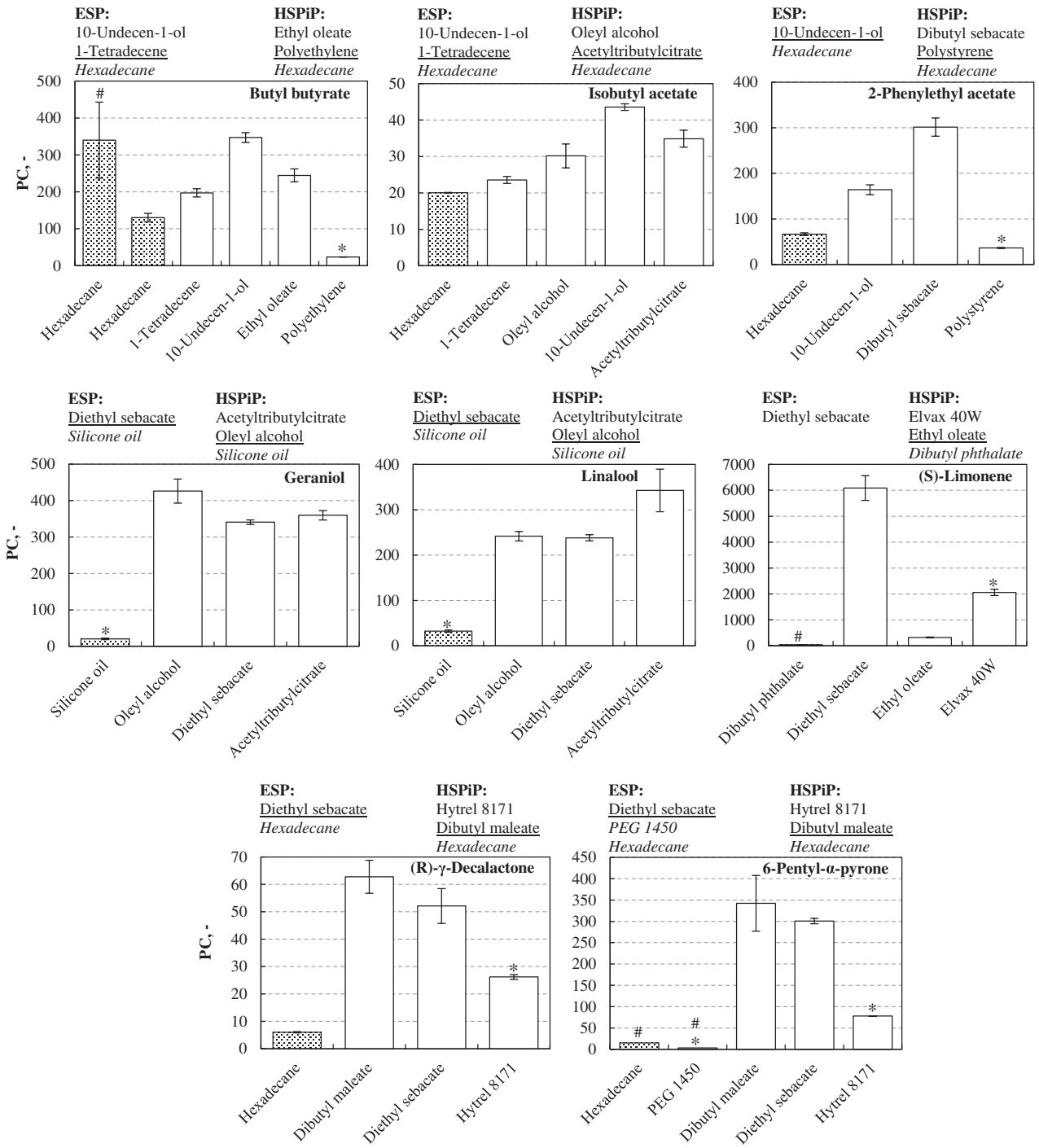

Figure 3. Partition coefficients with standard deviations (n = 3) for F&F compounds belonging to the chemical classes of esters, monoterpenes/-terpenoids and lactones in different biphasic systems with water as the aqueous phase. Above each chart, the sequestering phase rankings provided by ESP and HSPiP are listed, with sequestering phases printed in Roman being predicted as effective ones and with sequestering phases printed in *italic* being predicted as poor choices. Effective and unsuitable sequestering phases are separated by a horizontal line. White bars represent partition coefficients in biphasic system with sequestering phases predicted to be effective, whereas dotted bars are used for systems with sequestering phases predicted to be poor. Asterisks (*) highlight systems which involved a polymer as the sequestering phase, hashes (#) indicate that the respective partition coefficient values are reported in the literature and were therefore not determined experimentally in this work.

hexadecane and silicone oil, resulting in smaller *Ra* distances and thus higher affinities. This is especially true for silicone oil, whose δ_{tot} of 11.8 is much smaller than the δ_{tot} values of the target molecules. Patel *et al.* demonstrated that silicone oil cannot function as an efficient sequestering phase for the absorption of alcohols, ketones or esters – chemical classes which include many F&F compounds – and that only compounds belonging to the classes of alkanes, aromatic hydrocarbons and chlorinated substances partition into silicone oil with high PCs.[52] However, the latter do not constitute F&F compounds and our results, based on thermodynamic affinity studies, confirm that silicone oil is a poor sequestering phase for F&F molecules. In contrast, the use

Table 2. Hansen solubility parameters (atomic dispersion interactions: δ_D, molecular dipole interaction: interactions δ_P, hydrogen bonding: δ_H, total solubility parameter: δ_{tot}) of all target compounds and of selected sequestering phases. Oleyl alcohol, 10-undecen-1-ol, dibutyl maleate and diethyl sebacate were predicted by ESP and HSPiP to be effective sequestering phases for target compounds across all chemical classes, whereas the alkane hexadecane and the liquid polymer silicone oil were predicted to be poor extractants

		Hansen solubility parameter, $MPa^{0.5}$			
Compound	Description	δ_D	δ_P	δ_H	δ_{tot}
Benzyl alcohol	Target compounds	19.0	6.0	12.5	23.5
3-Methylbutanol		15.6	5.5	11.4	20.1
2-Phenylethanol		18.5	5.6	11.4	22.5
Benzaldehyde		18.9	8.0	6.3	21.4
Isobutyraldehyde		15.4	8.3	5.4	18.3
Vanillin		19.6	10.7	12.5	25.6
Acetoin		17.2	10.6	13.8	24.4
2-Heptanone		16.0	5.5	4.2	17.5
Raspberry ketone		18.7	7.6	9.7	22.4
Butyl butyrate		15.9	3.8	5.0	17.1
Isobutyl acetate		16.0	3.7	11.1	19.8
2-Phenylethyl acetate		17.7	4.1	5.6	19.0
Geraniol		16.9	4.2	7.6	19.0
Linalool		16.8	2.9	6.9	18.4
(S)-Limonene		16.7	1.9	3.2	17.1
(R)-γ-Decalactone		16.4	10.0	4.5	19.7
6-Pentyl-α-pyrone		16.8	10.3	5.4	20.4
Oleyl alcohol	Sequestering phase, alcohol	16.2	2.6	7.3	18.0
10-Undecen-1-ol	Sequestering phase, alcohol	16.2	3.8	7.7	18.3
Dibutyl maleate	Sequestering phase, ester	16.5	6.1	7.2	19.0
Diethyl sebacate	Sequestering phase, ester	16.0	4.0	4.7	17.2
Hexadecane	Sequestering phase, alkane	15.9	0.1	0.1	15.9
Silicone oil	Sequestering phase, liquid polymer	11.7	0.4	0.7	11.8

of solvents belonging to the classes of alcohols and esters as sequestering phases for F&F compounds (namely, benzaldehyde, geraniol, limonene and 2-phenylethanol) is already described in different articles in the literature,[11,33,41,44] however, with a lack of explanation for the suitability of such solvents based on thermodynamic first-principles methods, as it is proposed here.

The third trend can be best seen in Figs 4 and 5, where the data from Figs 2 and 3 is grouped by the chemical classes of the F&F target compounds, and the PC values are normalized to the highest value within the data series of each target compound. Here, it becomes very apparent that the applied approach of pre-selecting sequestering phases based on thermodynamic affinities predicted by first-principles methods was successful. Sequestering phases which were predicted by ESP and HSPiP to be ineffective for absorbing the target compounds were experimentally proven to indeed be poor, whereas sequestering phases predicted to be effective experimentally resulted in significantly higher PCs than those predicted to be unsuitable. This is true for all target compounds across all chemical classes, except for certain systems where polymers were involved, resulting in unexpectedly low PC values for systems with polymers predicted to be good sequestering phases. However, this phenomenon, as described earlier, can be attributed to the fact that these polymers contain substantial amounts of crystalline/hard segments which are not actively contributing to the absorption of the target compound. As also noted, selecting different polymer grades, or designing polymers with a higher proportion of soft segment may be a fruitful area of further ISPR/TPPB research 'driven' by the thermodynamic considerations described here, especially as inert and reusable polymers can meet the increasing call for eco-friendlier ('greener') biphasic bioprocesses.

The use of ionic liquids (ILs) as potentially 'green' sequestering phases in ISPR/TPPB systems has been proposed recently, however successful experimental confirmation of their efficacy has largely fallen short of expectations, primarily because of the cytotoxic nature of many ILs.[53] Due also to the significant potential for ionic interactions in the use of ILs, rather than functional group interactions as described here, the 'group contribution' approaches of Hansen and UNIFAC are not likely to predict IL behavior. However, the recurring cytotoxicity of ILs may have been addressed, as in a recent paper we have shown that ILs can be polymerized as *polyionic liquids*, thereby conferring complete biocompatibility, while retaining partitioning effectiveness.[54] We believe that the use of customized absorptive polymers as well as polyionic liquids is a promising avenue of research for future 'green' ISPR/TPPB applications.

Getting back to the analysis of our data presented in Figs 4 and 5, the ESP/HSPiP ranking order for sequestering phases predicted to be good is not consistently reflected by the order of the experimental PC values, which can be attributed to the fact that pre-selected sequestering phases often manifested very similar 'in-silico' PCs (ESP) or Hansen *Ra* distances (HSPiP). Although first-principles methods can never predict the behavior of real systems with full certainty, the main outcome is the fact that the applied approach successfully distinguished between effective and poor sequestering phases for F&F compounds. In this context it should be noted that this work can provide researchers working on the microbial production of F&F compounds with

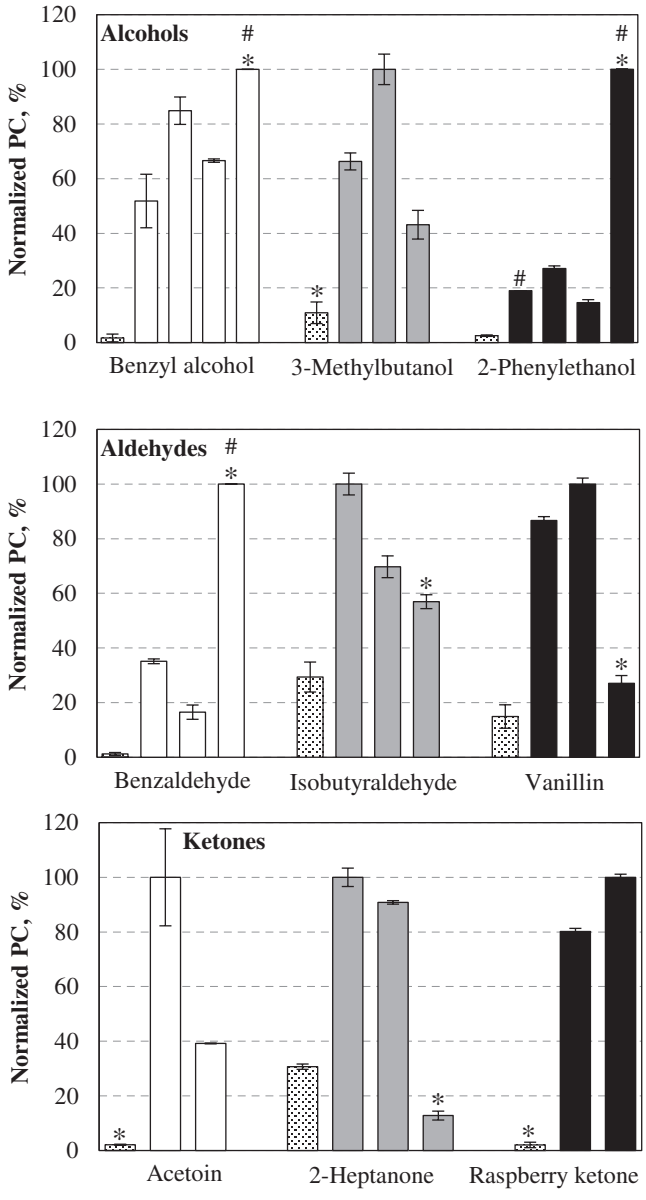

Figure 4. Partition coefficients with standard deviations (n = 3) for F&F compounds belonging to the chemical classes of alcohols, aldehydes and ketones in different biphasic systems with water as the aqueous phase. Each chart presents data from one chemical class of target compounds, different amounts of shading are used for different target compounds. The partition coefficient values are normalized to the highest value for each target compound. Dotted bars, always on the very left side of each data series, are used for systems with sequestering phases predicted to be poor. Asterisks (*) highlight systems that involved a polymer as sequestering phase, hashes (#) indicate that the respective partition coefficient value is reported in literature and was not determined experimentally in this work.

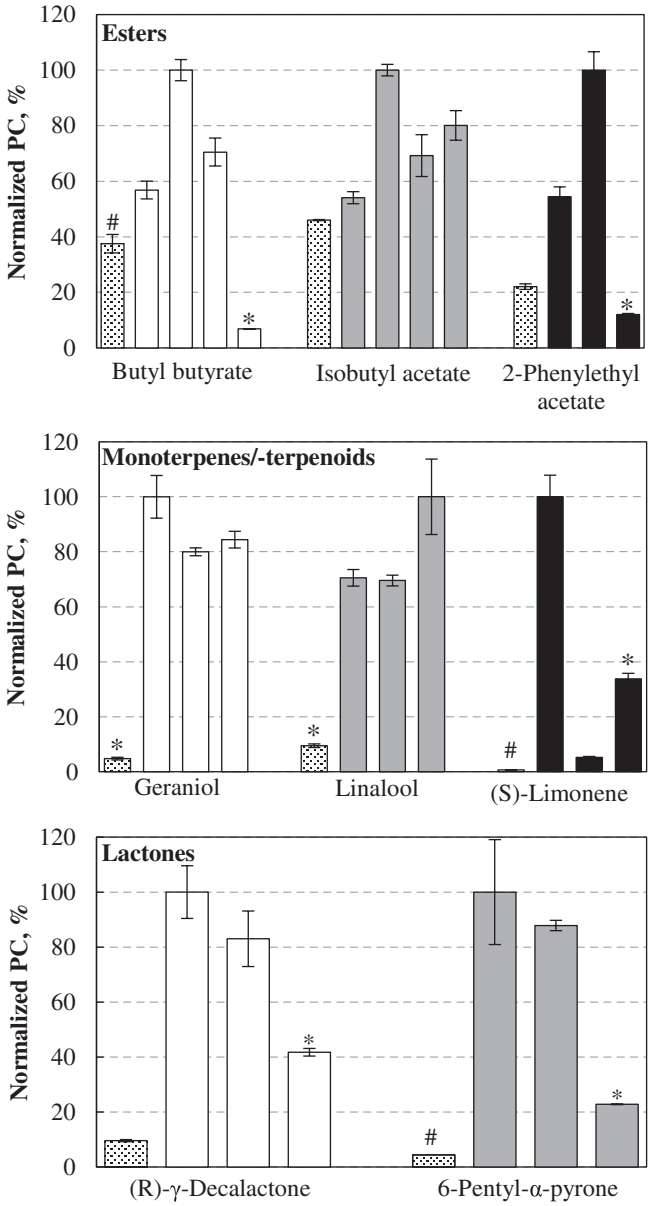

Figure 5. Partition coefficients with standard deviations (n = 3) for F&F compounds belonging to the chemical classes of esters, monoterpenes/-terpenoids and lactones in different biphasic systems with water as the aqueous phase. Each chart presents data from one chemical class of target compounds, different amounts of shading are used for different target compounds. The partition coefficient values are normalized to the highest value for each target compound. Dotted bars, always on the very left side of each data series, are used for systems with sequestering phases predicted to be poor. Asterisks (*) highlight systems that involved a polymer as sequestering phase, hashes (#) indicate that the respective partition coefficient value is reported in literature and was not determined experimentally in this work.

excellent choices for efficient sequestering phases. For several of the pre-selected molecules, outstanding absorbents resulting in high PCs could be identified, e.g. the ketones acetoin and 2-heptanone partition into 10-undecen-1-ol with experimental PCs of 94.83 ± 17.75 and 68.63 ± 2.31, respectively, the ester 2-phenylethyl acetate partitions into dibutyl sebacate with a PC of 301.56 ± 19.97, and the monoterpene (S)-limonene and the lactone 6-pentyl-α-pyrone are absorbed by diethyl sebacate with PCs of 6088.41 ± 478.67 and 300.67 ± 6.43, respectively (see Figs 2 and 3).

CONCLUSIONS

As many common F&F compounds exhibit severe cytotoxicity towards microorganisms, the microbial production of such substances requires ISPR approaches such as exemplified by TPPBs in which a second, non-aqueous phase acts as a product sink. In this work, thermodynamic first-principles methods (UNIFAC, HSPs) were applied to differentiate between effective and poor sequestering phases and experimental determination of PCs was

used to validate the 'in-silico' rankings of different liquid and solid absorbents. The partitioning behavior of 17 different F&F target compounds was investigated across a variety of both solvents and polymers serving as sequestering phases in biphasic systems, with a high degree of agreement between highly-ranked extractants and experimental PC values. Additional insights also emerged concerning generally effective solvent classes, and the influence of crystalline non-absorptive fractions of polymers, which can reduce the effectiveness of some polymers as TPPB sequestering phases. Our approach to discriminate between good and poor sequestering phases by applying thermodynamic first-principles methods emerged as a successful strategy and we identified outstanding extractants for several F&F compounds.

ACKNOWLEDGEMENTS

Xenia Priebe gratefully acknowledges support by the German Research Foundation (DFG) through the TUM International Graduate School of Science and Engineering (IGSSE), and thanks Dirk Weuster-Botz for providing the opportunity to work at Andrew Daugulis' research group for 4 months. Andrew Daugulis acknowledges financial support of the Natural Sciences and Engineering Research Council of Canada, and numerous stimulating conversations with Timothy Simon.

Supporting Information

Supporting information can be found in the online version of this article.

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