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Use of factorial design for the multivariate optimization of polypropylene membranes for the cleanup of environmental samples using the accelerated membrane-assisted cleanup approach

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ABSTRACT

Accelerated membrane-assisted cleanup (AMAC) is a recently developed method to purify extracts from matrix rich samples such as fish tissue and sediments. In this study, we tested the applicability of cast polypropylene (CPP) membranes in AMAC and evaluated the optimized dialysis procedure for the cleanup of extracts of fish tissue. Design of experiments was used to optimize the factors temperature, solvents and static time of dialysis. Main factors influencing dialysis procedure were solvents and temperature as well as the number of cycles. For the CPP membrane the optimal parameters were a temperature of 55 °C, a solvent mixture of *n*-hexane:acetone (90:10, v:v), a static time of dialysis of 6 min and 20 dialysis cycles. Comparing to the LDPE membrane this was a reduction of dialysis time from 160 to 120 min, but a higher solvent use of 150 ml per sample. However, compared to LDPE membranes CPP exhibited a lower retention of fish tissue matrix and thus reduced cleanup efficiency. Compound specific structural descriptors such as the molecular weight, the van der Waals volume and a shape factor were calculated to explain differences in diffusivity of the different model compounds. We concluded that the permeation of the molecules was related to molecular shapes and the availability of free solvent cavities in the membranes.

1. Introduction

Cleanup procedures are an inherent part of the workflow of bio- and chemical–analytical analysis of organic micro-pollutants in extracts of complex environmental sample matrices such as biological tissues, sediments, suspended particulate matter and soils. The separation of matrix compounds (e.g. lipids, humic acids, and pigments) is necessary to avoid their interferences with chemical analysis and bioassays as well as to achieve the requirements of high, replicable and reproducible analytes recoveries [1]. Established approaches for this purpose are size-exclusion methods like gel permeation chromatography (GPC), column chromatography using different sorbents (e.g. silica gel, alumina, Florisil®), and chemical treatment (saponification, oxidation) [1–5]. These methods are often optimized and selective for the analysis of specific target compounds. However, for chemical analysis in combination with bioanalytical approaches such as effect-directed analysis (EDA) and toxicity identification evaluation (TIE) [6] nonselective rather than compound specific cleanup procedures are recommended to recover as many potentially toxic compounds as possible [1,7].

Membrane-assisted cleanup techniques such as membrane dialysis extraction (MDE) [8], rapid dialysis procedure (RDP) [9] and accelerated membrane assisted cleanup (AMAC) [1] can help to bridge the gap between nonselectivity for different classes of small molecules and a significant retention of matrix macro molecules. AMAC – based on the RDP approach – is a cleanup method to purify lipid and organic matrix rich extracts of biota and sediment samples [1,10–15]. A further development of the method utilizes AMAC for the direct extraction and in-cell cleanup of extracts of sewage sludge with pressurized membrane-assisted liquid extraction (PMALE) [16]. Membranes are morphologically classified in microporous and nonporous ones referring to the presence or absence of pores in their structures [17]. The transport in microporous membranes is mostly influenced by viscous flow and sieving dependent on membrane pore characteristics (porous transport or flow model) and in nonporous membranes by molecular interactions of the permeating compound with the membrane material (solution-diffusion model) [17–19]. In the latter the transport of small molecules is a random and individual molecular motion influenced by the segment mobility of the polymer chains and the free

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solvent cavities of approximately 5–10 Å [19–21]. The driving force for the transport in membranes is a chemical potential by a gradient across the membrane [17,18], e.g., supported by a frequent renewal of the receiving or acceptor solvent phase [22,23]. Thus, the separation capability of the dialysis procedure depends on the nonselective transport of the small molecular analytes from a complex raw extract across the membrane with a significant or total retention of the matrix macro molecules due to size exclusion or slow diffusion referring in microporous and nonporous membranes, respectively.

In membrane-assisted cleanup methods or dialysis often commercially available low-density polyethylene (LDPE) tubes are used [1,8,16,24] because of easy handling, low costs, and stability in a variety of organic solvents [24]. As an alternative polypropylene membrane with a thickness of 0.03 mm and 0.05 mm have been applied in membrane-assisted solvent extraction (MASE) for the analysis of chlorophenols and triazines in water samples [25,26]. However, literature regarding the usage of polypropylene membranes for cleanup or dialysis purposes is limited.

Design of experiments (DOE) using the full factored central composite design (CCD) approach was used to optimize the recovery by varying the factors temperature, solvents and static time of dialysis. In contrast to univariate experiments where the factors are studied one by one, CCD considers all factors and factor levels at the same time [27,28]. CCD facilitates a reduction in the number of experiments with a complete coverage of the experimental space to be analyzed. It combines a core two-level factorial design (edge points) describing linear effects, a center point denoting the middle of all factor levels (or component ranges in a mixture) and axial star points representing quadratic effects resulting in an approximately spherical experimental space.

In our previous study we used LDPE membranes for the AMAC approach [1]. Thus, this paper evaluates the usability of polypropylene membranes for AMAC. Two different polypropylene membranes were studied and one was optimized for the parameters temperature, static time of dialysis, number of cycles and solvents. The optimized procedure was compared with the LDPE procedure for lipid removal efficiency.

2. Material and methods

2.1. Chemicals

Target analytes selected to develop AMAC represented different compound classes and physicochemical properties are listed in Table S1 (Supplementary data). All standards were purchased from LCG Promochem (Wesel, Germany), Dr. Ehrenstorfer (Augsburg, Germany) or Sigma Aldrich (Steinheim, Germany). The solvents acetone, *n*-hexane, and toluene (Suprasolv® or LiChrosolv® grade) were obtained from Merck (Darmstadt, Germany).

2.2. Gas chromatography–mass spectrometry

GC–MS analyses were carried out on a HP 6890 GC coupled to a HP MSD 5973 (Agilent, Palo Alto, USA), equipped with a 30 m × 0.25 mm i.d. × 0.25 μm film HP-17 MS or an HP-5 MS fused capillary silica column, a 5 m pre-column (Agilent J&W, Folsom, USA) and a splitless injector with deactivated glass wool. Chromatographic conditions were as follows: 280 °C injector temperature, 1 μl pulsed splitless injection at oven temperature of 60 °C (1 min isotherm), then programmed at 30 K/min to 150 °C, at 6 K/min to 186 °C and finally at 4 K/min to 280 °C (30 min isotherm). Carrier gas velocity (Helium 5.0, Air Liquide, Böhlen, Germany) was 1.2 ml/min at constant flow. The mass spectrometer was operated in electron impact ionization mode (EI+, 70 eV) with a source temperature of 250 °C scanning from 30 to 500 amu (full-scan mode) or in single ion monitoring (SIM mode) recording typical masses from compounds fragmentation patterns. Five-point external calibration in the linear range from 0.25 ng/μl to 5 ng/μl was used to quantify target analytes. Each sample was spiked with benzo[a]pyrene-d_{12} as internal standard to correct results for errors due to differences in sample-volumes and injection. The instrumental limits of detection (LOD) defined as three times the signal-to-noise ratio were in the range from 0.7 pg/μl to 117 pg/μl and the limit of quantification (LOQ) defined as ten times the signal-to-noise ratio was in the range from 2.4 pg/μl to 390 pg/μl.

2.3. Extraction of fish tissue and extracts processing

All experiments regarding matrix effects were conducted using extracts of tissue from two different fish species. Frozen rainbow trout (*Onchorhynchus mykiss*) and salmon (*Salmo salar*) were bought at a local supermarket. The thawed fish muscle tissues were minced and freeze dried. 25 g of each dried tissue was ground with 50 g of diatomaceous earth (Isolute HM-N, IST Ltd., Hengoed, UK) using a porcelain mortar and pestle. The mixtures were filled in 100 ml ASE cells and extracted by means of an ASE 300 device (Dionex, Sunnyvale, CA). The extraction was performed with *n*-hexane:acetone 50:50 (v/v) at 80 °C and 10 MPa for three static cycles of 5 min (Table S2, Supplementary data). The joint extracts were concentrated using rotary evaporation to a volume of approximately 20 ml, transferred to a measuring cylinder, refilled to 25 ml and stored in the freezer at −20 °C until usage.

The extracts of fish tissue were saponified and derivatized to quantify the recoveries of fatty acid lipids during dialysis procedure. The extracts were mixed with 0.5 ml 1 M potassium hydroxide (p.a. grade, Merck Darmstadt, Germany) in methanol and incubated for 2 h at 60 °C in an oven. After cooling to room temperature 200 μl of 6 M hydrochloric acid (p.a. grade, Merck Darmstadt, Germany) were added and the fatty acids were extracted two times with 1 ml of *n*-hexane. A solution of 30 ng nonadecanoic acid (Fluka, Steinheim, Germany) and of hexan:chloroform:methanol (95:3:2, v:v:v) was added to the extract. The mixture was reduced to dryness using nitrogen, reconstituted in 1 ml of a solution of methanol:chloroform:38% hydrochloric acid (10:1:1, v:v:v) and incubated overnight at 60 °C in an oven. Finally, the fatty acid methyl esters were extracted three times using 0.5 ml of *n*-hexane:toluol (1:1, v:v). Aliquots of the raw fish extracts were treated with the same procedure to estimate the raw content of fatty acids lipids in triplicate.

2.4. Accelerated membrane-assisted cleanup

2.4.1. General description of the AMAC

Briefly, dialysis bags were tailor-made using commercially available cast polypropylene (CPP) (procast®, Zeisberger Süd-Folie GmbH, Asperg, Germany) and LDPE (Polymer-Synthese-Werk GmbH, Rheinberg, Germany) (half-)tubes with a membrane thickness of 50 μm and 80 μm, respectively. Pieces with a length of 10 cm were cut from stock roll, each membrane was cleaned for 24 h in a mixture of *n*-hexane:acetone (50:50) to remove excess oligomers, slip agents, plasticizers, stabilizers and other impurities, rinsed with fresh solvent and air dried for not more than 10 min [1]. Remaining compounds in blank samples that could be assigned to polymer fabrication were for example phthalates, 1,2-diphenylethane, 1,1′-[dithiobis(methylene)]dibenzene, 1-dodecanol, caprolactam, 1-(octyloxy)octane, n-butylbenzenesulfonamide, 1,1′-[dithiobis(methylene)]dibenzene, bis(2-ethylhexyl) adipate, and 13-docosenamide. These compounds were not disturbing instrumental analysis in this study.

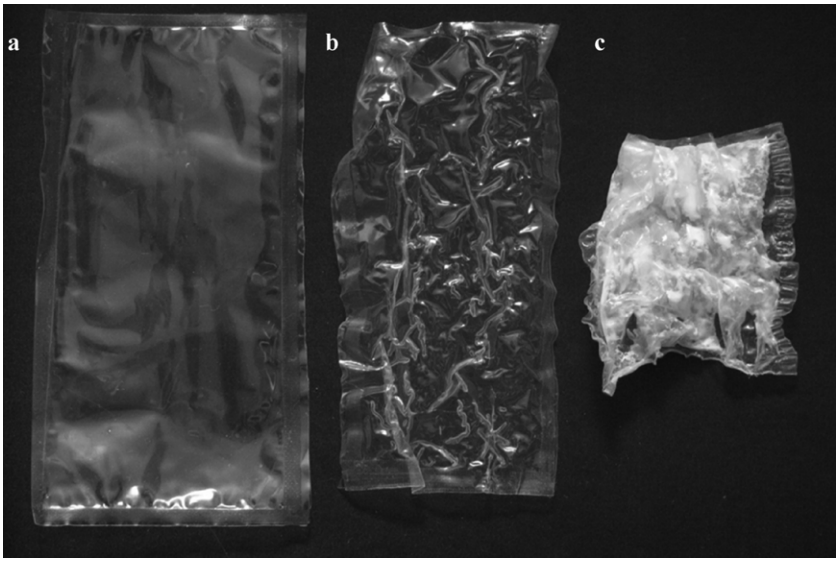

Fig. 1. Influence of temperature and solvent selection on disintegration of polypropylene membranes (ASE: 16 cycles á 10 min, pressure 3.5 MPa; (a) 100% acetone at 80 °C, (b) 100% *n*-hexane at 60 °C, (c) 100% *n*-hexane at 80 °C).

Table 1
Parameters for AMAC with LDPE membranes [1] and optimized parameter for AMAC with CPP membranes (this study) (Ac = acetone; Hx = *n*-hexane; min = minutes; MPa = megapascal; psi = pound-force per square inch; sec = seconds).

Parameter	AMAC with LDPE [1]	AMAC with CPP (optimized)
Pressure [MPa (psi)]	3.45 (500)	3.45 (500)
Temperature [°C]	40	55
Static time of dialysis [min]	10	6
Cycles	16	20
Solvent (Hx:Ac)	70:30 (v:v)	90:10 (v:v)
Solvent flushing [%]	60	60
Nitrogen flushing [sec]	60	60

The LDPE tubes were sealed at one end and the CPP half-tubes were sealed at the long side and one end (Fig. 1) using a heat-sealing apparatus (Sealboy 2-1038, Audion Elektro, Kleve, Germany) and stored in an air-tight amber borosilicate bottle at room temperature.

The dialysis bags were filled with 1.0 ml solvent, spiked with 500 ng of each model compound, or 0.5 ml of fish extract and the second end of the membrane were sealed, while carefully avoiding air cushions within. The bags were placed into a 33 ml ASE cell equipped with a stainless steel mesh to prevent the membranes from clinging to the inner wall of the extraction cell and to ensure free access of the solvent to the surface of the membrane during the procedure [1]. AMAC was performed using an ASE 200 device (Dionex, Sunnyvale, USA). The temperature, number of cycles, static time of dialysis and ratio of *n*-hexane to acetone was chosen according to Table 1. The pressure was 3.45 MPa during each experiment due to former optimization experiments [1].

2.4.2. Experimental design for optimization of polypropylene membranes for AMAC

A full factorial central composite design (CCD) with 2^3 factor combinations including two replicate center points and six star points placed at $\pm\alpha$ from the center point of the design space was performed. CCD was run in triplicate resulting in a total number of 48 experiments. The settings of the three independent factors temperature, solvent and static time of dialysis are listed in Table 2.

The limits of the temperatures are based on the possible settings of the ASE device (room temperature or in minimum 40 °C). Hence, the lower star point ($-\alpha$) of temperature was set to room temperature (RT), too. The upper star point ($+\alpha$) of 60 °C is given by preliminary investigations of membrane stability using different temperatures and solvents. Acetone and *n*-hexane were chosen as solvents due to good performance with AMAC as well as the static time of dialysis according to results of Wenzel et al. [9] and Streck et al. [1]. The resulting surface response design with a twofold repetition of the center is shown in Table 3.

Optimization of dialysis with CPP regarding maximal recoveries was performed using analysis of variance (ANOVA) and calculation of the desirability function. ANOVA was used to analyze the causalities between linear and quadratic effects as well as linear and quadratic interactions of the factors temperature, solvent and static time and target compounds' recoveries (variable *Y*) (Eq. (1)). Linear and quadratic effects and interactions, respectively, were computed using the second order polynomial regression model:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i \cdot X_i + \sum_{i=1}^{3} \beta_{ii} \cdot X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} \cdot X_i X_j \quad (1)$$

Table 2
Settings of factors for the optimization of AMAC using a full factorial central composite circumscribed design (Ac = acetone; Hx = *n*-hexane; min = minutes; RT = room temperature; solvent = content of *n*-hexane [%]).

Factor	Edge and central points			Star points	
	Lower limit (−)	Central point (o)	Upper limit (+)	$-\alpha$	α
Temperature [°C]	25 (RT)	40	55	25 (RT)	60
Static time of dialysis [min]	8	10	12	5	15
Solvent [%]	10:90 Hx:Ac	50:50 Hx:Ac	90:10 Hx:Ac	100 Ac	100 Hx

Table 3
Factor combinations of the central composite design for factors temperature, static time of dialysis and solvent (o = central point; + = upper limit; − = lower limit; α = upper star point; $-\alpha$ = lower star point; min = minutes; solvent = content of *n*-hexane [%]).

Run	Solvent [%]	Temperature [°C]	Static time of dialysis [min]	
1	o	o	O	Center points
2	o	o	O	
3	+	+	+	Edge points
4	−	+	+	
5	+	+	−	
6	+	−	+	
7	−	−	−	
8	+	−	−	
9	−	−	+	
10	−	+	−	
11	o	$-\alpha$	O	Star points
12	o	α	O	
13	$-\alpha$	o	O	
14	α	o	O	
15	o	o	$-\alpha$	
16	o	o	α	

where β_0 (center point of the system), b_i (coefficient of linear effects), b_{ii} (coefficient of quadratic effects) and b_{ij} (coefficient of interactive effects) are constants and X_i represents the factors. The experimental error was estimated using the residual sum of least squares:

$$SQ = \sum_{i=1}^{n} (y_i - \bar{y})^2 \tag{2}$$

where y_i is the experimental value of each factor level i and $\bar{y}$ is the mean of the experimental values of all factor levels.

The differences of the variances of the estimated effects of linear, quadratic and interactive effects and the overall mean of effects were tested using the *F*-test for their significance with $\alpha = 0.05$.

Since the regressions vary for the different compounds, multiple optimal factors settings are received. Hence, a methodology to decide on optimal conditions for a set or group of compounds is required. The desirability function modified by Derringer and Suich [29] is a way to proceed (Eq. (3)). A transformation of all responses into a desirability scale converts the recoveries into a dimensionless value between 0 and 1 such that the multivariate optimization problem is converted to an univariate one [29]. Further, it allows indicating values below or above predefined thresholds or to weigh recoveries. In brief, measured responses were transformed using the two-sided desirability function [29]:

$$d_i = \begin{cases} \left[\dfrac{\hat{Y}_i - Y_{i_{\min}}}{c_i - Y_{i_{\min}}}\right]^s & Y_{i_*} \le \hat{Y}_i \le c_i \\ \left[\dfrac{\hat{Y}_i - Y_{i_{\max}}}{c_i - Y_{i_{\max}}}\right]^t & c_i < \hat{Y}_i \le Y_{i_{\max}} \\ 0 & \hat{Y}_i < Y_{i_{\min}} \quad \text{or} \quad \hat{Y}_i > Y_{i_{\max}} \end{cases} \tag{3}$$

where $Y_{i_{\min}}$ and $Y_{i_{\max}}$ denote the minimum and maximum acceptable values of $\hat{Y}_i$, $Y_{i_{\min}}$ was set to the minimum recovery value and $Y_{i_{\max}}$ to the maximum recovery value of each analyzed compound. If $\hat{Y}_i$ is outside these limits, the value for d_i is zero. c_i is the most desirable value of $\hat{Y}_i$ and ranges between $Y_{i_{\min}}$ and $Y_{i_{\max}}$. The parameters s and t are weighing factors to adjust the desirability function. The parameters were set to $s = 1$ and $t = 1$ to obtain a linear fitted desirability function.

Table 4
Settings of factors for the full factorial experimental plan (2^{4-0}) for the estimation of effects of different fish species and membranes on lipid removal efficiency (CPP = cast polypropylene; LDPE = low density polyethylene; solvent = content of *n*-hexane [%]).

Factor	Level 1	Level 2
Fish species	Salmon	Trout
Membrane	LPDE	CPP
Temperature [%]	40	55
Solvent [%]	70	90

With transformed responses, a composite desirability for n compounds was obtained by calculating the geometric mean from d_i values:

$$D = \sqrt[n]{\prod_{i=1}^{n} d_i} \tag{4}$$

From the response surface curve of the composite desirability optimal factors settings for high recoveries for a group of compounds was deducted.

2.4.3. Experimental design for the assessment of the fatty acid lipid removal efficiency

A randomized two-level full factorial design with 2^4 factor combinations was used to estimate the effects of the factors fish species, membrane, temperature and content of *n*-hexane on the fatty acid lipid removal efficiency (LRE) during dialysis procedure resulting in a total number of 16 experiments (Table 4). The data was assessed using ANOVA with a linear regression model without interaction [30]:

$$Y = \mu + a_i + b_j + c_k + d_l + \varepsilon_{ijklm} \tag{5}$$

where μ denotes the overall mean, a_i denotes the effect of level i of factor A, b_j denotes the effect of level j of factor B, c_k denotes the level k of factor C and d_l denotes the level l of factor D. ε_{ijklm} denotes the experimental error estimated using Eq. (2).

The differences of the variances of the estimated effects and overall mean of effects were tested using the *F*-test with $\alpha = 0.05$ to obtain significant factors. Group mean values were calculated for each factor combination of significant factors to assess LRE. The results are given as response ratios between recovered fatty acid lipids and the amount of fatty acid lipids in the raw fish extracts measured using the GC–MS methods described above.

2.5. Data processing, modeling and statistical calculations

The software packages STATISTICA 8 [31] and GraphPad Prism® 5.03 [32] were used for design of experiments and statistical calculations. Calculation of geometrical molecules structure values was performed using ChemAxon Marvin 5.7.0 [33]. Calculated vapor pressure values and molar masses were obtained from EPISuite™ 4.1 [33].

3. Results and discussion

3.1. Influence of solvents and temperature on polypropylene membranes

Solvents and temperature have a potential impact on the integrity of a membrane resulting in swelling and in consequence disintegration of the membrane [34,35]. The swelling causes a reduction of bulk density and raising an increase of the molecular movement of polymer chains, and in combination with thermal influences an enhanced disintegration of the membrane occurs. In

Fig. 1 three examples of CPP membranes treated with different solvents at different temperatures are shown. *n*-Hexane and acetone impacted the membranes differently at a given temperature of 80 °C. While the membrane exposed to acetone was not macroscopically affected (Fig. 1a), the membrane used with *n*-hexane was disintegrated and exhibited shrinking and holes (Fig. 1c). With acetone, swelling of the membrane is lower due to lesser uptake of the polar solvent into the membrane. The swelling index of *n*-hexane in polypropylene is 7.29 cm^3/g and of acetone 0.05 cm^3/g, respectively [34,35]. The temperature was decreased to 60 °C with *n*-hexane as solvent (Fig. 1b). The membrane was shrunk without macroscopically visible disintegration, but it was closed with no solvent leakage. Therefore the upper temperature limit was set to 60 °C for further experiments. Biaxial oriented polypropylene film (profilm, Zeisberger Süd-Folie GmbH Asperg, Germany) was also tested for usability. The profilm foil was not suitable due to problems with handling and heat sealing.

3.2. Optimization experiments

3.2.1. Analysis of variance (ANOVA)

The interdependence of *n*-hexane (%) temperature (°C) and duration of dialysis cycles (min) was examined using ANOVA. Fig. 2 depicts the Pareto charts for analytes representing different classes of compounds and physical–chemical properties. The ratio of *n*-hexane to acetone shown by the percentage of *n*-hexane was the main factor controlling analytes recoveries significantly ($p < 0.05$). Hence, the selection of the correct solvent or solvent mixture is very important for the dialysis procedure. The second dominating factor was the temperature, but not significantly for each target compound. The static time of dialysis had no influence in the limits chosen in this study. Furthermore, ANOVA indicated the dominance of linear dependences. Quadratic and linear factor combinations were not significant (not shown).

3.2.2. Solvent

ANOVA unraveled that the ratio of *n*-hexane to acetone is the main controlling factor for analytes' recoveries. Fig. 3 depicts the desirability profiles of selected compounds with different physicochemical properties showing the dependencies from temperature (°C) and content of *n*-hexane (%) and their expected maximum recoveries (shown by the desirability profile). PAHs (Fig. 3a) and PCBs (Fig. 3b) are expected to recover in maximum with 100% of *n*-hexane content. Benzophenone (BP) was estimated to gain the maximum amount with *n*-hexane content of 100% as well (Fig. 3d). Octachlorodibenzodioxin (OCDD) (Fig. 3e) showed an optimum of recovery with an acetone content of 25% due to dipole–dipole effects between the carbonyl group of acetone and the dioxin. The optimization resulted in maximum recoveries using a *n*-hexane content of 100% for the most compounds. However, blending of *n*-hexane and a small amount of acetone had a noticeable influence on the recoveries of some compounds such as OCDD. Therefore a ratio of 90% *n*-hexane to 10% acetone was chosen and used for further experiments because it was within the prognosis interval of the desirability function of all model compounds and a negative effect was negligible.

3.2.3. Temperature and static time of dialysis

The ANOVA showed a positive effect of elevated temperatures on recoveries confirming previous results [1,9,16]. This can be explained by the thermodynamic temperature dependence of diffusion according to Fick's first law of diffusion and by the increasing segment mobility of the polymer chains resulting in the formation of more and larger cavities in the membrane supporting diffusion [20,36]. The impact of the duration of each extraction was not significant in ANOVA validating the findings of Rodil et al. [16]. Increasing temperature and longer dialysis times resulted in slightly higher expected recoveries for most compounds (Fig. 4a–f). However, the factor static time of dialysis could be neglected because longer dialysis times did not yielded higher recoveries. Considering these results there are two options how to decide between the best temperature and dialysis time to gain high recoveries. Firstly, a short dialysis time and a high temperature provide good recoveries due to enhanced diffusion at a reduced time needed for each dialysis cycle. Secondly, a longer dialysis time and decreased temperatures are resulting in lower recoveries, but theoretically in a higher selectivity of the dialysis for target compounds and matrix retention because of a lower segment mobility of the polymer chains leading to less and smaller cavities in the membrane. Hence, the duration of dialysis was set to 6 min and the temperature to 55 °C. However, the optimized temperature with high recoveries for most compounds was 60 °C, but the CPP membranes showed decomposition at this temperature resulting in turbidity of the reduced extract after dialysis due to co-extracted olefins, which did not occur at 55 °C.

3.2.4. Number of dialysis cycles

The number of consecutive dialysis cycles, i.e. the periodical exchange of the solvent after a static time of dialysis for example of 6 min, is an important factor to gain greater recoveries [1,9,16]. The periodical exchange of the dialysis solvent sustains steep chemical gradient between the inner membrane with the raw extracts and the receiving solvent surrounding the membrane such that the diffusion process is supported. In Fig. 5a the recoveries of different model compounds are plotted against the number of dialysis cycles. The results show that an increasing number of cycles caused higher recoveries. The top levels of recoveries were reached after 20 dialysis cycles. Remarkable are the low recoveries of less than 40% of small molecules, e.g. naphthalene, diphenylether, acenaphthylene and acenaphthene, due to evaporation during extract preparation for GC–MS analysis. This can be explained by their high vapor pressures (Table S1 in Supplementary data). Hence, these compounds were excluded from further statistical analysis (except cluster analysis).

3.2.5. Confirmation of the optimized method using CPP and comparison with LDPE method

The optimized AMAC method using CPP membranes was compared with the previously published AMAC procedure using LDPE membranes [1] to verify the new experimental settings. Fig. 5b depicts the results of the comparison of the procedures using CPP and LDPE. The recoveries of both experimental designs using CPP membranes with 16 and 20 dialysis cycles resulted in either significantly lower or significantly higher values compared to the standard procedure using LDPE membranes (Wilcoxon matched-pairs signed rank test, two-tailed, $p < 0.05$). Thus, the usage of CPP membranes with 20 cycles enhanced the crossover of the model compounds. Furthermore, usage of CPP membranes reduced the overall dialysis time by 25% (120 min CPP method vs. 160 min LDPE method). The precision of both approaches is listed in Table S3 in Supplementary data.

3.2.6. Comparison of compound and membrane specific properties on the recoveries

Diffusion through polymers depends on size and shape of the penetrating molecules [20,38] such that (1) increasing size degreases diffusion, (2) different substitutes (e.g., chlorine or methyl group) decrease or increase diffusion, and (3) linear, flexible, and symmetrical compounds have increased diffusion coefficients compared to rigid molecules [38,39]. Thus, the van der Waals volume (VWV) describing the molecule in its 3D projection, the minimal projections size (MiPS) and maximal projection size

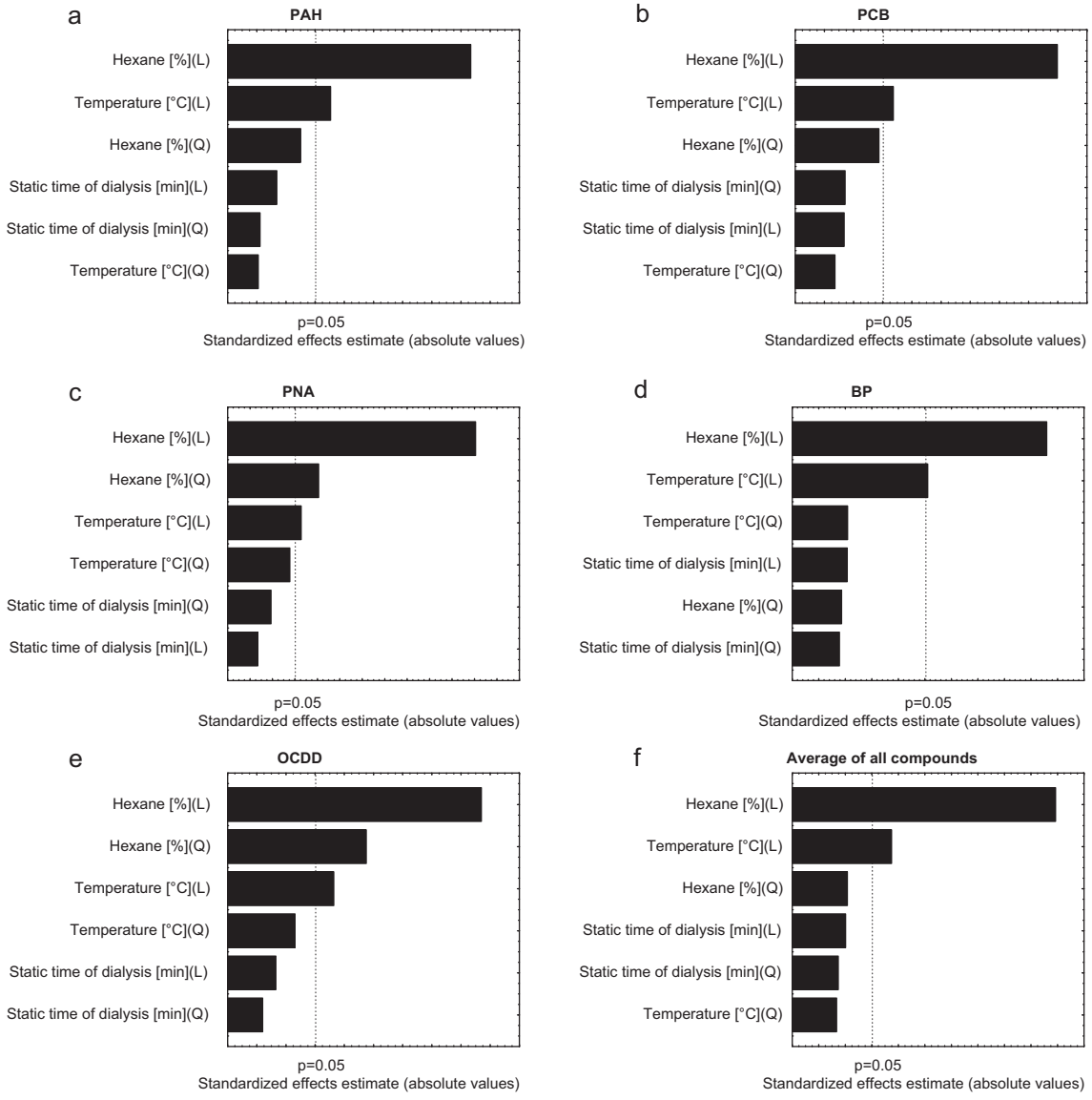

Fig. 2. Pareto charts for estimation of significance of the main factors temperature [°C], static time of dialysis [min] and ratio of *n*-hexane to acetone shown by percentage of *n*-hexane (hexane [%]); L = linear effects, Q = quadratic effects; dashed line: significance level of $p = 0.05$ (PAH = average of 13 EPA-PAH, PCB = sum of 6 PCB according to Ballschmiter and Zell [37], PNA = n-phenyl-2-naphthylamine, PROM = prometryn, OCDD = octachlorodibenzodioxin).

(MaPS) as well as the total number bonds and number of rotatable bonds were calculated using MarvinSketch [40]. VWV, MiPS and MaPS were optimized for their lowest energy conformation during computation. Nevertheless, molecules may take higher energy conformations while diffusing across polymers [39].

MiPS and MaPS were used to calculate the molecules shape expressed by a shape factor (φ) [39] that shows if a molecule has either a more spherical or a more cuboid-like shape (Eq. (6)):

$$\varphi = \frac{\text{surface of the parallelpiped volume box}}{\text{surface of a cube of same volume}} = \frac{ab + bc + ca}{3(abc)^{2/3}} \quad (6)$$

where a, b, and c are the dimensions of the parallelepiped volume box of each molecule from which the surface of a cube of same volume is calculated. The shape factors (φ_{lit}) provided by Reynier et al. [39] were compared with those modeled with MarvinSketch (φ_{est}) showing a linear correlation ($R^2 = 0.83$, rss = 0.05) (Table S4 in Supplementary data). However, φ_{est} were different from φ_{lit} (factor 1.2 ± 0.1) in order of divergent algorithms used for the estimation of the geometry parameters. The shape factors of our study increased from 1.63 (more spherical molecule) to 1.78 (more cuboid-like molecule) (see Table S1 in Supplementary data). The upper shape factors are related to the lower domain of φ_{est} of the aliphates (C11–C14), and thus, diffusion of the molecules used in our study is rather a movement from free solvent pore to another than the 'crawling mode of molecules having many degrees of freedom' such as aliphates [39]. The rotatable bond fractions [41] were calculated as the ratios of the number of rotatable bonds to the number of bonds in a molecule, and hence, these are descriptors for the molecular flexibility. Though, an increased degree of freedom could have positive as well as adverse effects on the diffusivity of a molecule [39,42].

In a first evaluation, MMs were plotted against the recoveries of the analytes using both membranes showing a linear regression for CPP ($R^2 = 0.34$, rss = 7.06) and a polynomial regression for LDPE ($R^2 = 0.38$, rss = 9.45) (Fig. 6a; Table S6 in Supplementary data). However, as presented above CPP gained higher recoveries than LDPE due to dialysis at a raised temperature and differences in the polymer properties such that diffusion was enhanced. The first group of compounds showed recoveries between 50% and 60% (LDPE) or 70% (CPP), respectively, including low to middle

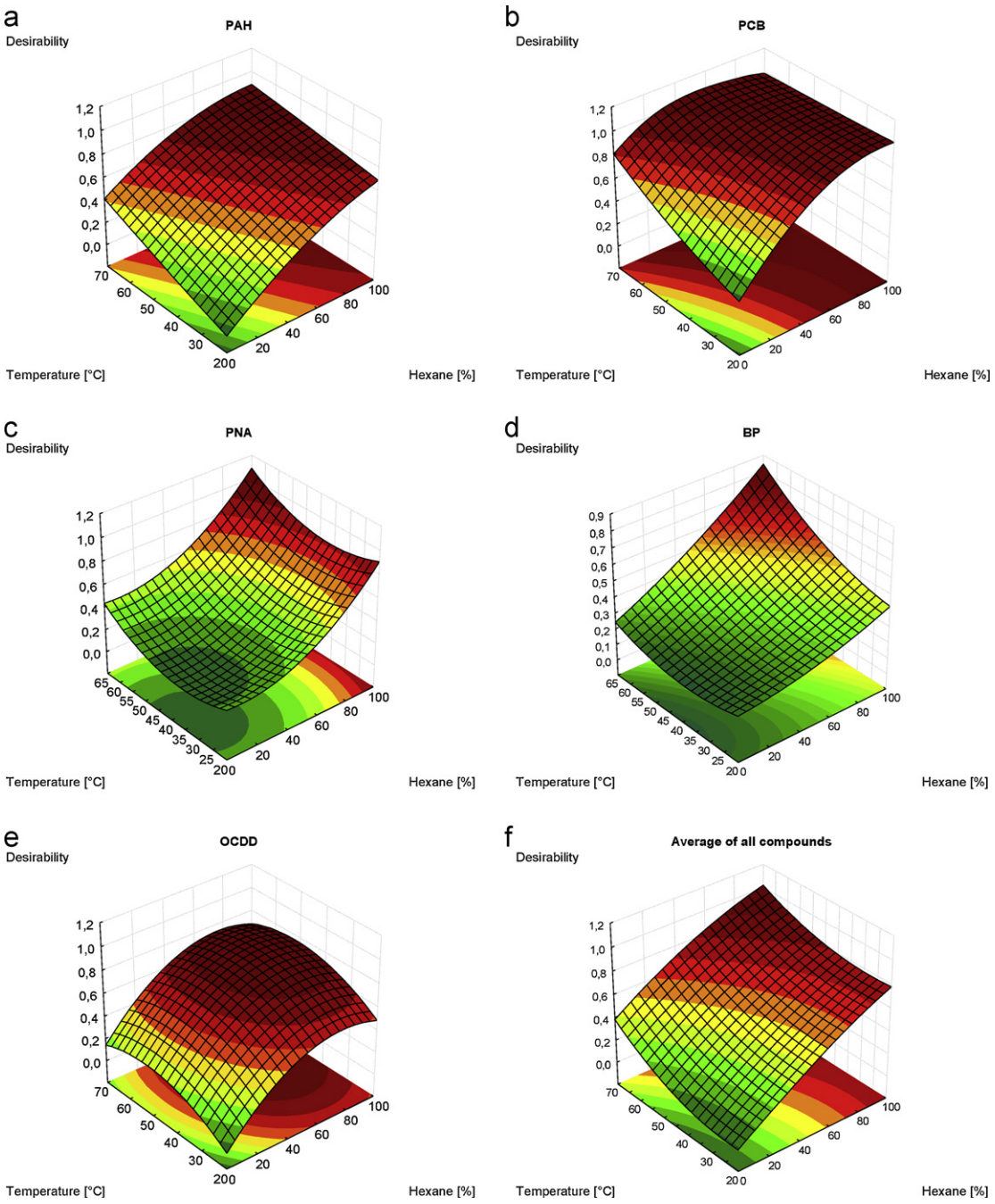

Fig. 3. Desirability surfaces for the optimization of the factors solvent composition (acetone:hexane given aspercentage of *n*-hexane) and temperature [°C] (PAH = average of 13 EPA-PAH, PCB = sum of 6 PCB according to Ballschmiter and Zell [37], PNA = n-phenyl-2-naphthylamine, BP = benzophenone, OCDD = octachlorodibenzodioxin).

mass compounds in the range from 150 g/mol to 325 g/mol (e.g., benzo[a]pyrene and hexachlorobenzene) but also OCDD in case of LDPE. The second group consists of compounds with recoveries above 60% (LDPE) or 70% (CPP) for example DDT, methylparathion, prometryn, and anthraquinone as well as OCDD in case of CPP.

Molecular geometry may disclose a more comprehensive explanation and confirmation of the compounds' recoveries. The regression of VWV vs. the recoveries yielded with the CPP and LDPE membranes, respectively, revealed only low regression values (CPP: linear regression, $R^2 = 0.25$, rss = 7.45; LDPE: polynomial regression, $R^2 = 0.32$, rss = 9.95) (Fig. 6b; Table S6 in Supplementary data). These results are in agreement with the study of Saleem et al. [38] showing that compounds with similar molecular volumes and structures such as benzene, toluene and xylene may have only small differences in their diffusivity. Nevertheless, the molecular volume did not fully explain the differences of recoveries.

Following the suggestions of Reynier et al. [39] that the shape of the molecules and other factors such as the rotatable bond fraction (RBF) [41] may give a better interpretation of differences in diffusivity. The factor RBF is rank-ordered, thus, hierarchical agglomeration (cluster analysis) was performed to classify the compounds according to recoveries, rotatable bond fractions and shape factors using Statistica [31]. Ward's method was used in combination with Euclidean distances. Cluster analysis showed that the compounds could be assigned to five main clusters (dendrograms see Figs. S1 and S2 in Supplementary data).

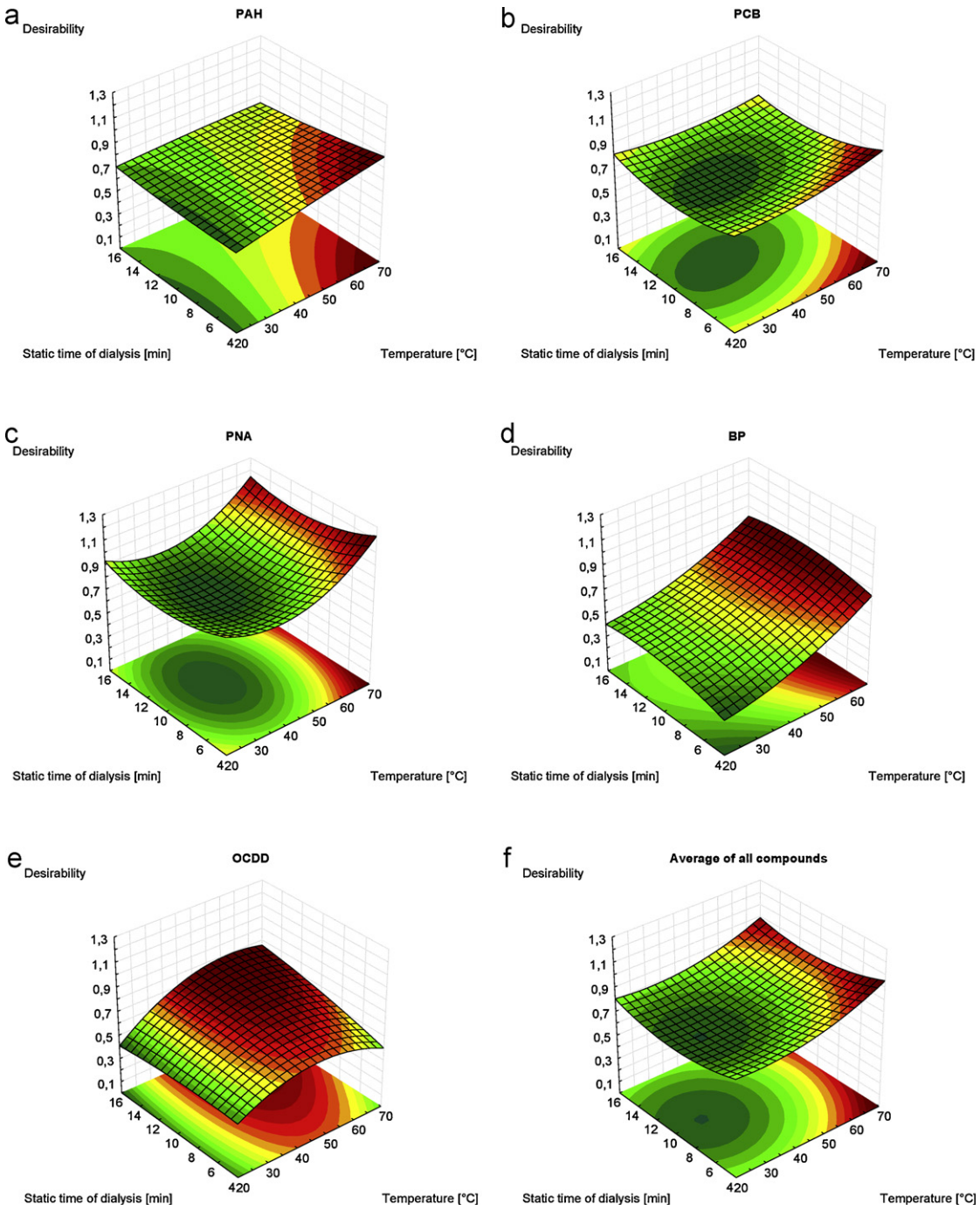

Fig. 4. Desirability surfaces for the optimization of the factors static time of dialysis [min] and temperature [°C] (PAH = average of 13 EPA-PAH, PCB = Sum of 6 PCB according to Ballschmiter and Zell [37], PNA = n-phenyl-2-naphthylamine, BP = benzophenone, OCDD = octachlorodibenzodioxin).

Cluster 1 (CPP/LDPE): acenaphthene (Ace), acenaphthylene (Acen), naphthalene (Nap), diphenylether (DPh); Cluster 2 (CPP/LDPE): benzophenone (BP), diphenylamine (DPA), di-p-tolymethane (DpT), and fluorene (Fluo); Cluster 3 (CPP): anthracene (Ant), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), diisopropylenenaphthaline (DIPN), γ-cyclohexane (HCH), hexachlorobenzene (HCB), n-phenyl-2-naphthylamine (PNA), and phenanthrene (Phe); Cluster 3 (LDPE): Ant, BaP, BbF, BkF, DIPN, fluoranthene (Flu), HCH, HCB, OCDD, and Phe; Cluster 4 (CPP): anthraquinone (AQN), benzo[a]anthracene (BaA), chrysene (Chry), DDT, Flu, OCDD, PCBs without PCB 28, pyrene (Pyr), and tonalide (Ton); Cluster 4 (LDPE): methylparathion (MeP), PNA, PCB 28, PCB 52, PBC 180, prometryn (Prom), Pyr, and Ton; Cluster 5 (CPP): MeP, Prom, and triphenylphosphate (TPP); and Cluster 5 (LDPE): ANQ, BaA, Chry, DDT, Flu, PCB 101, PCB 138, PCB 153, Pyr, and TPP.

Generally, the compounds could be assigned to different groups: (1) compounds with high vapor pressures, (2) compounds with rigid amine, carbonyl, condensed or substituted structures, and (3) compounds with more flexible structures.

Group 1: all molecules have high vapor pressures, and thus, are exposed to volatilization during analytical processing: Ace, Acen, DPh, and Nap.

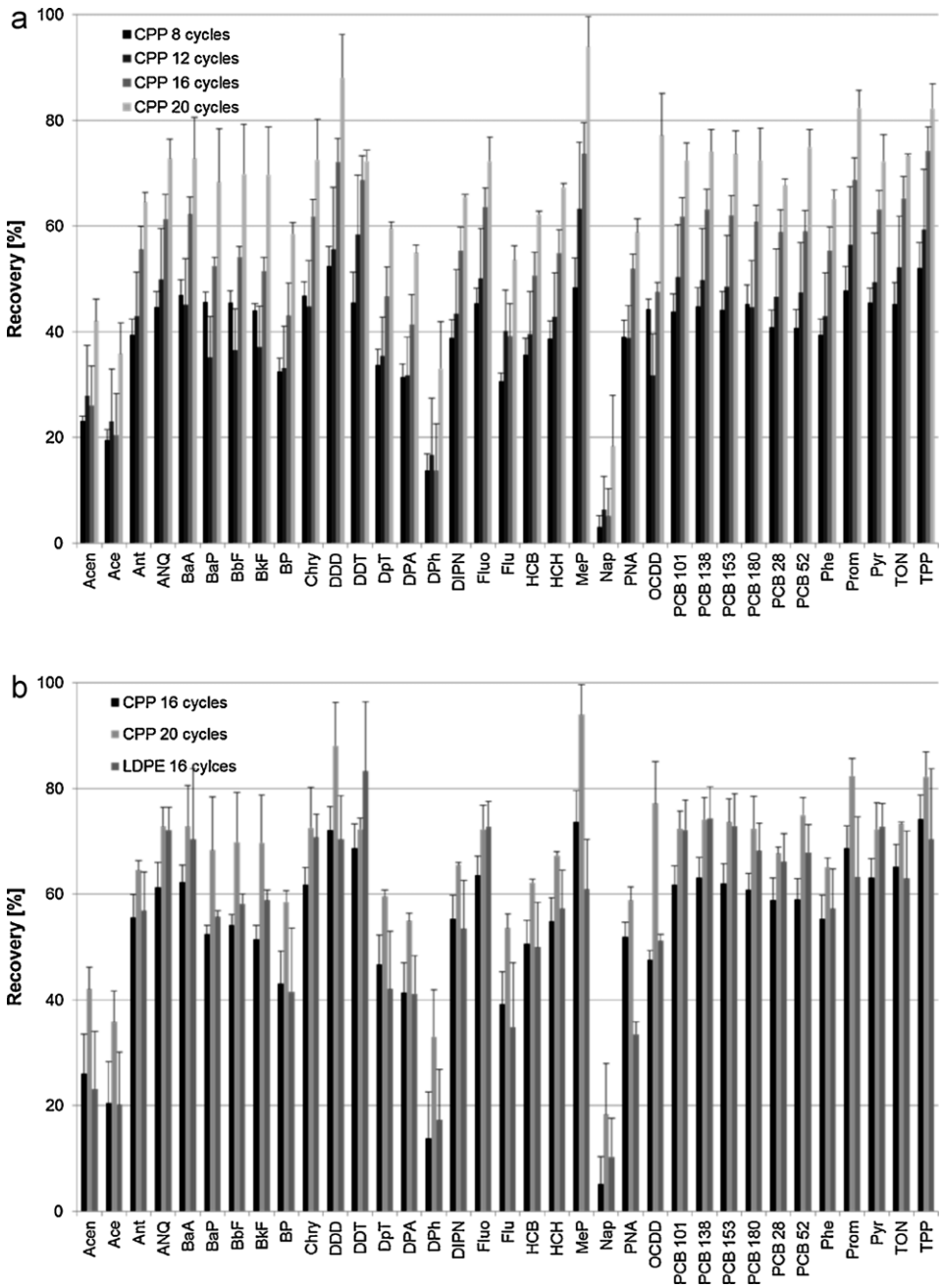

Fig. 5. (a) Recoveries depending on the number of dialysis cycles using cast polypropylene membranes (CPP) (n = 3; temperature = 55 °C, Hx:Ac = 90:10, static time of dialysis per cycle = 6 min); (b) comparison of recoveries using CPP with 16 and 20 cycles (n = 3; temperature = 55 °C, Hx:Ac = 90:10, static time of dialysis per cycle = 6 min and polyethylene membranes (LDPE) (*n* = 3; 16 static cycles á 10 min, temperature = 40 °C, Hx:Ac = 70:30); pressure in all experiments: 3.45 MPa (mega-pascal); Acen = acenaphthylene;Ace = acenaphthene; Ant = anthracene; ANQ = anthraquinone; BaA = benzo[a]anthracene; BaP = benzo[a]pyrene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BP = benzophenone; Chry = chrysene; DDD = dichlordiphenyldichlorethane; DDT = dichlorodiphenyltrichloroethane; DpT = Di-p-tolymethane; DPA = diphenylamine; DPh = diphenylether; DIPN = diisopropylenenaphthaline; Fluor = fluorene; Flu = fluoranthene; HCB = hexachlorobenzene; HCH = γ-cyclohexane; MeP = methylparathion; Nap = naphthalene; PNA = n-phenyl-2-naphthylamine; OCDD = octachlorodibenzodioxin; PCB = polychlorinated biphenyl; Phe = phenanthrene; Prom = prometryn; Pyr = pyrene; TON = tonalide; TPP = triphenylphosphate).

Group 2: the assigned compounds have different but rigid structural characteristics:

- Amine structure (intermediate shapes): DPA, and PNA
- Carbonyl structures (cuboid-like shapes): AQN and BP
- Condensed and/or substituted structures (intermediate to cuboid-like shapes): γ-cyclohexane, PAHs (except those in group 1), and OCDD
- Substituted structures (intermediate to spherical shapes): DDT, HCB, and PCBs,
- Other rigid structures (spherical shapes): DIPN, DpT, and Ton

Group 3: the substances have rigid and flexible (>3 rotatable bonds) structures as well as more spherical shapes: MeP, Prom, and TPP.

The diffusion behaviors of the most compounds were similarly comparing both membranes related to the groups defined above. A few compounds showed different behaviors, namely AQN, BaA, Chry, DDT, MeP, PNA, OCDD, PCB 28, PCB 101, PCB 138, PCB 153

Table 5
Group means of response ratios ($n = 2$) of effects of different fish species and membranes on lipid removal efficiency (CPP = cast polypropylene; LDPE = low density polyethylene; CI = confidence interval ± 95%; s = standard deviation).

Fish species	Membrane	Temperature [°C]	Group mean	$s_{estimated}$	CI (±95%)
Salmon	LDPE	40	0.04	0.03	[−0.27, 0.36]
Salmon	LDPE	55	0.18	0.01	[0.10, 0.26]
Salmon	CPP	40	0.37	0.31	[−2.46, 3.19]
Salmon	CPP	55	0.37	0.13	[−0.76, 1.49]
Trout	LDPE	40	0.09	0.03	[−0.16, 0.34]
Trout	LDPE	55	0.15	0.17	[−1.35, 1.64]
Trout	CPP	40	0.36	0.29	[−2.29, 3.0]
Trout	CPP	55	0.29	0.03	[−0.05, 052]

and Prom. The most remarkable compounds were DDT, MeP, Prom and OCDD. The variances of the other compounds are negligible (Fig. 5b). The dissimilarity of the DDT diffusion was may be related to the widely known problem of decomposition of DDT to its transformation product dichlordiphenyldichlorethane (DDD) during GC injection. While MeP, OCDD, and Prom yielded highest recoveries of all compounds using the CPP membrane, they reached only medial values using the LDPE membrane. MeP, Prom and TPP have almost the same φ, but maybe TPP is more flexible than the others, and hence, has a higher diffusivity even at lower temperature conditions using the LDPE approach. The temperature differences were probably the reason for the lower recoveries using the LDPE approach at all, also explaining the lower diffusivity of OCDD compared to the CPP approach. Furthermore, it was reported that the temperature and the membrane swelling effects mobility of the polymer chains due to plasticization effects [43,44], and thus, availability and shape of free solvent pores due to higher mobility of the polymer chains.

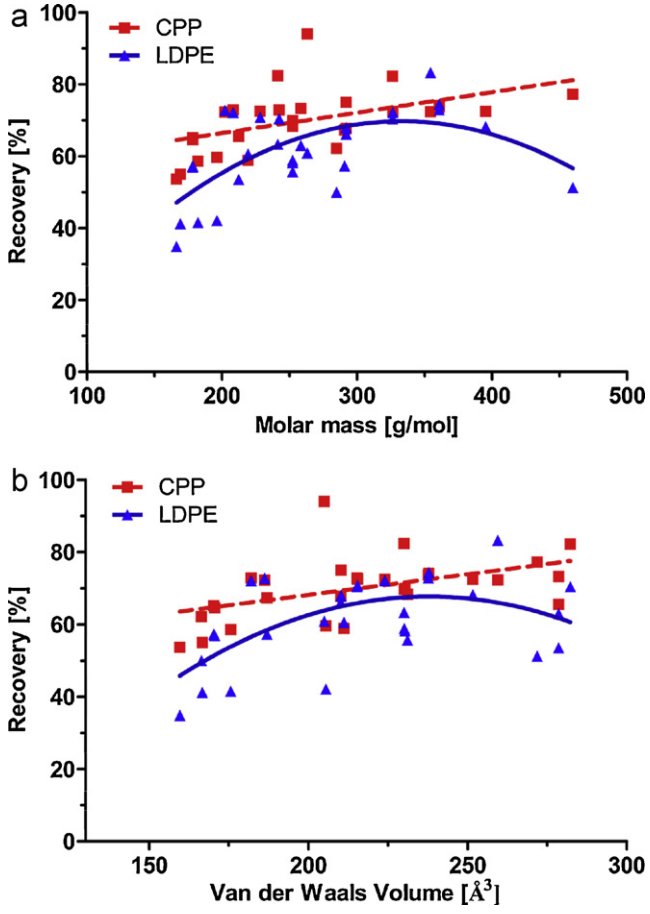

Fig. 6. Regressions of (a) molar masses [g/mol] and (b) van der Waals volumes [Å^3] with the recoveries of the model compounds (CPP = cast polypropylene membrane (squares (■), dashed lines); LDPE = low density polyethylene membrane (triangles (▲), solid lines)).

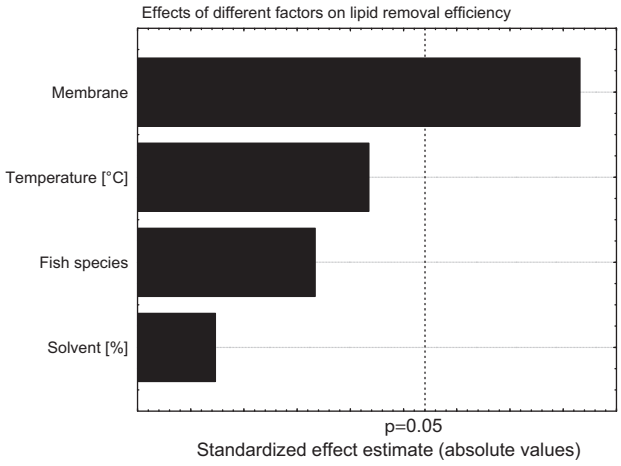

Fig. 7. Pareto chart for the estimation of effects of membranes (CPP/LDPE), temperatures (40/55 °C), fish species (salmon/trout) and solvent (content of *n*-hexane: 70/90%) on the lipid removal efficiency during dialysis procedure (dashed line: significance level of $p = 0.05$).

3.3. *Confirmation of effects of membranes on fatty acid lipid removal efficiency*

The effects of the different dialysis settings and membranes on fatty acid lipid removal efficiency (LRE) were assessed for two different fish species (salmon and rainbow trout) to verify the LRE of the new method. The selection of membranes (CPP vs. LDPE) had a significant effect ($p > 0.05$) in order of retention of the fish fatty acid lipids (Fig. 7). The other factors temperature and fish species had an important but not significant influence. The dependence from the percentage of content of *n*-hexane was negligible. Table 5 lists the group mean values regarding fish species, membrane and temperature. The lowest recoveries of 4% and 9%, and hence, best retention was achieved with the LDPE membrane at a temperature of 40 °C for both fish species, respectively. The retention of lipids using the CPP membrane was twofold lower than using the LDPE membrane. These results validate the previously published AMAC settings and procedure [1].

4. Conclusions

The accelerated membrane-assisted cleanup (AMAC) is a method for purifying lipid-rich extracts. Results of our previous study showed that the procedure mainly depends on the parameters temperature, pressure, the static dialysis time and the number of dialysis cycles to achieve a good separation of organic analytes and fatty acid-rich matrix. Optimization of these parameters

revealed that the selection of solvent and the temperature were the most important and significant parameters for the diffusion of small molecules across nonporous membranes. These findings can be explained in the first case by the thermodynamic temperature dependence of diffusion according to Fick's first law of diffusion and by increasing segment mobility of the polymer chains and in the latter case by an accelerated intrusion of *n*-hexane in the membrane than acetone, and thus better swelling of the membrane. The comparison of the analytical results with physical properties and molecule geometry parameters disclosed that the molecular shape mainly affected the compounds recoveries. Hence, we concluded that the diffusivity of the distinct compounds was strictly dependent from the availability of free solvent pores in the membranes. This study revealed that the LDPE membrane was more suitable to reach an optimal trade-off between the recoveries of analytes and the lipid removal efficiency than the CPP membrane. Therefore, the challenge in the future will be to facilitate the selectivity of the membranes for a broader range of compounds for example by developing composite membranes integrating different kind of polymeric materials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.069.

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