Analytica Chimica Acta 1301 (2024) 342387

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Solvent-free parallel artificial liquid membrane extraction for drugs of abuse in plasma samples using LC-MS/MS

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HIGHLIGHTS

- 96-well liquid-phase microextraction of drugs of abuse from plasma.
- Synthetic organic solvents replaced by microliter volumes of essential oil.
- Simple workflow, aqueous extracts injected directly in LC-MS.
- Performance data in compliance with forensic toxicology guideline requirements.
- A simple, fast, and efficient eco-friendly technique for routine analysis.

ARTICLE INFO

Keywords:
Parallel artificial liquid membrane extraction
PALME
New psychoactive substances
Drugs of abuse
Essential oil
LC-MS/MS

ABSTRACT

Background: Parallel artificial liquid membrane extraction (PALME) is a 96-well plate setup variant of liquid-phase microextraction. Basic or acidic analytes are extracted in neutral form from the sample, through a supported liquid membrane (SLM), and into aqueous acceptor. PALME is already considered a green extraction technique, but in the current conceptual work, we sought to make it even greener by replacing the use of organic solvents with essential oils (EO). PALME was combined with LC-MS/MS for analysis of plasma samples and multiple drugs of abuse with toxicological relevance (amphetamines, phenethylamines, synthetic cathinones, designer benzodiazepines, ayahuasca alkaloids, lysergic acid diethylamide, and ketamine).

Results: Fourteen EO were compared to organic solvents frequently used in PALME. The EO termed smart & sassy yielded the best analyte recovery for all drugs studied and was thus selected as SLM. Then, factorial screening and Box-Behnken were employed to optimize the technique. The extraction time, concentration of base, sample volume, and percentage of trioctylamine significantly impacted analyte recovery. The optimum values were defined as 120 min, 10 mmol/L of NaOH, 150 μL, and 0%, respectively. Once optimized, validation parameters were 1–100 ng mL-1 as linear range, accuracy ±16.4%, precision >83%, 1 ng mL-1 as limit of quantitation, 0.1–0.75 ng mL-1 as limit of detection, matrix effect <20%, and recovery 20–106%. Additionally, EO purchased from different production batches were tested and achieved acceptable reproducibility. Data were in compliance with requirements set by internationally accepted validation guidelines and the applicability of the technique was proven using authentic samples.

Significance: In this study, the use of an EO provided a solvent-free sample preparation technique suited to extract different classes of drugs of abuse from plasma samples, dismissing the use of hazardous organic solvents. The

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https://doi.org/10.1016/j.aca.2024.342387
Received 10 December 2023; Received in revised form 5 February 2024; Accepted 18 February 2024
Available online 26 February 2024

method also provided excellent sample clean-up, thus being a simple and efficient tool for toxicological applications that is in agreement with the principles of sustainable chemistry.

1. Introduction

In the early 2000s, the New Psychoactive Substances (NPS) began to be commercialized as legal alternatives to conventional drugs of abuse and thus drastically changed the landscape of the illicit drug market. These novel chemicals are designed to mimic the effect of classic psychoactive substances, such as amphetamines and lysergic acid diethylamide (LSD). However, as a result of chemical modifications, the potency of these new compounds might be considerably higher [1–3]. In addition to that, NPS are commonly sold mixed with other drugs and might be inadvertently consumed by drug users. Unwittingly taking substances of unknown potency can result in severe intoxication or even death [3–5]. Over 15 years after the beginning of this phenomenon, law enforcement, toxicologists, and other professionals are still being challenged with the ever-growing number of heterogeneous chemically diverse molecules that are introduced into the illicit drug market. For example, by November 2023, the total number of NPS reported to The United Nations Office on Drugs and Crime worldwide accounted for 1230 [1,2].

To face the NPS threat, the analysis of biological specimens is crucial in clinical and forensic toxicology. However, some NPS are found at low concentrations in biological samples due to their high potency and thus require the use of efficient extraction techniques combined with highly sensitive analytical instruments [3,5,6]. In fact, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been one of the instruments of choice for that purpose. In addition, although several extraction techniques have been proposed to analyze NPS in blood, urine, and other biological specimens, as new compounds are constantly appearing in the drug market, adapting these existing methodologies or developing new ones is often required [7–10]. These techniques must be also compatible with conventional drugs of abuse, which are still relevant [1].

Among the large number of works proposing approaches to analyze NPS and classic drugs of abuse in biological samples, some also attempt to reduce the environmental impact of such practices [10–13]. This can be accomplished, for instance, by choosing less hazardous organic solvents, reducing sample volume, processing multiple samples simultaneously, and improving overall cost-effectiveness. This goal of developing more eco-friendly methodologies in analytical chemistry and toxicology has been a trend in recent years that can rely on guidelines established specifically to that end [14,15]. In that regard, liquid-phase microextraction (LPME) is a fine example of a green alternative approach. Proposed by Pedersen-Bjergaard and Rasmussen, in 1999, LPME is a miniaturized version of classic liquid-liquid extraction that is able to avert some of its limitations, such as the high volumes of sample and organic solvents that are frequently employed [13,16,17].

In 2013, an adaptation of LPME to a 96-well plate setup was proposed by Gjelstad et al. named parallel artificial liquid membrane extraction (PALME) [18]. In this format, two 96-well plates are placed in parallel but the principle of LPME remains (Fig. 1). Basic or acidic analytes are extracted from aqueous sample, through a thin liquid membrane of a few microliters of organic solvent, and into aqueous acceptor. Essentially, PALME allows the simultaneous extraction of almost a hundred samples, making the technique simpler, more efficient, and of high-throughput. In addition, this setup is amenable to automation, and both sample and organic solvent volumes are substantially reduced, making PALME a remarkable improvement to conventional hollow-fibre LPME, which has been applied in different contexts as its predecessor [19–27].

PALME is already considered a green sample preparation approach

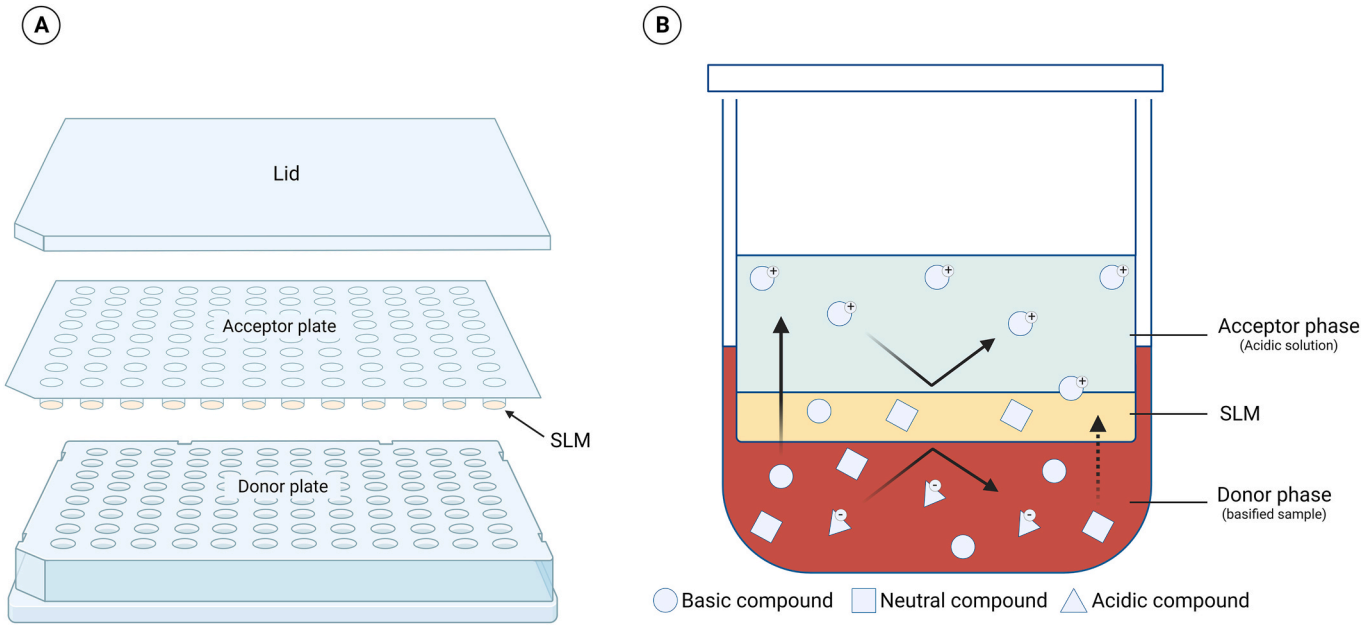

Fig. 1. Illustration of PALME setup and the principle of extracting basic analytes. First, the donor solution is added to the wells of the donor plate and the SLM is pipetted onto the membrane in the acceptor plate. Then, the two individual plates are sandwiched together and the acceptor solution is added to the wells of the acceptor plate. Finally, a lid is placed to prevent evaporation of the SLM (A). Once this setup is assembled, compounds are transferred from the donor to the acceptor phase under agitation, i.e. analyte extraction. For the extraction of basic substances, the donor phase is comprised of a base added to an aqueous sample, such as blood or plasma, to keep basic analytes in their neutral state. A hydrophobic organic solvent is used as SLM to serve as a barrier to charged compounds. The acidic solution protonates basic compounds once they reach this compartment, thus avoiding their back-extraction to the SLM or donor phase (B). SLM: supported liquid membrane.

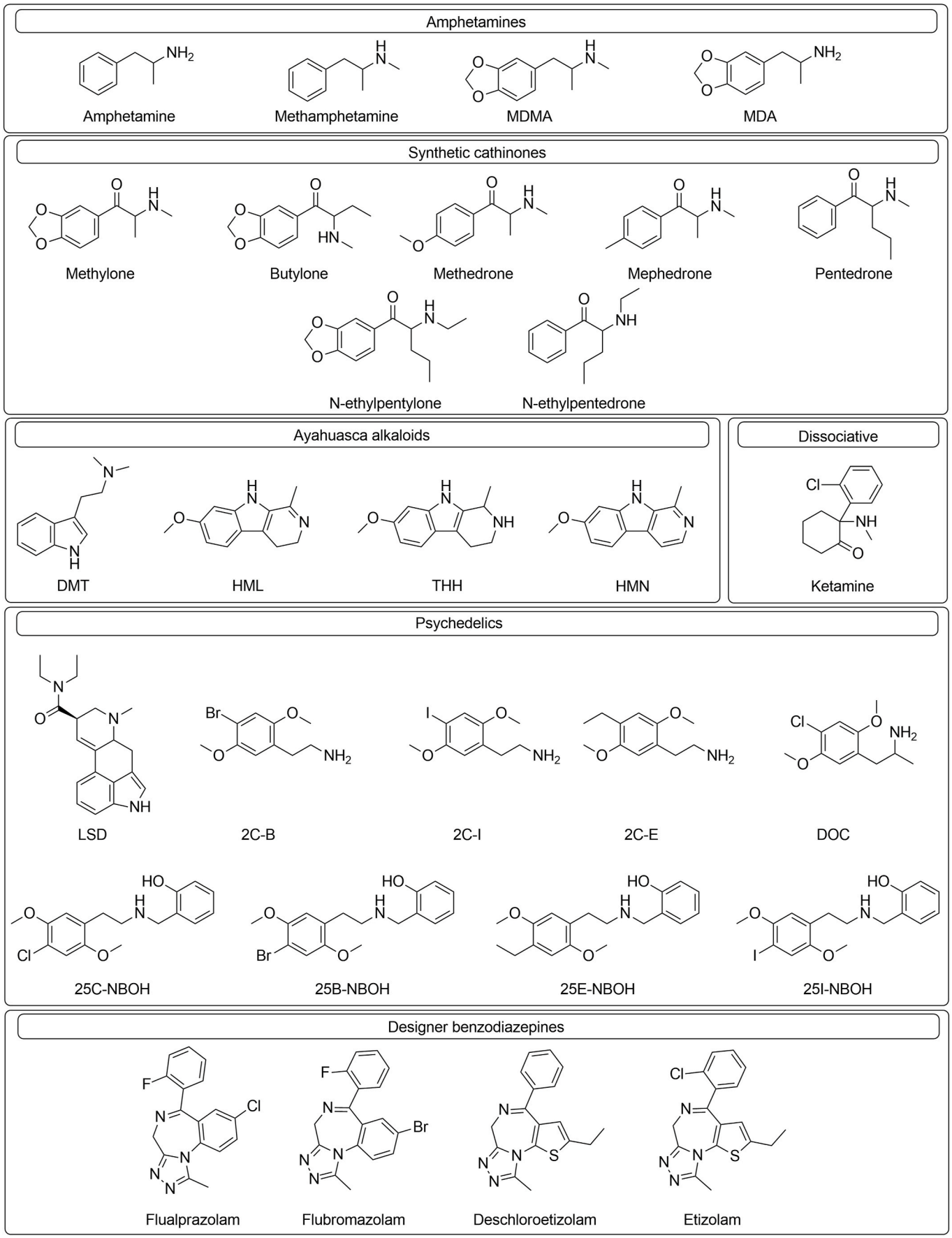

Fig. 2. Chemical structures of the analytes included in the present work.

but we sought to make it even greener by replacing the organic solvents used as supported liquid membrane (SLM) with more eco-friendly alternatives without sacrificing overall efficiency. Essential oils (EO) are hydrophobic concentrated plant extracts that have been previously used in extraction techniques, including LPME [11,13]. The main advantage of using EO is the reduced hazard for both the operator and the environment. With that in mind, we explored the efficiency of using these natural extracts as SLM in PALME in a method aimed at different classes of drugs of abuse, including NPS (Fig. 2). This conceptual article describes the development of this idea.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, formic acid, and ammonium formate of HPLC grade were purchased from Merck (Darmstadt, Germany). Citric acid, sodium hydroxide, sodium bicarbonate, 2-nitrophenyl octyl ether (NPOE), dihexyl ether, dodecyl acetate, and trioctylamine (TOA) were purchased from Merck (Darmstadt, Germany). The EO were purchased from different brands: cedar wood, clove, *Eucalyptus radiata*, lavender, lemon, lemon-grass, lime, peppermint, rosemary, and smart & sassy were purchased from dōTERRA International, LLC (Pleasant Grove, UT, USA); clove, *Eucalyptus globulus*, and peppermint were purchased from Bio-Essência® (São Paulo, Brazil); and a second brand of *Eucalyptus globulus* was purchased from LASZLO© (Minas Gerais, Brazil). Ultrapure water (resistivity 18.2 MΩ cm, total organic carbon (TOC) $\leq$ 5 ppb) was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

All analytes and internal standards were acquired from Cerilliant Corporation (Round Rock, TX, USA) and were available at 1.0 mg mL^{-1} and 100 μg mL^{-1}, respectively. Analytes were grouped accordingly to psychoactive effect, i.e. classic stimulants: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxyamphetamine (MDA); synthetic cathinones: methylone, methedrone, butylone, mephedrone, pentedrone, N-ethylpentedrone, and N-ethylpentylone; dissociative: ketamine; psychedelics: LSD, 2,5-dimethoxy-4-chloroamphetamine (DOC), 2,5-dimethoxy-4-iodophenethylamine (2C–I), 2,5-Dimethoxy-4-ethylphenethylamine (2C-E), 2-(((2-(4-chloro-2,5-dimethoxyphenyl)ethyl)amino)methyl)phenol (25C–NBOH), 2-(((4-Bromo-2,5-dimethoxyphenethyl)amino)methyl) phenol (25B–NBOH), 2-((2-(4-Iodo-2,5-dimethoxyphenyl)ethylamino) methyl)phenol (25I–NBOH), and 2-(((4-Ethyl-2,5-dimethoxyphenethyl) amino)methyl)phenol (25E-NBOH); ayahuasca alkaloids: N, N-dimethyltryptamine (DMT), harmaline (HML), harmine (HMN), and tetrahydroharmine (THH); and designer benzodiazepines: deschloroetizolam, flubromazolam, flualprazolam, and etizolam. Dilutions of these stock solutions were prepared for optimization and validation studies. All solutions were in methanol or acetonitrile and stored at −20 °C.

2.2. Samples

Both blank and authentic human plasma samples used in this work were provided by the Laboratory of Analytical Toxicology from the Toxicological Assistance and Information Centre located at the University of Campinas. Samples were collected in blood collection tubes containing EDTA and subsequently centrifuged (5 min at 3500×*g*) to allow plasma separation. Plasma samples were then stored at −20 °C before analysis. The use of human specimens in the present study was approved by the Ethics Committees of both the University of São Paulo and the University of Campinas (CAAE: 46404121.8.3001.0067).

2.3. Sample preparation procedure

A 96-well donor plate of polypropylene with 0.33 mL wells from Agilent (Santa Clara, CA, USA) and a 96-well acceptor plate from Millipore (Billerica, MA, USA) with polyvinylidene fluoride (PVDF) as support for the SLM were used. The PVDF material has 0.45 μm and 6.0 mm of pore size and internal diameter, respectively. A lid provided by the manufacturer was used to reduce or prevent evaporation of the solutions during the procedure. This same setup was described previously (Fig. 1) [21–23,28]. Once assembled, the PALME setup was placed on a ThermoMixer® C (Eppendorf® EP5382000023, Hamburg, Germany) for agitation.

For the extraction procedure, 100 μL of NaOH 25 mM were pipetted into the donor wells in the donor plate. Then, 150 μL of plasma aliquots previously spiked with the analytes were added to each well. Next, 3 μL of the smart & sassy EO were added to each of the PVDF filters to create the SLM, and the two plates were then sandwiched together. Finally, 50 μL of citric acid 50 mM was pipetted into the acceptor wells in the acceptor plate, a lid was placed, and the system was kept for 120 min under 500 rpm of agitation. Once the extraction was finished, the acceptor solutions were simply transferred to autosampler vials, and 7.5 μL were injected into the LC-MS/MS system.

2.4. LC-MS/MS

All analyses were performed using an ultra-performance LC-MS/MS instrument. The LC was an Acquity System equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) and the MS was a Quattro Premier XE model, both from Waters Corporation (Milford, MA, USA). Ammonium formate (pH 3.1, 1 mM) was used as mobile phase A and acetonitrile as mobile phase B, both containing 0.1% of formic acid (v/v). The method was kept at a constant flow rate of 0.3 mL/min with column oven temperature at 40 °C. The chromatographic gradient was as follows: initially, 10% of B was kept for 2 min, followed by a ramp to 20% within 5 min, and a second ramp to 100% of B within 3 min. Then, the initial conditions were restored in 0.5 min and kept for 1.5 min to re-equilibrate the system with a total run time of 12 min. The MS was operated in positive ionization mode with electrospray capillary voltage set to 2.5 kV, desolvation gas flow rate to 1100 L/h, cone gas flow rate to 200 L/h, desolvation temperature to 450 °C, and source temperature to 120 °C. The multiple reaction monitoring approach was used and the specific conditions to each analyte are displayed in Table S1.

2.5. Method optimization

The Design of Experiment statistical approach was used for both univariate and multivariate analysis of the extraction procedure where a fractional factorial screening 2^{k-1} and a central composite design (CCD) were used. Significance was considered when $p < 0.05$ and the adjusted coefficient of determination obtained by Analysis of Variance (ANOVA) served to determine the percentage of data variance explained by the model. All data were processed using GraphPad Prism® 8 and Statistica® 10 software.

2.6. Method validation

The ANSI/ASB standard 036, 1st Edition 2019 guide for method validation in Forensic Toxicology was used with the following parameters assessed: linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, imprecision, selectivity, and carryover. The matrix effect (ME), recovery (RE), and process efficiency (PE) study was performed according to Matuszewski et al. [29,30].

2.6.1. Calibration model

The linear range was assessed from six calibrators with five replicates. Heteroscedasticity weighting (1/x) was applied and the linear regression was acceptable when the coefficient of correlation (r^2) $\geq$ 0.99.

2.6.2. LOD and LOQ

LOD was experimentally determined as the lowest concentration

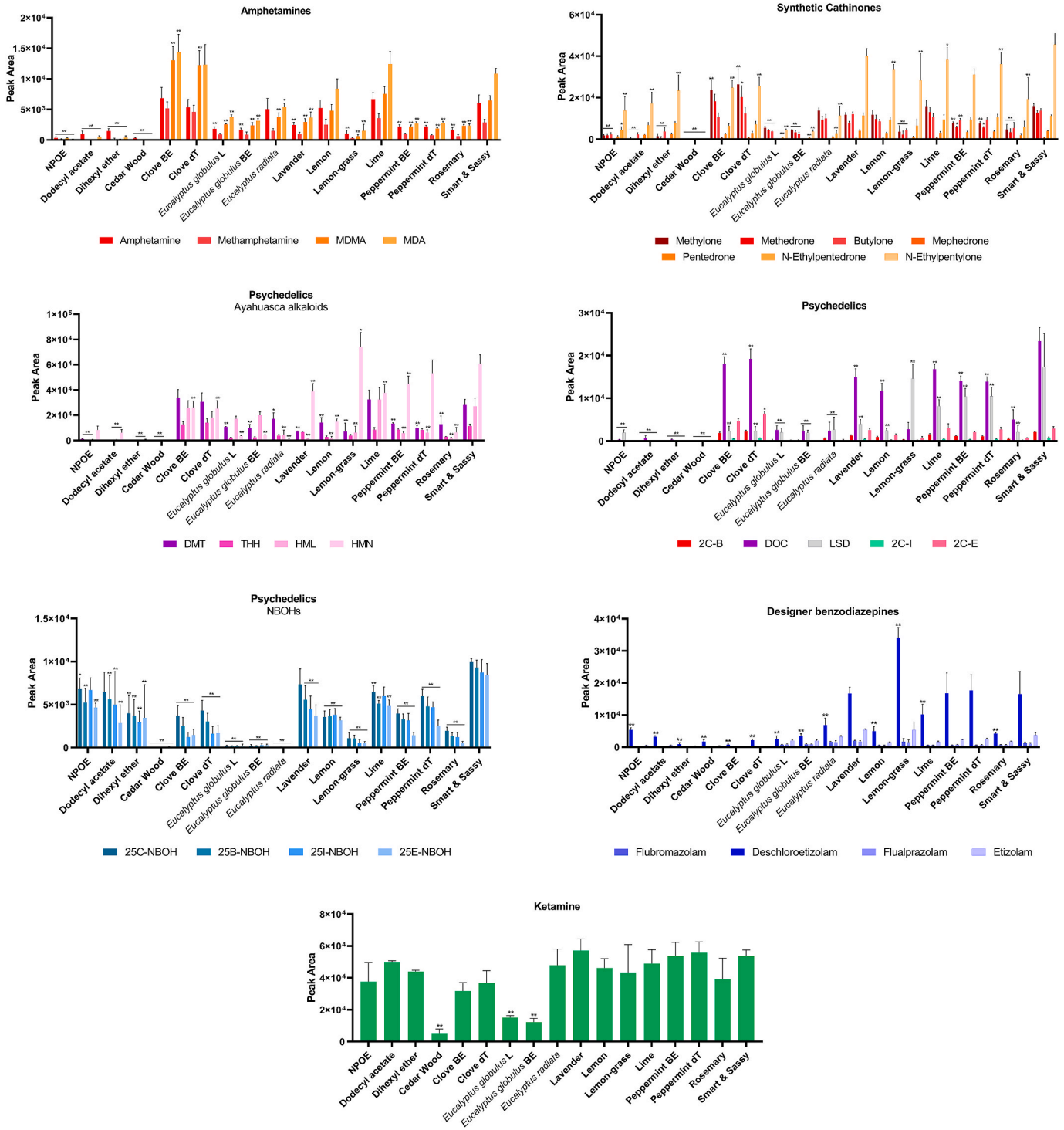

Fig. 3. Comparison between EO and other organic solvents as SLM. The mean of the absolute peak areas is shown (n = 4). Two-way ANOVA with post-hoc Tukey Test was used to determine the difference among groups. *p < 0.05 vs smart & sassy; **p < 0.01 vs smart & sassy. Clove BE: from BioEssência®; clove dT: from dōTERRA International; Eucalyptus globulus L: from LASZLO©; Eucalyptus globulus BE: from BioEssência®; peppermint BE: from BioEssência®; peppermint dT: from dōTERRA International; SLM: supported liquid membrane.

capable of providing a signal-to-noise ratio above three with retention times ±0.05 min. LOQ was set as the first concentration of the calibration curve by analyzing three samples from different donors on three different days with freshly prepared calibration curves. To determine LOQ, all identification and quantitation criteria should be met: accuracy (±20%), imprecision (≤20%), signal-to-noise ratio (≥10:1), and retention times (±0.05 min).

2.6.3. Accuracy and imprecision

Three QC levels were analyzed with a freshly prepared calibration curve on five independent days. The accuracy and imprecision of the method were then considered acceptable when ±20% and ≤20%, respectively. Accuracy was reported as bias and imprecision was expressed as coefficient of variation (CV) for both within-run and between-run imprecisions.

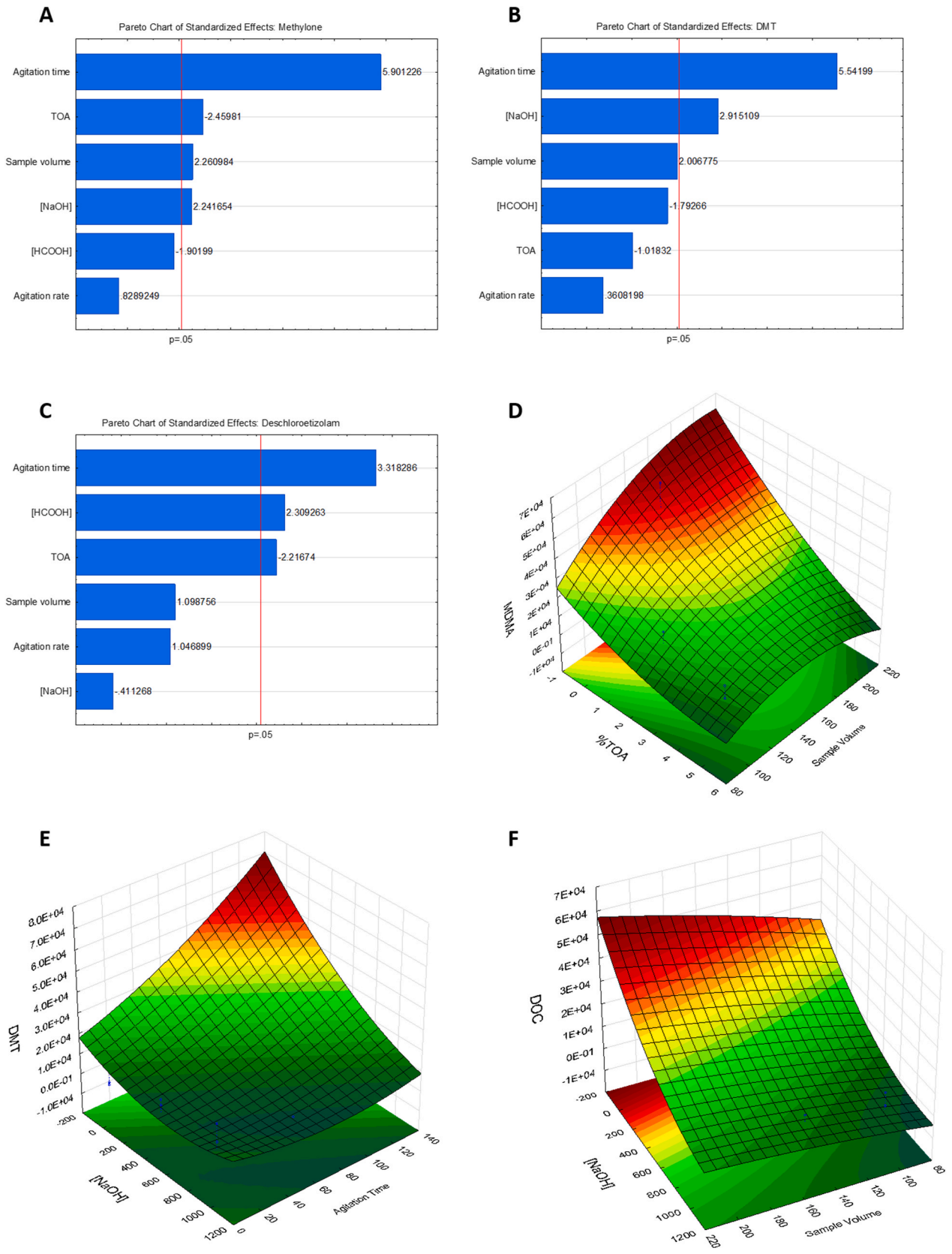

Fig. 4. Optimization study of the EO-based PALME herein proposed. A, B, and C are Pareto charts representing the statistically significant variables in the fractional factorial screening (bars crossing the red line: $p < 0.05$). D, E, and F are surface response graphs showing the optimum combination (red areas) of the statistically significant variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Linearity, LOD, LOQ, accuracy, and imprecision results.

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Calibration range (ng mL^{-1})	Linearity (r^2)	QC (ng mL^{-1})	Bias (%)	Imprecision (%)	
							Within-run	Between-run
Amphetamine	0.2	1	1–100	0.9928	2	10.9	8.9	11.9
					40	−4.3	8.7	12.8
					80	1.4	6.2	7.7
Methamphetamine	0.1	1	1–100	0.9903	2	6.4	12.5	10.4
					40	−9.4	5.6	9.5
					80	−4.7	6.6	6.6
MDMA	0.1	1	1–100	0.9978	2	14.2	5.1	5.4
					40	−8.5	4.4	8.7
					80	−1.2	4.8	4.9
MDA	0.2	1	1–100	0.9966	2	16.1	7.7	7.16
					40	−0.1	6.1	12.3
					80	−0.3	5.9	7.2
Methylone	0.1	1	1–100	0.9966	2	6.9	13.6	14.8
					40	4.0	7.0	9.5
					80	5.8	11.7	9.9
Methedrone	0.1	1	1–100	0.9959	2	3.0	10.7	11.7
					40	−0.5	9.1	9.2
					80	2.2	6.8	5.9
Butylone	0.1	1	1–100	0.9953	2	11.1	9.7	9.4
					40	−0.5	6.1	6.2
					80	2.9	4.2	5.2
Mephedrone	0.5	1	1–100	0.9955	2	3.2	9.0	14.8
					40	0.5	6.7	8.8
					80	5.0	4.6	4.5
Pentedrone	0.2	1	1–100	0.9981	2	4.4	5.8	11.8
					40	−0.5	8.5	10.5
					80	2.9	3.3	8.3
N-ethylpentedrone	0.5	1	1–100	0.9998	2	4.5	11.8	13.4
					40	−1.4	7.5	9.6
					80	0.8	3.9	5.3
N-ethylpentylone	0.1	1	1–100	0.9984	2	14.8	4.7	6.7
					40	−8.3	2.6	3.7
					80	−3.2	2.0	6.7
DMT	0.1	1	1–100	0.9962	2	10.8	10.8	12.0
					40	2.2	6.4	9.8
					80	2.1	6.4	9.3
THH	0.2	1	1–100	0.9961	2	12.3	11.0	14.8
					40	−2.8	9.8	14.7
					80	−2.3	12.2	14.0
HML	0.75	1	1–100	0.9970	2	−0.6	13.8	16.4
					40	2.3	12.9	12.5
					80	−1.9	8.5	7.0
HMN	0.1	1	1–100	0.9974	2	5.0	7.7	13.4
					40	0.7	10.2	13.2
					80	−9.0	8.2	11.3
Ketamine	0.1	1	1–100	0.9965	2	14.8	5.6	5.2
					40	−6.5	3.9	3.7
					80	−5.4	2.2	4.4
2C–B	0.75	1	1–100	0.9950	2	9.8	11.7	12.4
					40	−1.1	10.6	12.2
					80	0.9	7.9	9.9
DOC	0.2	1	1–100	0.9977	2	16.4	5.7	5.2
					40	1.2	6.9	12.5
					80	−1.5	4.1	8.9
LSD	0.1	1	1–100	0.9984	2	12.1	11.4	10.9
					40	−4.9	5.3	6.6
					80	0.5	3.1	4.6
2C–I	0.5	1	1–100	0.9995	2	8.0	10.5	13.3
					40	−5.3	11.5	12.3
					80	−1.4	8.4	11.0
2C-E	0.2	1	1–100	0.9990	2	3.0	7.6	13.0
					40	7.2	11.6	11.7
					80	−5.5	9.4	11.0
25C–NBOH	0.5	1	1–100	0.9941	2	1.1	12.2	14.3
					40	−3.9	13.8	13.7
					80	−4.4	10.3	10.2
25B–NBOH	0.5	1	1–100	0.9913	2	−0.4	14.3	13.3
					40	−4.7	0.7	0.7
					80	−2.3	0.4	0.3
25I–NBOH	0.2	1	1–100	0.9975	2	−3.3	10.7	12.9
					40	−1.5	13.6	14.9
					80	−6.1	12.5	11.7
25E-NBOH	0.2	1	1–100	0.9974	2	−2.3	15.0	12.9

(*continued on next page*)

Table 1 (*continued*)

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Calibration range (ng mL^{-1})	Linearity (r^2)	QC (ng mL^{-1})	Bias (%)	Imprecision (%)	
							Within-run	Between-run
					40	4.9	12.0	13.0
					80	−2.5	7.0	9.3
Deschloroetizolam	0.2	1	1–100	0.9927	2	8.0	13.4	14.5
					40	−0.5	5.4	12.6
					80	−6.6	6.9	7.1
Flualprazolam	0.75	1	1–100	0.9949	2	−0.2	14.3	14.0
					40	−1.4	8.8	9.3
					80	−2.3	5.5	6.9
Flubromazolam	0.75	1	1–100	0.9935	2	0.6	10.6	10.9
					40	−1.1	6.8	11.8
					80	−2.2	5.2	5.1
Etizolam	0.2	1	1–100	0.9979	2	−8.4	15.9	15.5
					40	−4.5	7.8	12.9
					80	−10.9	14.8	12.6

LOD: limit of detection; LOQ: limit of quantitation; QC: quality control.

2.6.4. ME, RE, and PE

The ME was the calculated ratio between post-spiked extracts with neat analyte injections; RE was calculated as the ratio between spiked plasma samples with post-spiked extracts; and PE was obtained considering the ratio between spiked plasma samples with neat analyte injections [30].

2.6.5. Intermediate imprecision for EO

Five blank plasma samples were spiked at each of the three QC levels and extracted simultaneously with three different batches of the smart & sassy EO. These samples were analyzed in terms of within- and between-batch imprecisions. Values were considered acceptable when ≤20% [11, 29].

2.6.6. Selectivity

The presence of common endogenous substances and exogenous compounds was studied as potential interferents. To that end, ten blank plasma samples from different subjects were extracted, and neat standards of common substances were directly injected into the instrument and analyzed by the method (Table S3). In both studies, no interfering peaks should be visualized in the same retention times as the target analytes.

3. Results and discussion

3.1. Selection of EO as liquid membrane

In a first set of experiments, fourteen different EO were tested as liquid membranes. The model analytes were amphetamines (four compounds), synthetic cathinones (seven compounds), ayahuasca alkaloids (four compounds), psychedelics (nine compounds), designer benzodiazepines (four compounds), and ketamine. These were selected to represent a wide range of compounds in terms of molecular weight (135.2–413.3 g/mol), polarity ($1.23 \leq \log P \leq 4.06$), and basicity ($4.02 \leq pKa \leq 10.26$) (Table S2). In addition to the EO, extractions were also conducted with NPOE, dodecyl acetate, and dihexyl ether for comparison, as these are pure synthetic solvents frequently used for LPME (Fig. 3). Interestingly, for most of the substance classes, the EO were more efficient than the synthetic solvents but none were highly efficient for all the model analytes. The reason behind that could be plenty fold. For example, the composition of the SLM is known to play a major role on extraction efficiency, as it affects mass transfer from the donor to acceptor compartment. Thus, the presence of chemicals in the SLM that allow formation of ionic, hydrogen bond, and π-π interactions, are likely to impact analyte recovery [31]. EO are comprised of multiple natural compounds that have hydrogen-bond donor or acceptor groups and

Table 2
ME, RE, and PE values.

Analyte	ME (%)			RE (%)			PE (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Amphetamine	97.2	95.4	105.7	70.4	70.2	69.1	68.5	67.0	73.1
Methamphetamine	102.4	93.3	106.2	67.5	77.2	68.4	69.1	72.0	72.6
MDMA	91.0	92.0	102.3	75.1	73.6	79.3	68.4	67.7	81.1
Methylone	86.4	83.5	86.6	60.6	72.0	76.3	52.4	60.1	66.1
Mephedrone	91.1	98.0	103.6	72.1	80.7	81.8	65.6	79.1	84.8
Pentedrone	85.6	80.0	109.1	95.3	90.9	91.8	81.5	72.7	100.2
N-ethylpentedrone	91.2	94.8	91.2	89.6	99.4	94.9	81.7	94.2	86.6
DMT	101.0	100.6	99.7	66.0	70.2	79.8	66.6	70.6	79.6
HML	101.9	102.6	109.8	43.7	38.9	42.0	44.5	39.9	46.1
HMN	105.9	96.9	109.2	76.4	78.5	82.8	80.9	76.1	90.4
Ketamine	101.7	93.7	105.6	95.4	88.5	106.1	97.1	82.9	112.0
DOC	98.4	108.7	107.2	53.4	58.2	63.6	52.6	63.3	68.2
LSD	109.6	115.4	119.1	67.8	82.6	82.3	74.3	95.3	98.0
2C–I	102.9	101.2	107.1	49.3	59.0	55.5	50.7	59.7	59.4
2C-E	104.7	118.4	112.2	64.7	55.8	62.3	67.7	66.1	69.9
25C–NBOH	88.3	94.9	103.1	78.3	76.4	77.6	69.2	72.5	80.0
25I–NBOH	96.0	118.0	117.0	55.8	63.6	60.2	53.6	75.1	70.4
25E-NBOH	109.4	103.8	113.7	77.5	88.3	86.3	84.7	91.6	98.1
Flualprazolam	111.4	104.6	110.4	28.4	33.4	34.2	31.6	34.9	37.7
Flubromazolam	110.8	116.0	110.8	20.6	25.3	27.3	22.8	29.4	30.2
Etizolam	119.9	118.8	116.9	30.6	31.3	26.0	36.6	37.2	30.4

ME: matrix effect; RE: recovery; PE: process efficiency.

Table 3
Imprecision assessment of EO from different production batches.

Analyte, QC (ng mL^{-1})	Imprecision (%)	
	Within-batch	Between-batch
Amphetamine		
2	7.4	6.3
40	7.9	9.7
80	3.5	3.3
Methamphetamine		
2	6.8	6.7
40	7.9	12.8
80	2.9	4.8
MDMA		
2	4.8	4.4
40	3.1	7.8
80	4.1	3.7
MDA		
2	10.8	8.9
40	8.3	12.6
80	6.3	7.2
Methylone		
2	12.6	17.2
40	5.5	8.4
80	12.9	10.6
Methedrone		
2	13.6	11.4
40	10.9	9.3
80	7.0	6.3
Butylone		
2	8.0	8.0
40	8.0	6.5
80	2.7	4.4
Mephedrone		
2	7.5	6.4
40	4.5	11.8
80	2.6	2.1
Pentedrone		
2	3.3	16.4
40	6.3	5.1
80	4.0	11.3
N-ethylpentedrone		
2	3.2	10.3
40	8.3	6.8
80	2.3	7.5
N-ethylpentylone		
2	3.4	3.5
40	2.7	3.2
80	2.1	4.2
DMT		
2	14.5	15.1
40	9.5	11.1
80	6.6	7.7
THH		
2	8.4	17.4
40	13.2	16.8
80	14.0	16.3
HML		
2	9.3	17.3
40	7.1	8.3
80	6.1	5.3
HMN		
2	8.8	12.0
40	11.0	9.1
80	5.7	5.8
Ketamine		
2	3.5	2.9
40	2.0	2.4
80	2.3	2.5
2C–B		
2	4.8	4.2
40	10.4	8.6
80	4.9	9.9
DOC		
2	4.0	3.4
40	7.2	11.1
80	2.3	3.8
LSD		
2	11.2	10.7
40	2.8	2.6
80	3.2	5.3
2C–I		
2	12.1	13.2
40	12.4	13.0
80	5.1	5.4
2C-E		
2	10.2	14.9
40	11.6	10.8
80	13.0	12.9
25C–NBOH		
2	14.2	17.1
40	7.9	10.3
80	7.7	6.3
25B–NBOH		
2	15.3	12.6
40	8.9	7.5
80	13.5	11.0
25I–NBOH		
2	13.9	12.3
40	11.0	12.1
80	11.9	12.5
25E-NBOH		
2	14.1	14.7
40	13.9	16.2
80	4.5	7.8
Deschloroetizolam		
2	4.8	5.4
40	4.4	7.7
80	2.2	3.0
Flualprazolam		
2	12.4	12.7
40	5.6	4.7
80	6.6	6.5
Flubromazolam		
2	9.3	10.7
40	5.6	13.7
80	3.1	3.2
Etizolam		
2	8.9	10.3
40	6.8	7.7
80	5.7	5.6

QC: quality control.

Table 4
Real case samples analyzed by the proposed technique.

Samples	Analyte	Concentration (ng mL^{-1})
01	Butylone	8.9
02	N-ethylpentedrone	19.2
03	N-ethylpentylone	1.1
04	DMT	3.3
	THH	51.3
	HML	26.3
	HMN	26.3
05	DMT	< LOQ
	THH	26.2
	HML	17.6
	HMN	10.6
06	DMT	6.6
	THH	38.4
	HML	12.4
	HMN	9.9
07	25B–NBOH	< LOQ

LOQ: limit of quantitation.

aromatic rings, hence directly affecting the interaction between SLM and target analytes [32–34]. In addition to those chemical interactions, different classes of drugs of abuse were used as model analytes (Fig. 2 and Table S2). Thus, the result of combining a complex SLM composition

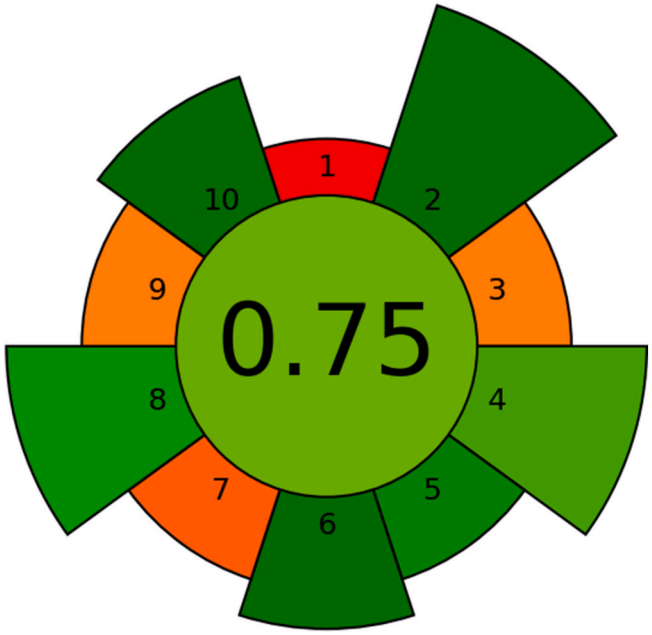

Fig. 5. AGREEprep assessment of the EO-based PALME proposed in the present work. The value in the inner circle represents the overall score of the technique (0–1.0). Each of the ten parameters evaluated are placed around the inner circle and the length of each criterion represents the weight on the final score. The darker the green tones in each of the ten criteria, closer to the desired sustainable performance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to extract different groups of drugs with chemically diverse properties seemed to have been the cause for the differences in extraction efficiency observed in Fig. 3. To pinpoint how each EO constituent might be influencing analyte extraction, these EO molecules would have to be studied in isolated or in defined mixtures [35]. For the present work, the EO termed smart & sassy was selected as the best compromise for simultaneous extraction of all compounds and, therefore, was optimized as described below.

3.2. Optimization of operational parameters

Based on previous works, the sample pH, acceptor pH, sample volume, agitation rate, and extraction time were considered the major operational parameters. In addition, TOA was added to the liquid membrane to suppress secondary interactions with the solid support membrane [18,19,22–24]. Therefore, the concentration of TOA was considered an important operational parameter as well. In total, six parameters were included in this first optimization step. The fractional factorial screening 2^{6-1} was performed considering the concentration of base in the sample (10–100 mM), the concentration of acid in the acceptor (10–100 mM), sample volume (100–200 μL), percentage of TOA added to the liquid membrane (0–5%), agitation rate (300–700 rpm), and time (5–120 min). The outcome of the optimization experiments is summarized in Fig. 4.

The agitation time was the most important operational parameter, followed by the concentration of base in the sample, sample volume, and percentage of TOA (Fig. 4A–C). The concentration of acid in the acceptor was critical only for the designer benzodiazepines (Fig. 4C). These compounds are extremely weak bases and, therefore, required strongly acidic conditions in the acceptor (Table S2).

After this screening step, a CCD was performed to find the optimum extraction conditions. Extraction recoveries increased with increasing extraction time up to 120 min. Extractions for 180 min were also tested but these failed due to stability issues with the EO liquid membrane in the final stage. At 120 min, equilibrium was still not obtained. Thus, compared with LPME using synthetic organic solvents as the liquid membrane [18,20–24], the extraction kinetics in the current EO-based system was relatively slow. This was likely attributed to the higher viscosity of the smart & sassy EO.

The extraction recoveries were also affected by the concentration of base in the sample. As seen from Fig. 4E and F, the extraction efficiency decreased upon increasing the concentration of NaOH in the sample. This is not typical for LPME with pure synthetic liquid membranes and indicates that high levels of NaOH affected the chemical stability of the EO-based liquid membrane. Most probably, basic hydrolysis may explain this observation and may also explain why the EO liquid membrane cannot be used for very long extractions. Therefore, 10 mM NaOH was selected for pH adjustment of the sample.

Extraction recoveries increased with increasing sample volume up to 150 μL. Above this level, the convection in the sample was reduced despite strong agitation and the extraction efficiency decreased (Fig. 4D and F). This observation was in accordance with previous studies [18,19,23]. In addition, the performance was not affected by the addition of TOA to the liquid membrane. Most probably, natural constituents of the EO suppressed secondary interactions and, therefore, no additional effect was obtained with TOA [19–22,24,32–34].

In a separate set of experiments, NaOH and formic acid were replaced with sodium bicarbonate and citric acid. The two latter substances are less hazardous and greener [36]. The results are summarized in Fig. S1 in supporting information. Generally, recoveries with sodium bicarbonate were lower than with NaOH, due to pH. For most model analytes, extractions with citric acid and formic acid were comparable, but for the four designer benzodiazepines, the green alternative provided improved extraction recovery. Therefore, in the final method, NaOH and citric acid were selected.

3.3. Method validation

The calibration curves used for all the analytes ranged from 1 to 100 ng mL^{-1} with $r^2 \geq 0.99$ (Table 1). This range was chosen as a compromise based on the typical plasma concentrations of the drugs covered by the method [4,5,10,11]. The established LOD for most analytes varied between 0.1 and 0.5 ng mL^{-1}, while the LOQ was set to 1 ng mL^{-1} for all compounds (Table 1). Although the model analytes were not extracted exhaustively, the LOD were comparable to those obtained in similar PALME setups for methylone, pentedrone, 2C-E, deschloroetizolam, etizolam, and flubromazolam [21,23]. Thus, although the current work used an EO-based liquid membrane and slower kinetics were observed, detection and quantification limits were not seriously affected.

The accuracy and imprecision of the method were also within acceptable limits established by validation guidelines, that is ±20% and ≤20%, respectively (Table 1 and Section 2.5.3) [29]. The ME study showed no ion suppression nor enhancement ±20%, which is in agreement with previous studies that demonstrate PALME provides excellent sample clean-up (Table 2) [19,22,26,37]. In addition, despite the slow kinetics observed, the RE values were high for most model analytes. As for the poorly extracted compounds (RE < 60%), the LOD achieved suffices for the application to real case samples (Table 2). Thus, the technique showed to be efficient even for the analytes with poor recoveries.

In a final validation step, extraction with EO from different production batches was tested. This was based on the fact that EO are plant extracts, and their composition may vary depending on several factors including the harvest, season, and extraction conditions [32–34]. In a new set of experiments, smart & sassy EO of different batches were acquired and evaluated in terms of intermediate precision [11]. As shown in Table 3, the imprecision for most of the analytes was ≤15% at the three QC levels. A closer investigation of these results revealed that the older EO bottle included in this experiment was responsible for producing the higher values observed in the between-batch imprecision.

Table 5
Comparison of the proposed PALME method with previous applications.

Donor phase		SLM	Acceptor phase*	Extraction time (min)	Analytes	RE (%)	ME (%)	Ref.
pH adjustment	Sample							
200 µL of NaOH 20 mM	400 µL of plasma	2 µL of dihexyl ether	Formic acid 20 mM	30	Pethidine, haloperidol, methadone, and nortriptyline	55–89	–	[18]
125 µL of HCl 250 mM	125 µL of plasma	2 µL of dihexyl ether	NH_3 25 mM	60	Ketoprofen, fenoprofen, diclofenac, flurbiprofen, ibuprofen, and gemfibrozil	59–108	–	[20]
115 µL of NaOH 40 mM	125 µL of plasma	4 µL of hexadecane	Formic acid 20 mM	120	Fluoxetine, fluvoxamine, and quetiapine	91–96	86–109	[37]
115 µL of HCl 250 mM		4 µL of isopentylbenzene	NH_3 25 mM					
125 µL of phosphate buffer 50 mM	125 µL of plasma	2.5 µL of 2-nonanone + 15% DEHP	Trifluoro acetic acid 150 mM	45	Hydralazine, ephedrine, metaraminol, salbutamol, and cimetidine	2–89	–	[19]
115 µL of NaOH 40 mM	125 µL of plasma	5 µL of dodecyl acetate + 1% TOA	Formic acid 20 mM	120	Methylone, 4-Fluoroamphetamine, pentedrone, 3,4-Methylenedioxypyrovalerone, *meta*-	25–117	–	[21]
75 µL of NaOH 80 mM	150 µL of whole blood				Chlorophenylpiperazine, 6-(2-aminopropyl) benzofuran, methoxetamine, ethlyphenidate, methylenedioxy-2-aminoindane 2C-E, bromo-dragonfly, and AH-7921	3–86		
250 µL of NaOH 10 mM	DBS	4 µL of dodecyl acetate + 1% TOA	Formic acid 20 mM	60	Ketoprofen, fenoprofen, diclofenac, and flurbiprofen	58–74	–	[22]
250 µL of formic acid 20 mM			NH_3 25 mM		Quetiapine and amitriptyline	74–88	88–102	
115 µL of NaOH 40 mM	125 µL of plasma	5 µL of dodecyl acetate + 1% TOA	Formic acid 20 mM	120	Citalopram, fluoxetine, venlafaxine, *o*-desmethylvenlafaxine, sertraline, norfluoxetine, and paroxetine	72–111	86–112	[24]
130 of phosphate buffer 50 mM	100 µL of whole blood	2-undecanone and dihexyl ether (1:1) + 1% TOA	150 µL of DMSO + formic acid 200 mM (75:25)	60	Alprazolam, bromazepam, deschloroetizolam, diazepam, diclazepam, etizolam, phenazepam, flubromazepam, flubromazolam, flunitrazepam, clonazepam, clonazolam, lorazepam, meclonazepam, midazolam, N-desmethyldiazepam, nitrazepam, oxazepam, zolpidem, and zopiclone	52–104	87–107	[23]
115 µL of NaOH 40 mM	125 µL of plasma	4 µL of dodecyl acetate + 1% TOA	Formic acid 20 mM	60	Amitriptyline, nortriptyline, quetiapine, venlafaxine, *O*-desmethyl-venlafaxine, and fluoxetine	47–89	–	[28]
125 µL of NaOH 50 mM or $NaHCO_3$ 500 mM	125 µL of plasma	3 µL of dihexyl ether or sesame oil	Formic acid 20 mM or citric acid 10 mM	120	90 basic compounds (drugs and endogenous metabolites)	0–103	-	[36]
125 µL of NaOH 40 mM	125 µL of breast milk	3 µL of dihexyl ether + 1% TOA	100 of µL formic acid 20 mM	50	Amphetamine, methamphetamine, and MDMA	40–89	88–105	[26]
HCl	Water samples	4 µL of 1-Octanol	NaOH	120	Soman acid, ethyl methylphosphonic acid, sarin acid, cyclohexyl-sarin acid, and isobutyl methylphosphonic acid	0–100	–	[44]
Drops of HCl (32%)	350 µL of urine	4 µL of n-Octanol	NaOH 1 M	120	Soman acid, ethyl methylphosphonic acid, sarin acid, cyclohexyl-sarin acid, and isobutyl methylphosphonic acid	–	–	[45]
140 µL of phosphate buffer 50 mM	100 µL of plasma	5 µL of dodecyl acetate + 1% TOA	DMSO and formic acid 200 mM (50:50)	30	Repaglinide	97–101	98–102	[46]
100 µL of NaOH 25 mM	150 µL of plasma	3 µL of EO	Citric acid 10 mM	120	29 basic drugs of abuse	20–106	80–119	This work.

*Acceptor volume was 50 µL if not specified. EO: essential oil; SLM: supported liquid membrane; RE: recovery; ME: matrix effect; TOA: trioctylamine; DEHP: 2-di(ethyl-hexyl) phosphate; DBS: dried blood spot; DMSO: Dimethyl sulfoxide.

Nevertheless, all values were ≤20% and, therefore, within accepted limits by validation guidelines [29]. In conclusion from this experiment, the extraction performance with different batches of EO varied to some extent but is avoidable by using the same batch for series of extractions. This could be attributed to loss of stability of the compounds present in the EO.

As a final proof of the applicability of the proposed technique, seven real case samples were analyzed and the results are displayed in Table 4.

3.4. Greenness score

As the sole purpose for studying the use of EO as liquid membrane was environmental consciousness, the greenness of the method was evaluated. The AGREEprep approach was chosen for it allows a thorough evaluation of different aspects of the sample preparation procedure [38,39].

In this work, the focus was on replacing pure synthetic solvents used in PALME with greener alternatives. The use of hazardous solvents in a sample preparation procedure is evaluated by criterion 2 in AGREEprep. As shown in Fig. 5, this parameter was considered highly green. In combination with other features, such as using only 150 µL of plasma and replacing formic acid with citric acid, the overall score was slightly improved in comparison to previous works (Fig. 5) [36]. In contrast, the lowest scores obtained in this evaluation were related to sample preparation placement (1), reusability of the materials (3), lack of automation (7), and type of analytical instrumentation (9). Some of these four limitations are easier to address than others. For example, the liquid membrane is not reusable (3), but the technique performed in this study

is amenable to automation [27,40,41] (7), and other instrument can be used for analysis [20,31] (9). This illustrates that PALME is indeed a green alternative and improvements can still be explored to further reduce the environmental impact of this technique.

3.5. Feasibility of PALME for routine analysis

In this work, LPME in the 96-well plate format was studied as a tool for investigating the consumption of drugs of abuse by patients. With the increasing number of NPS, this has been a troublesome task in routine laboratories dealing with intoxicated patients. To address such cases, a reliable sample preparation technique that is fit to both traditional drugs of abuse and NPS is warranted. Moreover, this technique should also be fast and simple allowing the rapid analysis of urgent cases. The overall extraction of basic drugs of abuse from plasma samples using the herein proposed PALME proved to meet those requirements as it is considered a single-step procedure [19]. In addition to that, this setup allows the simultaneous processing of up to 96 samples, which is excellent for laboratories with massive routine casework.

Another important feature that has a major impact on the implementation of a technique in routine analysis is automation because it minimizes human handling further simplifying the analytical pipeline. In this regard, LPME automation has been reported, although such approaches tend to require sophisticated systems [42,43]. In contrast, PALME is simpler by default and so are the automated systems [27,40,41]. Thus, even though the PALME setup used in the present work was entirely manual, the advantage of being amenable to automation brings this technique closer to implementation in routine analysis. Of note, PALME automation would meet even closer today's standards of environmental consciousness (Fig. 5) [14].

In combination, the fast and simultaneous analysis of almost a hundred samples makes PALME a valuable tool not only for cases of drug consumption. Table 5 summarizes previously published articles using PALME for different applications. In terms of donor phase composition (NaOH) and extraction time (120 min), the technique described herein uses parameters similar to other works. In contrast, this study stands out in terms of acceptor phase and SLM by replacing formic acid with citric acid and completely dismissing the use of organic solvents as extractors, respectively. Moreover, most studies have focussed on medications, such as antidepressants and anti-inflammatory drugs, while only few have investigated PALME for the analysis of drugs of abuse and included different classes or even NPS (Table 5).

4. Conclusion

In this conceptual work, an EO-based PALME to extract model analytes from plasma samples was explored. A total of 29 illicit substances were chosen due to their toxicological relevance and heterogeneous chemical properties.

Among all SLM tested, the smart & sassy EO was the best candidate for all compounds covered by the method. However, compared to pure synthetic solvents, this EO had higher viscosity resulting in slower extraction rates. Nevertheless, the sensitivity required for the applicability of this method to real cases was still achieved for all analytes, even the ones that were poorly extracted.

Importantly, the natural variabilities of the smart & sassy EO were also considered during this study and were found not to impact the analytical robustness of the method. However, results suggest that EO bottles should be used for series of experiments, as extraction efficiency might decrease over time likely due to stability issues.

From an eco-friendly perspective, using only 3 μL of EO,150 μL of sample, citric acid as acceptor, and being able to simultaneously extract up to 96 samples makes PALME a valuable tool for toxicological analyses – especially considering that the technique is fit for different classes of compounds. This feature is particularly relevant in the current NPS scenario in which novel drugs are constantly appearing in the illicit drug market.

CRediT authorship contribution statement

André Luis Fabris: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Stig Pedersen-Bjergaard:** Writing – review & editing, Visualization, Formal analysis, Conceptualization. **Elisabeth Leere Øiestad:** Writing – review & editing, Resources. **Giordano Novak Rossi:** Resources. **Jaime E.Cecílio Hallak:** Resources. **Rafael Guimarães dos Santos:** Resources. **Jose Luiz Costa:** Writing – review & editing, Resources, Funding acquisition. **Mauricio Yonamine:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo [grant numbers 2021/09857–8, 2020/10809–5], Conselho Nacional de Desenvolvimento Científico e Tecnológico [grant numbers 142056/2020–0, 303275/2017–0, 315640/2021–9], and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES/PROCAD [INSPEQT Project, grant number 16/2020].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.342387.

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