Green Analytical Chemistry 3 (2022) 100028

Contents lists available at ScienceDirect

Green Analytical Chemistry

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

Liquid-phase microextraction in bioanalysis – How green can it be?

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ARTICLE INFO

Keywords:
Green analytical chemistry
Green sample preparation
Liquid-phase microextraction
Supported liquid membrane
AGREEprep

ABSTRACT

Fundamental experiments were conducted with three-phase liquid-phase microextraction (LPME) to optimize greenness, followed by evaluation with the AGREEprep metric system. Extractions were from human plasma, and recoveries were studied for 90 different basic substances (drugs and endogenous metabolites) in the log P-range from -4.2 to 8.1. In a first set of experiments, LPME was performed in a 96-well system using traditional LPME chemicals. Extraction was from 125 µL of human plasma, diluted with 125 µL of 10 mM NaOH, through 3.0 µL of dihexyl ether as liquid membrane, and into 50 µL of 20 mM HCOOH as acceptor. The optimal extraction window (OEW) with this system ranged from log P 2.2 to 5.0, and the majority of drug substances within OEW were extracted exhaustively (average recovery 71%). For this procedure, the AGREEprep metric score for greenness was 0.62. In a next set of experiments, NaOH, dihexyl ether, and HCOOH were replaced with $NaHCO_3$, sesame oil, and citric acid to improve greenness. Even with sesame oil, the majority of the drug substances with log P from 2.2 to 5.0 were extracted with high efficiency, and the AGREEprep score increased to 0.68 due to the highly green nature of the chemicals. In a third set of experiments, where LPME was conducted in a vial-based approach, greenness was improved further by recycling and washing equipment in hot water, except the liquid membrane. Extraction performance was unaffected by washing and recycling, and no carry-over was observed from the extraction equipment. Upon equipment washing and recycling, the total material waste was reduced to less than 10 mg per sample, and the AGREEprep score increased to 0.72. Because experiments were conducted manually and in laboratory, and because LC-MS was used for final analysis, we were unable to improve the AGREEprep score further. The experiments illustrate that LPME procedures may be developed to a very high level of greenness, under assistance by the AGREEprep metric assessment approach.

1. Introduction

Green Chemistry (GC) emerged in the 1990s to address the impact of chemicals and chemical processes on human health and environment [1]. Analytical chemistry plays an important role in GC, because it contributes with the chemical measurements required to develop, implement, and assess the GC activities. On the other hand, chemicals and energy are consumed in analytical chemistry as well, and therefore Green Analytical Chemistry (GAC) was introduced to minimize the negative environmental impact of chemical measurements through reducing energy demand, toxic laboratory waste, and use of hazardous solvents and chemicals [2].

While GAC emerged as a branch of GC, most of the original principles of GC were loosely related to analytical chemistry. For this reason, the twelve principles of GAC were introduced almost a decade ago [3]. The principles focused on minimizing sample volumes, in-situ measurements, integration and automation of analytical processes, avoiding derivatization, reducing chemical waste, implementing multi-analyte methods, low energy consumption, chemicals from renewable sources, replacing toxic chemicals, and increasing the safety for the operator. In addition, the GAC principles stated that direct analytical techniques should be applied to avoid sample pretreatment. This statement somewhat neglected the importance of sample preparation, and the important green technological advances in sample preparation. For this reason, Green Sample Preparation (GSP) was defined recently in the context of GC and GAC, based on ten principles [4]. The GSP principles emphasize in-situ activity, safe solvents and chemicals, reusable and sustainable materials, minimum amounts and waste, high sample throughput, integration and automation, low energy consumption, green instrumental methods, and operator safety. To assess the greenness of analytical methods in relation to the GAC principles, different approaches have been proposed and used up to date [5–15]. However, with the introduc-

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https://doi.org/10.1016/j.greeac.2022.100028
Received 6 July 2022; Received in revised form 18 August 2022; Accepted 25 August 2022

tion of the ten principles for GSP, a corresponding metric assessment system was required. This was termed AGREEprep and was presented very recently [16].

Green technological advances in the field of sample preparation include a variety of different microextraction techniques. Microextraction in analytical chemistry was initiated by the introduction of solid-phase microextraction (SPME) [17], and was followed among others by introduction of stir bar sorptive extraction (SBSE) [18], single drop microextraction (SDME) [19, 20], dispersive liquid-liquid microextraction (DLLME) [21, 22], hollow-fiber liquid-phase microextraction (HF-LPME) [23], 96-well liquid-phase microextraction (96-well LPME) [24], and electromembrane extraction (EME) [25]. All these microextraction methods can be assessed by the AGREEprep metric tool, but such data are currently very limited. With AGREEprep, different methods can be compared in terms of greenness. Furthermore, AGREEprep can assist in increasing the greenness of microextraction methods.

In the current paper, we report on a fundamental set of bioanalytical experiments with liquid-phase microextraction, where greenness was enhanced, assisted by AGREEprep. In contrast to previous work [23–25], traditional chemicals were replaced with highly green alternatives, and equipment was recycled after washing with hot water. In each experiment, extraction performance was documented with recoveries measured for 90 different substances (drugs and endogenous metabolites). Based on data from such a large number of substances, we were able also to define the optimal extraction window (OEW) [26]. The OEW is the log P range where the LPME system is expected to extract with high recovery. In parallel, greenness was assessed using the AGREEprep metric system. We used liquid membrane-based extraction systems, because such LPME systems are compatible even with very complex biological and environmental samples [27]. The majority of extractions were conducted in a 96-well system, because this type of technical format is preferred by industry and routine laboratories. We emphasize that procedures were subjected to fundamental experiments only, with the purpose of illustrating different approaches to increase greenness, and their impact on extraction performance. Procedures were not validated, as this was outside the scope of the current paper.

2. Experimental

The following sections describe the chemicals used, preparation of solutions, and LPME.

2.1. Chemicals

90 basic substances (drugs and endogenous metabolites), with log P values ranging from −4.2 to 8.1 were extracted (listed in Supplementary Material). Dihexyl ether, sodium bicarbonate, formic acid, and citric acid were obtained from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from VWR (Radnor, PA, USA). Olive oil, rapeseed oil, sunflower oil, and sesame oil were purchased from a local grocery store. Human plasma was obtained from Oslo University Hospital (Oslo, Norway), and stored at –28 ˚C.

Acetonitrile (LC-MS grade) and formic acid (LC-MS grade) were purchased from VWR (Radnor, PA, USA). Deionized water was obtained with a Millipak® (0.22 μm filter) purification system from Milli-Q (Molsheim, France).

2.2. Preparation of solutions

The 90 basic substances (drugs and endogenous metabolites) were dissolved individually at concentrations ranging from 1.0 to 9.0 mg/ml. They were then mixed and diluted to prepare a spiking solution at 5.0 μg/ml per compound. The spiking solution was added to human plasma to a concentration of 100 ng/ml. Spiked plasma was further diluted 1:1 with 50 mM NaOH or 500 mM sodium bicarbonate to maintain alkaline conditions in the sample.

2.3. 96-Well liquid-phase microextraction (96-well LPME)

The equipment used for 96-well LPME has been described previously [28]. Extractions were performed with a 96-well polypropylene deep well plate, with 0.5 mL wells from Agilent (Santa Clara, CA, USA) as sample plate. The acceptor plate was a 96-well MultiScreen-IP filter plate from Merck Millipore (Carrigtwohill, Ireland). The filter material was polyvinylidene fluoride (PVDF) with a pore size of 0.45 μm. Adhesive Platemax Pierceable Aluminum Sealing Film (Axygen, Union City, CA, USA) was used to seal the acceptor plate. Agitation at 900 rpm was accomplished during extraction with a Vibramax 100 agitation system from Heidolph (Kellheim, Germany).

The procedure included pipetting 125 μL of spiked plasma and 125 μL of either 50 mM sodium hydroxide or 500 mM sodium bicarbonate into a well in the sample plate (Fig. 1A). The corresponding filter on the acceptor plate was impregnated with 3.0 μL dihexyl ether or 2.7 μL sesame oil (Fig. 1B), which served as liquid membrane. 50 μL of 20 mM formic acid or 10 mM citric acid was pipetted above the liquid membrane, and served as acceptor (Fig. 1C). The acceptor and sample plate were clamped together and sealed with the adhesive aluminum foil, and was agitated at 900 rpm for 120 min to complete extraction. After extraction, the acceptor solution was collected and analyzed with UHPLC-MS/MS.

2.4. LPME in commercial equipment intended for electromembrane extraction (vial-based LPME)

Vial-based LPME (Fig. 1D) was performed with prototype device from Extraction Technologies Norway (Ski, Norway), intended for electromembrane extraction and described previously [29]. We used this equipment without the external power supply. Up to 10 samples were extracted simultaneously, and the vials holding the sample and acceptor solutions were produced in conducting polymer. The liquid membrane was immobilized in a flat porous polypropylene membrane (Extraction Technologies Norway), and this was located in a union connecting the sample vial and the acceptor vial.

Extraction was performed by pipetting 150 μL spiked plasma and 150 μL 500 mM sodium bicarbonate into the sample vial, and 300 μL 10 mM citric acid into the acceptor vial. Sesame oil (7.0 μL) was pipetted onto the polypropylene membrane. The sample and acceptor vials were connected by the union, housing the membrane. The connected vials were placed on a shaker (Extraction Technologies Norway), and extraction was performed for 120 min at 900 rpm. After extraction, acceptors were analyzed with UHPLC-MS/MS.

2.5. Liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

Acceptors were analyzed using an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA), consisting of a binary pump, an auto-sampler, and a column compartment with controllable temperature. Mobile phase A consisted of 95:5 v/v deionized water and acetonitrile containing 0.1% formic acid. Mobile phase B consisted of 5:95 v/v deionized water and acetonitrile containing 0.1% formic acid. The column was an Eclipse Plus C18 (2.1 × 50 mm, 1.8 μm, Agilent Technologies, Santa Clara, CA, USA) and was operated at 40 ˚C. The injection volume was 1.0 μL. The following gradient was applied: mobile phase B was kept at 0% from 0.0 to 1.0 min, ramped linearly to 53% from 1.0 to 6.0 min, 75% at 7.0 min, and 100% at 7.01 min. 100% mobile phase B was kept for 0.5 min, and was then set to 0% for a final 1.5 min re-equilibration. The flow of mobile phase was 0.4 mL/min during 7.0 min, 0.7 mL/min during 7.0–8.5 min, and 0.4 mL/min during 8.5–9.0 min.

Mass spectrometric detection was performed by an Agilent 6495 LC/TQ (Agilent Technologies, Santa Clara, CA, USA) with positive electrospray ionization at 3 kV and a gas temperature of 200 ˚C. The system

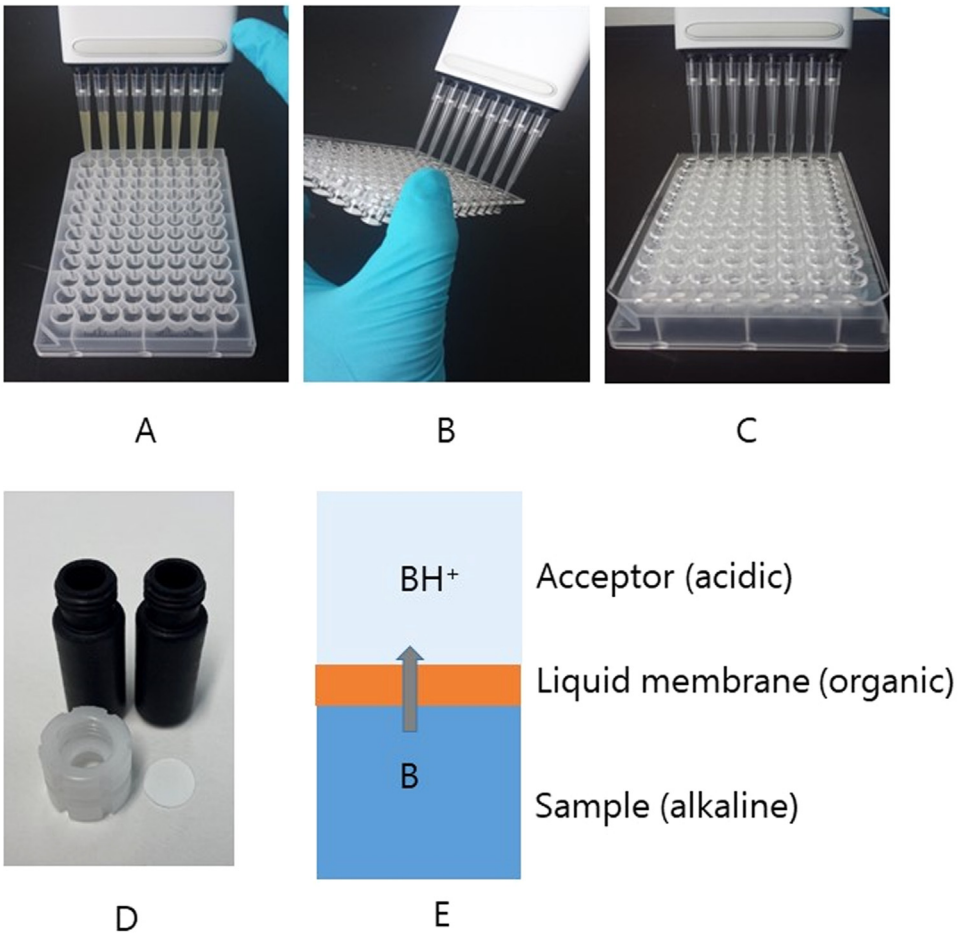

Fig. 1. LPME configurations, equipment, and principles. A) Pipetting samples into sample plate for 96-well LPME, B) pipetting liquid membrane solvent into filters in acceptor plate for 96-well LPME, C) pipetting acceptor into acceptor plate for 96-well LPME, D) equipment for vial-based LPME, E) extraction principles for 96-well and vial-based LPME.

was operated in dynamic MRM-mode, with a cycle time of 300 ms, resulting in a minimum dwell time of 4.52 ms. The MRM-transitions and the collision energies are listed in the Supplementary Material.

2.6. Calculations

Extraction recovery was calculated by Eq. 1:

$$R = \frac{C_{a,final}}{C_{s,initial}} \times \frac{V_a}{V_s} \times 100\% \quad (1)$$

Here, $C_{a,final}$ and $C_{s,initial}$ are the concentrations in the acceptor after the extraction and the concentration of analyte in the sample before the extraction, respectively. The terms V_a and V_s denote the volume of the acceptor and sample, respectively.

3. Results and discussion

In this work, all extractions were based on liquid-phase microextraction (LPME) and were initially conducted in 96-well technology, with potential for automation and high-throughput operation. Extraction performance was characterized for 90 different basic drug substances ($-4.2 < \log P < 8.1$) present in human plasma samples, and UHPLC-MS/MS was used as instrumental method. Two 96-well plates, as shown in Fig. 1A-C, were used as extraction device; samples were located in a deep well plate (termed sample plate), while a filter plate was used to hold the liquid membrane and acceptor (termed acceptor plate). The sample plate was made of polypropylene. The acceptor plate comprised 96 porous membranes of polyvinylidene fluoride, attached to acrylic glass (poly(methyl methacrylate). The extraction principle is shown in Fig. 1E. The basic drug substances were extracted as neutral species; from human plasma adjusted to pH ≥ 8,5, through a thin layer of organic solvent immobilized in the pores of the PVDF membrane (termed liquid membrane), and into acidified water (acceptor).

3.1. Performance and AGREEprep assessment of traditional 96-well LPME procedure

In a first set of experiments, a traditional procedure for non-polar basic compounds was evaluated with respect to extraction performance and greenness [28]. Operational parameters affecting recovery, including the composition and volume of the liquid membrane, volumes and pH of the sample and acceptor, time, and agitation, were selected based on previous experience from our laboratory. In this procedure, there was no recycling, and all chemicals and material inputs were wasted. For each plasma sample (125 µL), 3.0 µL dihexyl ether was used as liquid membrane (equivalent to 2.4 mg solvent). Sample pH was adjusted from 7.4 to approximately 10 by addition of 125 µL of 10 mM sodium hydroxide in water. This corresponded to 0.05 mg of pure NaOH per sample. The acceptor was 50 µL of 20 mM formic acid, and this corresponded to 0.05 mg of pure HCOOH per sample. The 96-well sample plate comprised 28.4 g of polypropylene, and the material waste per sample was equivalent to 296 mg. The 96-well acceptor plate consisted of acrylic glass and PVDF. The total weight was 36.6 g, and this plate contributed with 381 mg waste per sample.

96-Well LPME was performed for 120 min, and this long extraction time was selected to obtain equilibrium data. Extraction recoveries are plotted in Fig. 2A for the 90 basic substances (drugs and endogenous metabolites), as function of their log P. Drug substances in the range $-4.2 < \log P < 2.2$ suffered as expected from poor partition into the liquid membrane, due to polarity, and recoveries were practically zero.

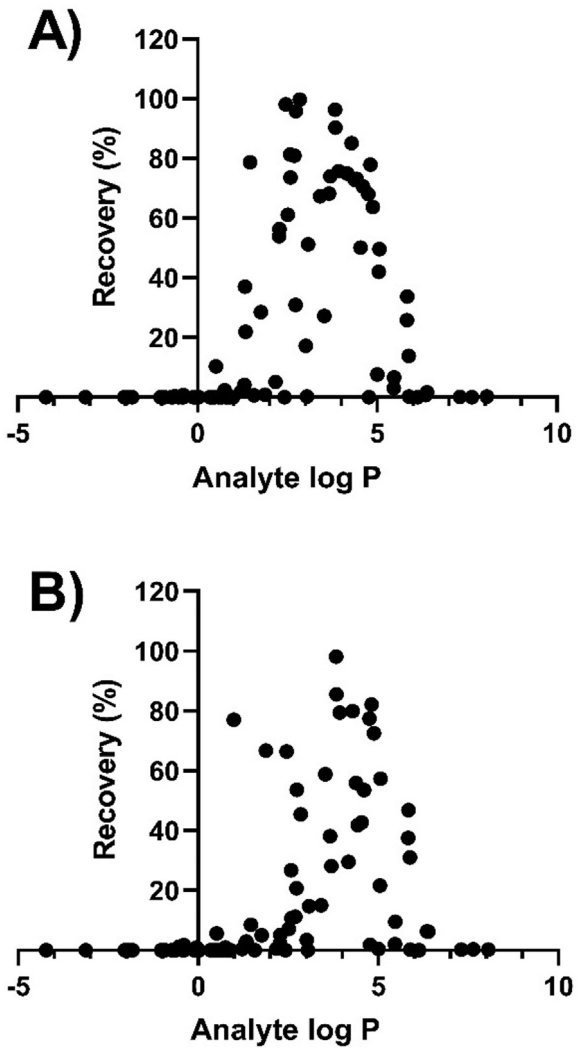

Fig. 2. Recoveries for 90 basic substances (drugs and endogenous metabolites) after 96-well LPME using A) traditional chemicals and B) green chemicals.

For drug substances with log $P > 2.2$, partition into the liquid membrane was favored. However, the most hydrophobic ones, with log $P > 5.0$, were partly trapped in the liquid membrane. Therefore, the optimal extraction window (OEW) [26] with dihexyl ether as liquid membrane was in the range $2.2 < \log P < 5.0$. Among 31 drug substances in this log P-range, 27 were extracted with recoveries exceeding 40%, and their average recovery was 71%. Four drug substances with log P within the defined OEW were not extracted, due to zwitterionic properties.

In a next step, the greenness of the 96-well LPME procedure was assessed using the AGREEprep metric tool [16]. This is based on ten different criteria (Table 1) with associated weighed score values ranging from zero to 1.0. The first criterion (criterion 1) favors in situ sample preparation to minimize time, materials, and energy. Our experiments were performed in laboratory (*ex situ*), and accordingly the score value was set to zero. The weight factor of criterion 1 is 1.0. Criterion 2 is related to the consumption of toxic and hazardous substances. This individual score calculation is logarithmic, and is based on total mass of hazardous substances. By definition, this score is set to 1.0 when the total mass of hazardous substances is less than 10 mg per sample. In the present case, the total mass of hazardous substances per sample were equal to the sum of the consumptions of pure dihexyl ether, sodium hydroxide, and formic acid:

Table 1
AGREEprep criteria [16].

Criterion 1	Sample preparation placement
Criterion 2	Hazardous materials
Criterion 3	Sustainability and renewability of materials
Criterion 4	Waste
Criterion 5	Size economy of sample
Criterion 6	Sample throughput
Criterion 7	Integration and automation
Criterion 8	Energy consumption
Criterion 9	Post-sample preparation configuration for analysis
Criterion 10	Operators safety

2.4. mg dihexyl ether + 0.05 mg sodium hydroxide + 0.05 mg formic acid = 2.5 mg per sample

The total mass of hazardous substances was less than 10 mg per sample, and therefore the score was set to 1.0. The weight factor for criterion 2 is 5.0. Criterion 3 concerns sustainability, reusability, and renewability of materials. In the current context, the two 96-well plates were defined as materials. Because they were not reused, and because they were produced from polypropylene and acrylic glass, the individual score was set to zero (weight factor 2.0).

Criterion 4 is related to the waste of material per sample. The material waste per sample was:

296 mg sample plate + 381 mg acceptor plate + 2.5 mg substances = 680 mg

The corresponding score value (logarithmic function) was 0.69 (weight factor 4.0). Greenness is favored by small sample sizes, and this is covered by criterion 5. The sample size was 125 μL in our case, and the logarithmic score value was calculated accordingly to 0.94 (weight factor 2.0). Extraction time was 120 min and during this period, we were able to process up to 96 samples in parallel. The potential sample throughput was therefore 48 samples per hour, and the score value for criterion 6 was calculated to 0.91 (weight factor 3.0).

Criterion 7 is related to the number of individual steps and to the level of automation. This criterion counts unitary steps, and our 96-well LPME procedure comprised only a single unitary step since acceptors were analyzed directly by LC-MS/MS after extraction. This resulted in a score of 1.0, but since all work was manual, this was multiplied with 0.25, and the total score for criterion 7 was 0.25 (weight factor 2.0). Criterion 8 addresses energy consumption, and in our extraction procedure, energy consumption was connected to the use of agitation. We used a 31 W agitation system, and two-hour operation consumed 62 Wh. With maximum load of samples, this corresponds to 0.66 Wh per sample. Based on this value, the individual score was set to 1.0 (weight factor 4.0).

The final analysis of acceptors was by LC-MS/MS, and the score for criterion 9 was set accordingly to 0.25 (weight factor 2.0). The final criterion 10 counts the pictogram labeling of the substances used. Dihexyl ether contributes two pictograms, sodium hydroxide has one pictogram, and formic acid has three pictograms. With six pictograms in total, the individual score was set to zero (weight factor 3.0).

The total AGREEprep assessment is summarized in Fig. 3A in the form of a pictogram showing a final score of 0.62. Greenness scores were high for minimal use of hazardous substances (criterion 2), low total mass of waste (criterion 4), small sample volume (criterion 5), high sample throughput (criterion 6), and low energy consumption (criterion 8). On the other hand, scores were low for criteria 1, 3, 7, 9, and 10. The score for criterion 1 was poor because extraction was performed in laboratory (ex situ), and the score for criterion 7 was low because extraction was done manually. Modern bioanalytical laboratories prefer LC-MS for the final analysis, and therefore LC-MS was used in the current set of experiments. This decision inevitably affected the score on criterion 9. While scores for criteria 1, 7, and 9 were not considered further, we addressed criteria 10 and 3 by new experiments as discussed below.

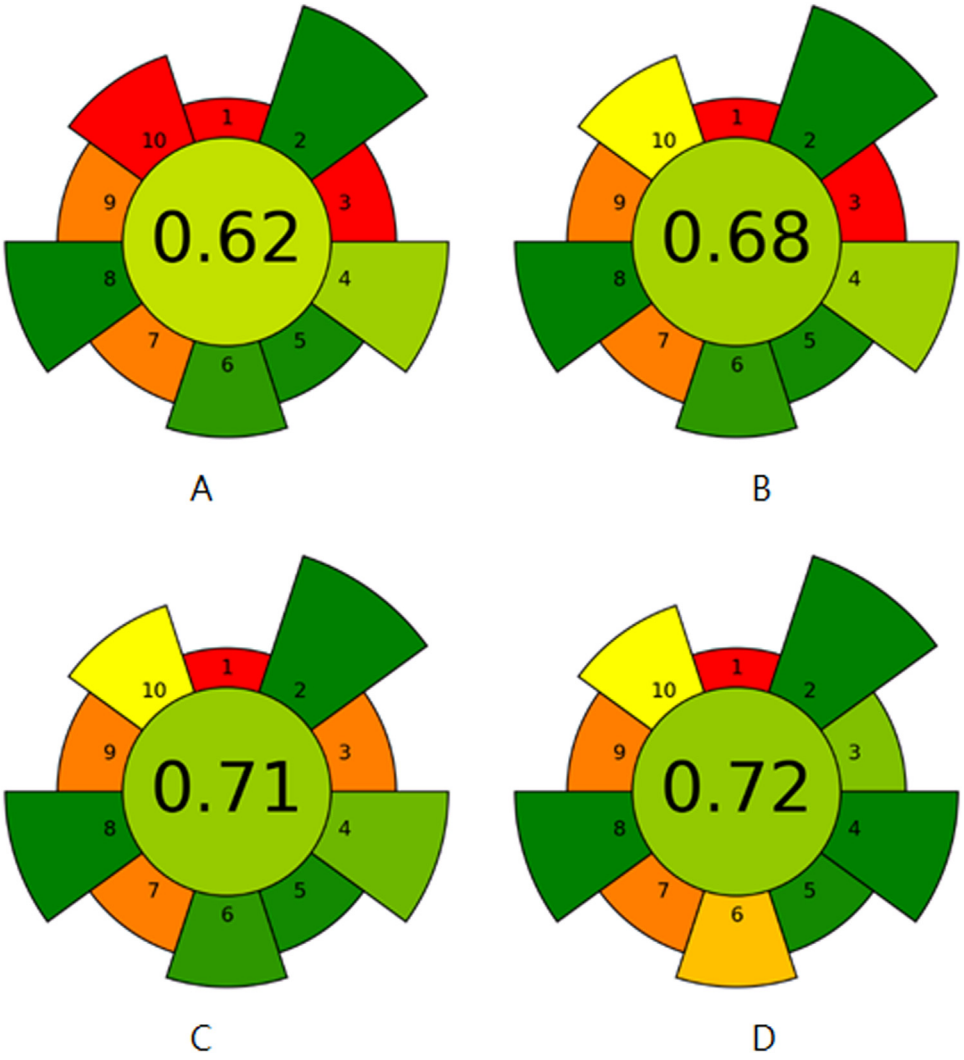

Fig. 3. AGREEprep assessment of LPME procedure. A) 96-well LPME with traditional chemicals, B) 96-well LPME with green chemicals, C) 96-well LPME with green chemicals and with recycling of the sample plate, and D) vial-based LPME with green chemicals and with recycling of equipment.

3.2. Improving greenness by substitution of chemicals

Green metrics allow the evaluation of the environmental impact of methods but at the same time identify the weak and strong points of the procedure. In a subsequent set of experiments, efforts were placed in improving the greenness of the method. The poor score on criterion 10 in Fig. 3A was related to the sum of pictograms for sodium hydroxide, dihexyl ether, and formic acid used during extraction. In a next set of experiments, these chemicals were replaced with substances used in the kitchen (green chemicals). Sodium hydroxide (one pictogram) was replaced with sodium bicarbonate (one pictogram). The number of pictograms was not reduced, but we still consider sodium bicarbonate more appropriate than sodium hydroxide. Formic acid (three pictograms) was replaced with citric acid (one pictogram), and dihexyl ether (two pictograms) was replaced with sesame oil. We found no pictograms for sesame oil, and we consider sesame oil as an ultimately green liquid membrane.

Sesame oil definitely showed potential as liquid membrane, and extraction performance is summarized in Fig. 2B. Looking into the log P-range from 2.2 to 5.0, 17 of 31 drug substances were extracted with recoveries above 40%, and their average recovery was 71%. Sesame oil provided somewhat higher selectivity than dihexyl ether, and recoveries appeared to be more structure dependent within the OEW. We also tested olive oil, rapeseed oil, and sunflower oil as liquid membrane. All of them functioned as liquid membrane, but they were less effective than sesame oil. In terms of AGREEprep scores, the substitution of substances improved the score for criterion 10. The assessment is summarized in Fig. 3B, and the total score increased from 0.62 to 0.68.

3.3. Improving greenness by reusing equipment

In a next set of experiments, we tried to improve the score related to criteria 3 (and 4) by washing and recycling the 96-well plates. The sample plate was easily washed in hot water without any use of organic solvent or surfactants. However, washing the acceptor plate was difficult because the liquid membranes were immobilized in the filters. Washing with ethanol and hot water was tested, but we were not able to remove the liquid membrane. Therefore, with the current 96-well plates, we were only able to recycle the sample plate. This reduced the waste of material under criterion 4 from 680 mg to 381 mg per sample. Due to this, the criterion 3 score increased from zero to 0.5, the criterion 4 score increased from 0.69 to 0.78, and the total AGREEprep score increased from 0.68 to 0.71 (Fig. 3C). Extractions with sample plates used previously, and washed in hot water, were successful; recoveries were the same as with new plates, and carry over was not observed.

In a final set of experiments, we performed extractions with vial-based LPME as illustrated in Fig. 1D. With this setup, LPME was done using commercial equipment for electromembrane extraction [30]. We used this equipment without the external power supply. The vials containing sample and acceptor were washed in hot water and recycled, and only the membranes, comprising 1.9 mg polypropylene and 6.4 mg sesame oil, were wasted. Thus, the material loss was reduced to 8.3 mg

per sample. Vial-based LPME provided recoveries comparable with 96-well LPME. Carry over using recycled vials were not exceeding 1%, and this originated from the UHPLC-MS/MS system and not from the recycled LPME vials. In terms of recycling and low material waste, vial-based LPME was superior to 96-well LPME. With vial-based LPME, the scores for criteria 3 and 4 therefore increased to 0.75 and 1.0, respectively. On the other hand, sample throughput was lower with vial-based LPME, because the equipment was designed for ten samples only. In 96-well LPME, the maximum sample throughput was 48 sample per hour; while this number was reduced to five for vial-based LPME. Equipment for vial-based LPME was approximately ten times heavier than for 96-well LPME, and this limited the capacity of the agitation system. Because of this, the score value for criterion 6 decreased from 0.91 to 0.38, and the total AGREEprep score was 0.72 (Fig. 3D)

3.4. Maximum AGREEprep score for LPME

In bioanalysis, LC-MS is currently the golden standard due to specificity and sensitivity, and such instrumentation is operated in specialized laboratories (ex situ). As long as LC-MS analysis has to be carried out ex situ, and because sample volumes are small and easily transportable, bioanalytical sample preparation will remain ex situ in the near future. Correspondingly, score values for criterion 1 and 9 will normally be zero and 0.25, respectively, in bioanalytical methods. With LPME as demonstrated above, reusability cannot be 100% because the liquid membrane and the solid support cannot be recycled [31]. Although the waste from this is less than 10 mg, the score value for criterion 3 will be limited to 0.75. In addition, as long as LPME is semi-automated, the score value for criterion 7 is limited to 0.25. Given the restrictions related to criteria 1, 3, 7, and 9, the maximum score value for LPME in bioanalysis is limited to 0.86. Our LPME experiments reported in Sections 3.1-3.3 were close to this value. However, to reach the limit, we need to adjust pH in sample and acceptor with chemicals without pictograms, and we need 96-well plates with replaceable membranes.

4. Conclusions

In the current paper, a three-phase liquid-phase microextraction (LPME) procedure for basic analytes was optimized with focus on greenness, assisted and assessed by the recent AGREEprep metric system. The LPME procedure provided a greenness score of 0.62 when traditional LPME chemicals were used for extraction. AGREEprep is a “strict” evaluation tool that is based on conventional and state-of-the-art sample preparation techniques. With this in mind, an impact score of the order of 0.62 is considered a high greenness score. Replacing the traditional chemicals with sesame oil as organic phase, sodium bicarbonate to control alkalinity, and citric acid to control acidity, the greenness score increased to 0.68. Washing equipment in hot water enabled recycling, and material waste was reduced to less than 10 mg per sample. This increased the greenness score to 0.72. Because LC-MS was used for analysis, and because sample preparation was performed manually in 96-well formate in our laboratory, maximum greenness score was limited to 0.86 in our bioanalytical framework. We expect that green microextraction methods, as exemplified in this work, will be important in the future, and we expect greenness metrics will be a common parameter documented for analytical methods.

Declaration of Competing Interests

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2022.100028.

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