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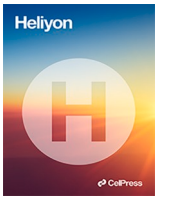

Optimisation of dispersive liquid-liquid microextraction for plasma sample preparation in bioanalysis of CDK4/6 inhibitors in therapeutic combinations for breast cancer treatment

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ABSTRACT

Cyclin D dependent kinase 4 and 6 (CDK 4/6) inhibitors are novel anticancer drugs used in therapeutic combinations with endocrine therapy for breast cancer treatment. Their determination in patient plasma is of high interest as a prerequisite for possible therapeutic drug monitoring. Dispersive liquid-liquid microextraction (DLLME) shows great potential in bioanalytical sample preparation. Its simplicity and speed, along with the suitability for using small amounts of sample and hazardous solvents are some of its main advantages. However, its application on plasma samples is scarce and requires further development. The aim of this work was to explore the applicability of DLLME in the simultaneous extraction of six drugs of interest from human plasma, with an emphasis placed on achieving high extraction recoveries with low sample and solvent consumption. To tackle the low availability and amount of the plasma sample, as well as the complexity of the biological matrix, three novel DLLME modes are proposed: organic sample DLLME (OrS-DLLME), aqueous sample DLLME (AqS-DLLME), and a modified air-assisted DLLME (AA-DLLME). The extractant and disperser type and volume, volume ratios of all the components in the ternary system, effect of pH and salting out were optimised for all three proposed modes of DLLME. Optimised representative DLLME-HPLC-DAD-FLD method was validated and shown to be linear ($R > 0.994$), precise (RSD $\leq$13.8%, interday), accurate (bias −13.1–13.1%, interday) and robust (relative effect −3.34–6.08%). Simultaneous extraction of all six drugs with high recoveries (81.65–95.58%) was achieved. Sample volumes used were as low as 50–100 μL, with necessary organic solvent volumes in μL ranges. Greenness scores obtained using the AGREE software were between 0.63 and 0.66, demonstrating compliance with green analytical chemistry principles. Finally, the validated method was successfully applied on breast cancer patient plasma samples.

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1. Introduction

Cyclin D dependent kinase 4 and 6 (CDK 4/6) inhibitors abemaciclib (ABE), palbociclib (PAL), and ribociclib (RIB) are novel anticancer agents, considered targeted therapy. They are used in therapeutic combinations with endocrine therapy anastrozole (ANA), letrozole (LET) or fulvestrant (FUL) for the treatment of breast cancer [1]. These drugs possess a wide array of physical-chemical properties, ranging from the hydrophilic and weakly basic RIB and PAL, over the more lipophilic weakly basic ABE, the intermediately lipophilic neutral ANA and LET, to the highly lipophilic, weakly acidic FUL. Their selected physical-chemical properties are shown in the Supplementary Table S1.

To improve clinical outcomes and reduce toxicity, these drugs may benefit from therapeutic drug monitoring (TDM). Several novel bioanalytical methods aimed at their determination in human plasma for the purposes of TDM have recently been published [1–6]. Most of these methods employ either non-selective protein precipitation (PPT) or lengthy and costly solid-phase extraction (SPE) as the sample preparation procedures, and only a few of them for the simultaneous determination of all six drugs of interest.

In the recent years, different types of solid and liquid phase microextractions have started to evolve, as a result of a growing effort to reduce the ecological impact of laboratory procedures. Dispersive liquid-liquid microextraction (DLLME) is a technique which relies on the use of a combination of organic solvents: a non-polar extractant immiscible with water, and a polar disperser miscible with both other phases [7]. Most commonly used extractants are high density chlorinated solvents, while different alcohols, acetonitrile (ACN) or acetone are among the often used dispersers [8]. When stirred, the disperser facilitates the extraction procedure via formation of an emulsion that increases the contact area between the phases. A smaller volume of the extractant than necessary for traditional LLE can consequently be used, and the procedure can be completed in a single extraction step, without the need for successive collection of fractions [9]. When compared to SPE, along with requiring fewer extraction steps and smaller volumes in general, the required equipment is also significantly cheaper and more accessible to the average laboratory.

In the previously published literature, DLLME has often been used for the analysis of pesticides, mycotoxins, metals, and drugs in large volume aqueous samples, such as water, fruit juice, wine, milk, but there are also several reports of its use on urine, plasma, or tissue [9–16]. The main differences between environmental and bioanalytical samples are in the availability and the amount of the sample, as well as the presence and type of matrix components. Attempts to optimise the DLLME procedure for bioanalytical samples were made in the mentioned reports, but further improvement with regard to reducing the necessary sample volume and improving extraction recoveries is still much needed.

In the published methods on plasma samples, PPT by a concentrated aqueous solution of salt or acid, or a polar organic solvent was conducted prior to DLLME. When an aqueous salt or acid solution was used, DLLME was performed in the same way as with aqueous environmental samples: a mixture of organic extractant and disperser was added to the aqueous sample and stirred [8]. On the other hand, when a polar organic precipitation agent such as ACN was used, it subsequently also acted as a disperser for DLLME [15,17,18].

One of the main reported drawbacks of DLLME is the use of relatively large volumes of dispersers, since they are often environmentally hazardous and can even pose a limit to the achievable extraction efficacy [17,19,20]. Namely, the disperser can increase the solubility of the analytes in the aqueous phase, thus reducing their extractability into the organic phase [16,17,19,21–23]. To overcome this disadvantage, air-assisted DLLME was developed. Successful dispersion is achieved by vigorous stirring of the extraction mixture without a disperser, usually with a pipette or a syringe, to introduce air bubbles [24].

The aim of this work was to explore the possibilities of DLLME in the simultaneous extraction of the six diverse drugs of interest from human plasma samples, as a prerequisite for bioanalytical application in a clinical setting. Emphasis was placed on achieving high extraction recoveries of all the analytes with low sample and solvent consumption by adjusting the DLLME procedure for the specific requirements of the scarce and complex plasma sample.

2. Materials and methods

2.1. Chemicals

HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid was obtained from Supelco (Bellefonte, PA, USA). Ultrapure water (conductivity 0.055 μS/cm) used throughout this work was purified by a Merck Millipore Milli-Q IQ 7015 system (Darmstadt, Germany). HPLC grade isopropanol (iPrOH) was obtained from Fisher Chemicals (Illkirch, France), acetone and ethanol (96%) from Sigma-Aldrich (St. Gallen, Switzerland), chloroform (CLF) was from Carlo Erba (Milano, Italy), while dichloromethane (DCM) and ethyl acetate were from T.T.T. (Sveta Nedjelja, Croatia). Ammonium sulphate (99.5%) was acquired from Sigma-Aldrich (St. Gallen, Switzerland). Sodium chloride (NaCl) was obtained from Merck (Darmstadt, Germany). Perchloric acid (70%) was purchased from Fluka (Buchs, Switzerland), glacial acetic acid from Panreac (Barcelona, Spain), and borate buffer (50 mM) from Agilent technologies (Santa Clara, CA, USA).

Standards of ANA and LET (purity >98%) were purchased from Tokyo Chemical Industry (Tokyo, Japan), FUL (purity >97%) from Sigma-Aldrich (St. Gallen, Switzerland), PAL and ABE (purity >98%) from Toronto Research Chemicals (Toronto, Canada), and RIB (purity >98%) from BioVision (San Francisco, CA, USA).

2.2. Analyte standard solutions

Primary stock solutions of ABE, RIB, ANA, LET and FUL were prepared at 1000 μg/mL in MeOH. Primary stock solution of PAL was prepared at 225 μg/mL in ACN:H_2O 50:50 *v/v*. A working solution of 100 μg/mL of all the analytes was prepared by mixing the primary

stock solutions and adequately diluting in MeOH. All solutions were stored at +4 °C and were stable for at least 3 months.

2.3. Reagent solutions

Ammonium sulphate was prepared as a 4 M solution in water. NaCl was prepared as a 20% *w/v* solution in water. Acetic acid and NaCl solutions were diluted with water to achieve the ionic strength of 1000 mM (1000 mM acetic acid, 5.8% *w/v* NaCl). Borate buffer was prepared as a 7.5 mM solution (ionic strength 45 mM) and a mixture of 7.5 mM with 5.6% *w/v* NaCl (ionic strength 1000 mM).

2.4. Plasma collection and pre-treatment

Venous blood from healthy volunteers (drug-free) and patients (treated with the drugs of interest) was collected, with informed consent, in tubes with K_2-EDTA anticoagulant. Plasma, obtained after centrifugation at 1500*g* for 10 min, was stored at −18 °C. Prior to any experiments it was thawed at room temperature for 30 min. The research was approved by the Ethics Committee of University of Zagreb Faculty of Pharmacy and Biochemistry (approval number 251-62-03-19-30) and by the Ethics Committee of University Hospital Centre Zagreb (approval number 02/21-JG).

2.5. Preparation of spiked plasma samples

Spiked plasma samples were prepared by adding the analyte working solution to drug-free plasma in the volume ratio plasma: standard solution = 9:1. A blank plasma sample was always prepared in parallel with the spiked samples, for the analytes to be added after the extraction process (post-extraction spiked samples).

2.6. Protein precipitation

Different agents – ACN, acetone, MeOH, 70% *v/v* perchloric acid, and 4 M ammonium sulphate were added to 100 µL of plasma in a volume which ensured complete precipitation of proteins. These volumes corresponded to 5 µL of perchloric acid, 400 µL of ACN or acetone, 1000 µL of MeOH, and more than 1000 µL of ammonium sulphate. The samples were vortexed for 10 s and centrifuged at 1200 *g* for 10 min. The clear supernatants were retrieved and, in the case of organic solvents, evaporated to dryness using Eppendorf Concentrator Plus (Hamburg, Germany), or directly analysed in the case of perchloric acid. The evaporated residues were reconstituted in an adequate volume of 65% *v/v* MeOH for the analysis.

2.7. Dispersive liquid-liquid microextraction procedure

During method development, 200 µL of ACN was added to 50 µL of spiked plasma sample for PPT. The supernatant (200 µL) was withdrawn. It was evaporated to dryness and reconstituted in 50–500 µL of aqueous phase, into which a mixture of a disperser (0–1000 µL) and extractant (50–500 µL) was injected. Alternatively, the supernatant after PPT was directly mixed with an extractant solvent (50–500 µL) and an aqueous phase (50–2500 µL). In all cases the organic and the aqueous phase were vigorously mixed 10 times with a pipette and vortexed for 10 s. After 5 min of centrifugation, the whole bottom organic layer was withdrawn, evaporated to dryness, dissolved in 40 µL of 65% *v/v* MeOH and analysed by liquid chromatography.

For method application on real patient samples, the initial and final sample volumes were adjusted. To 100 µL of plasma, 400 µL of

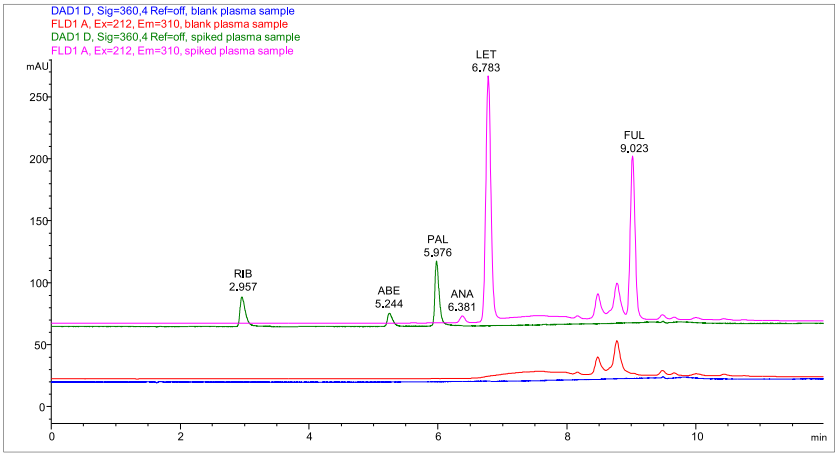

Fig. 1. Overlaid representative chromatograms of a spiked (green – DAD, and pink – FLD) and blank plasma sample (blue – DAD, and red – FLD), prepared using AqS-DLLME.

ACN was added for PPT, and 475 μL of the resulting supernatant was evaporated. The residue was dissolved in 100 μL of water, followed by DLLME with iPrOH:CLF = 50:100 μL. The collected organic fraction was evaporated and dissolved in 38 μL of 65% *v/v* MeOH.

2.8. Chromatographic method

Analyses were carried out on an Agilent 1100 Series HPLC system (Santa Clara, CA, USA) coupled to a diode array (DAD) and a fluorescence detector (FLD), using a Waters XBridge phenyl column, 150 × 4.6 mm, 3.5 μm (Milford, MA, USA). The column was thermostated at 30 °C. The mobile phase consisted of water with 0.1% *v/v* formic acid (mobile phase A) and MeOH with 0.1% *v/v* formic acid (mobile phase B) at a flow rate of 1 mL/min. Gradient elution was applied: 45–49% phase B in 2 min, followed by an increase to 100% phase B during 5.1 min, which was held for 2.9 min before returning to the initial conditions. The total method run time was 15 min, with all analytes eluting in 9 min. The autosampler was thermostated at 10 °C, 10 μL of sample was injected into the system and the needle was washed with 50% MeOH. The detection wavelengths were set to 360 nm for ABE, PAL and RIB on DAD, and 212 nm excitation with 310 nm emission wavelength for ANA, LET and FUL on FLD. A representative chromatogram of a blank and spiked plasma sample is shown in Fig. 1.

2.9. Method validation

Robustness was tested using one-variable-at-a-time approach. The volumes of water and CLF were varied 100 ± 10 μL, the volume of iPrOH 50 ± 10 μL, the number of suction-injection cycles 10 ± 5, and the vortex stirring time 10 ± 5 s. All samples were prepared in triplicate (total number of samples = 30). Robustness was evaluated by calculating the mean effect and standard deviation of positive and negative conditions (n = 6 samples per condition). Method validation was carried out in terms of linearity, precision, accuracy, and extraction recoveries. Precision and accuracy were calculated using 15 samples within-day and 27 samples in three days, linearity was assessed on three separate days on eight concentration levels shown in Table 1, and the obtained calibration curves were weighted by $1/x^2$ [25].

2.10. Data analysis

Data were collected using Agilent ChemStation 10 software (Santa Clara, CA, USA) and analysed using Microsoft Office 365 Excel (Redmond, WA, USA) and GraphPad Prism 8 (San Diego, CA, USA). Green analytical chemistry assessment was performed using AGREE v.0.5 beta (Gdansk, Poland) [26]. Samples were prepared in triplicate for each tested condition and the extraction recoveries were calculated from post-extraction spiked samples, using Equation (1). The results are presented in figures as the median and range of the three values.

$$\textit{Extraction recovery}\ (\%) = \frac{\text{Signal in the pre} - \text{extraction spiked sample}}{\text{Signal in the post} - \text{extraction spiked sample}} * 100\% \quad (1)$$

3. Results and discussion

3.1. Protein precipitation

Precipitation with ACN, MeOH, acetone, 4 M ammonium sulphate and 70% *v/v* perchloric acid was tested, on 100 μL of plasma sample. The necessary volume ratios of PPT agent:plasma for complete precipitation were 1:20 for perchloric acid, 4:1 for ACN and acetone, 10:1 for MeOH, and more than 10:1 for ammonium sulphate.

Ammonium sulphate could not cause complete precipitation even in high volumes and was therefore dismissed. MeOH was found unsuitable due to its low precipitation efficacy which required extensive sample dilution and long evaporation times. Perchloric acid showed great precipitation efficacy at a very low volume, but the extraction recoveries of all analytes, especially FUL, suffered due to low aqueous solubility and high protein binding [27].

ACN and acetone both showed good precipitation efficacy in an equal volume, with high analyte recoveries. However, the sample

Table 1
Calibration levels for each analyte.

Calibrant	PAL/μg/mL	RIB/μg/mL	ABE/μg/mL	FUL/μg/mL	ANA/μg/mL	LET/μg/mL
1	0.08	0.25	0.11	0.50	2.51	0.04
2	0.16	0.50	0.22	1.00	5.03	0.08
3	0.32	0.99	0.44	2.01	10.05	0.17
4	0.48	1.49	0.65	3.01	15.08	0.25
5	0.96	2.98	1.31	6.02	30.15	0.51
6	1.08	3.35	1.47	6.77	33.92	0.57
7	1.44	4.46	1.96	9.03	45.23	0.76
8	1.92	5.95	2.61	12.04	60.30	1.01

treated with acetone had a more pronounced tendency of foaming, probably due to co-extracted plasma components, which rendered sample handling difficult and indicated poorer sample clean-up. Thus, ACN was chosen as the optimal precipitation agent, in the volume ratio to plasma 4:1.

3.2. Preliminary DLLME experiments

In this work, PPT with an organic solvent was followed by DLLME for additional sample clean-up. Three distinct modes of DLLME were performed, as depicted in Fig. 2, to evaluate their suitability for bioanalytical sample preparation.

Firstly, an approach similar to the previously published DLLME procedures involving PPT with an organic solvent [15,17,18], further referred to as "organic sample DLLME" (OrS-DLLME). An aqueous phase and an extractant solvent are added to the acetonitrile supernatant obtained from PPT.

Secondly, "aqueous sample DLLME" (AqS-DLLME), a novel approach that encompasses evaporation of the acetonitrile supernatant from PPT, dissolution of the dry residue in water and extraction with a mixture of a selected disperser and extractant.

And finally, a modified air-assisted DLLME (AA-DLLME). In contrast to the previously published AA-DLLME applications on plasma samples which perform PPT with an aqueous salt solution [24], here an organic solvent is used and evaporated to dryness. The extraction procedure is then carried out only by multiple pipette suction-injection cycles and vortex stirring, without the addition of a disperser.

3.2.1. Sample volume and starting conditions

As reported in the previously published works, the most often used volume ratios of each component in the ternary system water: disperser:extractant are around 5:1:0.1. I.e., for 5–10 mL of aqueous phase, 0.5–3 mL of disperser and 10–200 μL of extractant are employed [15,20,23,28]. In this scenario, the required starting sample volumes are usually larger than 500 μL, which is unsuitable in

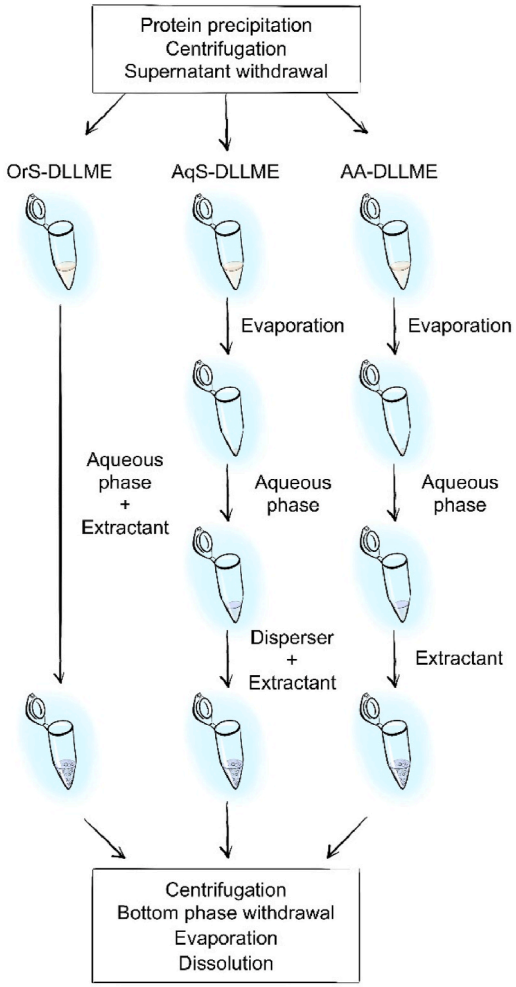

Fig. 2. General protocol for OrS-DLLME, AqS-DLLME and AA-DLLME procedures.

the bioanalytical context, where limited amounts of sample are available. Therefore, the goal of this work was to develop a DLLME method applicable for significantly smaller sample volumes.

During the optimisation of DLLME, the necessary sample volume was first determined, followed by testing the types and volume ratios of the extractant and disperser solvents. The effects of assisting conditions – pH adjustment and salting out, were then evaluated for each mode of DLLME. Finally, the lowest necessary volumes of organic solvents were fine-tuned for the optimised conditions.

In the first preliminary experiments, extraction of analytes from a neat aqueous standard solution was tested, using ACN and CLF, the most common disperser and extractant combination, in order to assess whether any recovery would be obtained. Since all the analytes' peaks were visible in the chromatogram, it was continued onto spiked plasma samples.

An OrS-DLLME procedure was performed, using the volume ratios of water:disperser:extractant resembling those found in the literature – 2500:500:100 μL. Smaller volumes of water and disperser would allow for less sample dilution and better environmental properties of the method, as well as reduce the possible negative effect of excess disperser in the mixture [20,22]. Therefore, extraction with the volume ratios of H_2O:ACN:CLF 140:160:100 and 240:160:100 μL was also tested. Namely, proteins were precipitated with 200 μL of ACN added to 50 μL of sample, and 200 μL of the supernatant was collected. It was assumed that this supernatant contained 160 μL ACNACN and 40 μL of the aqueous phase. The supernatant was diluted with ACN up to 500 μL and combined with 2500 μL of water and 100 μL of CLF, or mixed with 100 or 200 μL of water and 100 μL of CLF. The results are shown in Supplementary Fig. S1. Median recoveries were 75.5–85.3%, with ranges up to 6.2% for H_2O:ACN:CLF = 140:160:100 μL, 74.2–85.0% with ranges up to 8.6% for 240:160:100 μL, and 15.1–66.3% with ranges up to 18.1% for 2500:500:100 μL. The difference between using 100 and 200 μL of added water can be considered insignificant, whereas it is evident that the recoveries and the precision obtained using high aqueous and ACN volumes were remarkably lower.

Thus it was concluded that lower volumes of the disperser and the aqueous phase may be favourable. Since the minimal volume of the disperser in OrS-DLLME is dictated by the volume leftover from PPT, an AqS-DLLME procedure was introduced. Here, due to the evaporation of the supernatant, the choice of the type and volume of the disperser is less limited. This opens space for a potentially more efficient or selective extraction. Furthermore, extraction without a disperser (AA-DLLME) can thus also be performed, to assess both the necessity of using a disperser, its possible advantages, and its potential of reducing the extraction recoveries.

3.2.2. *Type of extractant and disperser solvents*

Before further optimising the exact volume ratios of the components, the types of extractant and disperser were determined using the novel AqS-DLLME approach, using a preliminarily determined volume ratio water:disperser:extractant 100:200:200 μL. This volume ratio was selected based on satisfactory emulsion formation and phase separation, as well as low and mutually similar volumes of all the components.

The extractant solvent yielding highest recoveries was tested with ACN as the disperser, since it was also used as the PPT agent, thus the results could be easily compared to the OrS-DLLME mode. CLF, DCM, ethyl acetate, CLF:DCM 1:1, and CLF:ethyl acetate 1:1 *v/v* were assessed as extractants. The results are shown in Fig. 3a. CLF showed highest recoveries and least imprecision due to easy withdrawal of the sedimented organic fraction. With ethyl acetate, the reproducibility of the procedure suffered because of its lower density which impeded the discrimination between the two layers. DCM showed significantly smaller extraction yields, which were improved in the CLF-DCM combination, nevertheless using only CLF alone was still superior.

Having selected CLF as the optimal extractant, the use of different dispersers – EtOH, MeOH, iPrOH, acetone and ACN was assessed. The results are shown in Fig. 3b. ACN and acetone had relatively similar effects, however, iPrOH, as a greener solvent [29], also showed high recoveries, especially for RIB and PAL. EtOH was visibly the least suitable. The use of iPrOH was later considered for further optimisation, in order to provide a less harmful alternative to ACN.

3.2.3. *Volume ratios of extractant, disperser, and aqueous sample*

As was implied in the preliminary OrS-DLLME experiment, the volumes and volume ratios of the components in the mixture play an important role in the extraction process. It was already reported that phase inversion and even complete phase miscibility can occur at low aqueous and high ACN volumes in H_2O:ACN:CLF mixtures [30]. Therefore, a working range of the components' volume ratios yielding acceptable extraction recoveries without hindering the phase separation had to be explored in more detail, while keeping in mind the described ternary system behaviour. Since a ternary diagram explaining their behaviour already exists, and since they

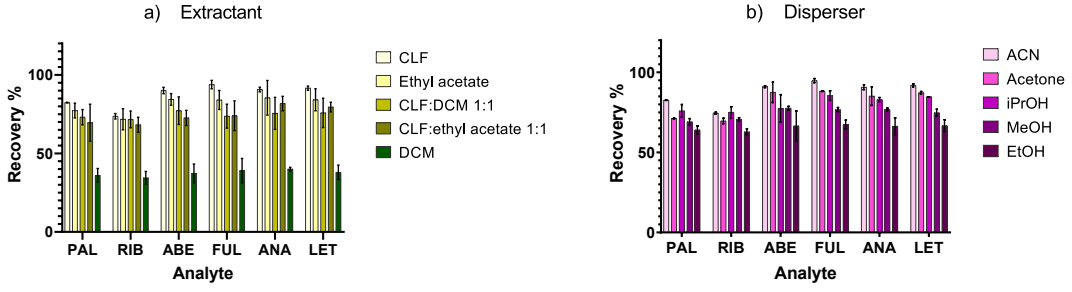

Fig. 3. Extraction recoveries obtained with: a) different extractants and ACN as disperser, b) different dispersers and CLF as extractant.

showed the best extraction recoveries as described above, ACN and CLF were selected as the disperser and extractant in this phase.

These experiments were performed in two stages. First, only aqueous solutions of the representative analytes RIB, LET, and FUL were considered, with the organic phase consisting of ACN as disperser and CLF as extractant. To determine the effect of the aqueous: organic volume ratio, the ratio of ACN:CLF was kept at 1:1, and the absolute volumes of the organic and aqueous phase were varied. The results – extraction recoveries and the volumes of the collected organic fractions for each of the conditions tested – are shown in Supplementary Fig. S2.

Lower recoveries were obtained with higher aqueous volumes. In this case, the volume of the formed organic fraction equals the volume of only the extractant solvent, while most of ACN likely remains dissolved in the aqueous phase, lowering its polarity. The analytes, especially the hydrophilic RIB, are freely soluble in H_2O:ACN mixtures, therefore their extraction to CLF is hindered. On the other hand, high extraction recoveries are observed at lower volumes of the aqueous phase, when the volume of the formed organic fraction corresponds to the sum of the initially added volumes of both CLF and ACN.

Additional volume ratios were then tested on plasma samples spiked with all six analytes of interest. All samples were prepared from the same initial volume of plasma (50 μL). The supernatant after PPT was evaporated to dryness and the samples were dissolved in different volumes of water. The extraction was performed with various volumes of ACN and CLF, yielding different final volumes of the obtained organic fraction, as shown in Supplementary Table S2. The whole organic fractions were evaporated and redissolved in 40 μL 65% *v/v* MeOH. The results are presented in Fig. 4.

As is evident from Fig. 4a, where low aqueous volumes are used, low volumes of the organic phase show impaired precision compared to higher organic volumes. However, in Fig. 4b, where varying aqueous volumes with low organic volumes are compared, a trend of increasing precision with increasing aqueous phase volume is observed. This is likely due to a more pronounced distinction between the phases. In Fig. 4c, where high aqueous volumes are shown, it is visible that the recoveries of all analytes except for FUL are in an acceptable range. Nevertheless, when placed side by side with the results obtained using low aqueous volumes, in Fig. 4d, it is apparent that the overall highest recoveries of all six analytes are obtained with lower volumes of water (H_2O:ACN:CLF 50:250:250 and 50:50:100 μL). These results imply that different conditions than those previously described in the literature [20,23,28] may be more suitable for using DLLME in plasma sample preparation.

Low volumes of water (50–100 μL) together with slightly higher volumes of extractant and disperser (200 μL each) were kept for subsequent experiments. The volume ratio disperser:extractant should be $\leq$ 1:1 to ensure successful phase separation in this setting [30]. The final volumes of extractant and disperser were fine-tuned after assessing other conditions that can significantly affect the extraction efficacy.

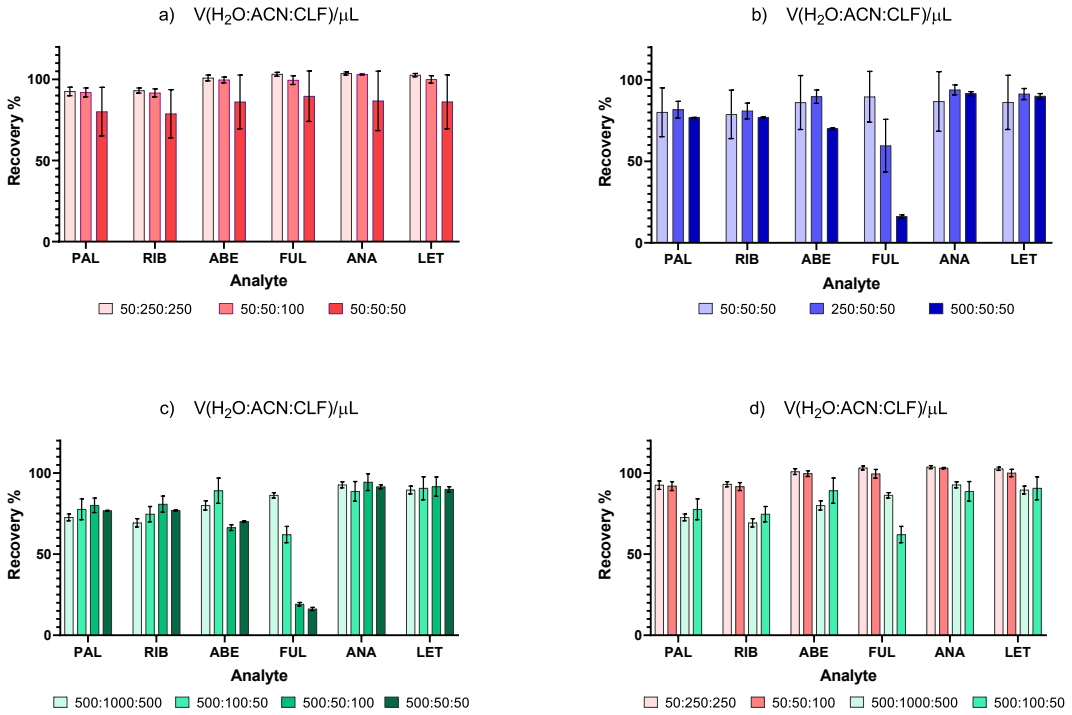

Fig. 4. Cross-comparisons of extraction recoveries obtained with different tested volumes and volume ratios: a) low aqueous volumes with different volumes of the organic phase; b) different volumes of the aqueous phase with low volumes of the organic phase; c) high aqueous volumes with different volumes of the organic phase; d) the conditions with the highest observed extraction recoveries.

3.3. Ionic strength and pH

Adjusting the pH of the aqueous phase can enhance extraction efficacy in the case of ionisable analytes. Since PAL, RIB, and ABE are all weakly basic, with the highest degrees of ionisation below pH 5, as described in our previous work [31], neutral to basic pH may be favourable for their extraction. ANA, LET and FUL do not ionise in a wide range of relevant pH values, therefore they might only benefit from the effect of salting out.

Experiments were conducted either at neglectable or elevated ionic strength (adjusted to I = 1 M). Ultrapure water and 7.5 mM trisodium borate buffer pH 9.3 were used to test low ionic strength at neutral and alkaline pH, while 5.8% *w/v* NaCl (pH 7), 1 M acetic acid (pH 2.4) and a mixture of 7.5 mM trisodium borate with 5.6% *w/v* NaCl (pH 9.3) were used to achieve salting out at different pH values.

These conditions were tested for each mode of DLLME, and the results are shown in Fig. 5a–c.

As discernible from Fig. 5a–c, acidic conditions mostly hindered the extraction recoveries of PAL, RIB, and ABE. On the other hand, alkaline conditions did not benefit their recoveries as much as was expected compared to neutral pH, with differences mostly in the margin of experimental error. Only in AqS-DLLME, extraction recoveries of RIB and PAL were slightly greater with higher pH, however the recovery of ABE was lower. It is important to note that the reported therapeutic concentrations of RIB in plasma are an order of magnitude greater than of all other analytes (1 μg/mL, as opposed to 100 ng/mL) [1], therefore it is of higher priority to maximise their recoveries instead.

Ionic strength had little to no effect on any of the analytes in AqS and OrS-DLLME, yet it proved vital in AA-DLLME. Namely, plasma samples are abundant in phospholipids, natural emulsifiers which are not successfully removed using PPT. Consequently, excessive foaming is observed at the interphase, which renders quantitative and clean extract removal difficult. Furthermore, it is likely some of the analytes remain trapped in this interphase, causing their lower extraction recoveries. In AqS and OrS-DLLME, the disperser solvent disrupts micelle formation, whereas in AA-DLLME the same effect can be achieved by the addition of salt, which lowers the zeta potential at the interphase. Indeed it was shown that increasing the ionic strength yielded significantly more precise results (discernible from the narrower ranges) and increased the extraction recoveries.

Low ionic strength in alkaline conditions had comparable effect to pure water. The results obtained at high ionic strength in neutral and alkaline pH are also mutually similar. This additionally underlines the importance of salting out over pH for the analytes of interest in AA-DLLME.

Different concentrations of NaCl were further assessed for AA-DLLME. Concentrations of NaCl higher than 10% *w/v* were not included, since it was observed that the increased density and viscosity of the aqueous phase led to aggravated phase separation (the aqueous phase would form a floating sphere in the middle of the organic fraction, which posed an increased risk of its unwanted transfer). The results, shown in Fig. 5d, indicate a proportional increase in recoveries with increasing ionic strength.

Based on the obtained results, performing DLLME in a neutral medium, without additives for both the AqS and OrS-DLLME modes and with 10% *w/v* NaCl for AA-DLLME, can be considered optimal for the extraction of the six drugs of interest from plasma samples.

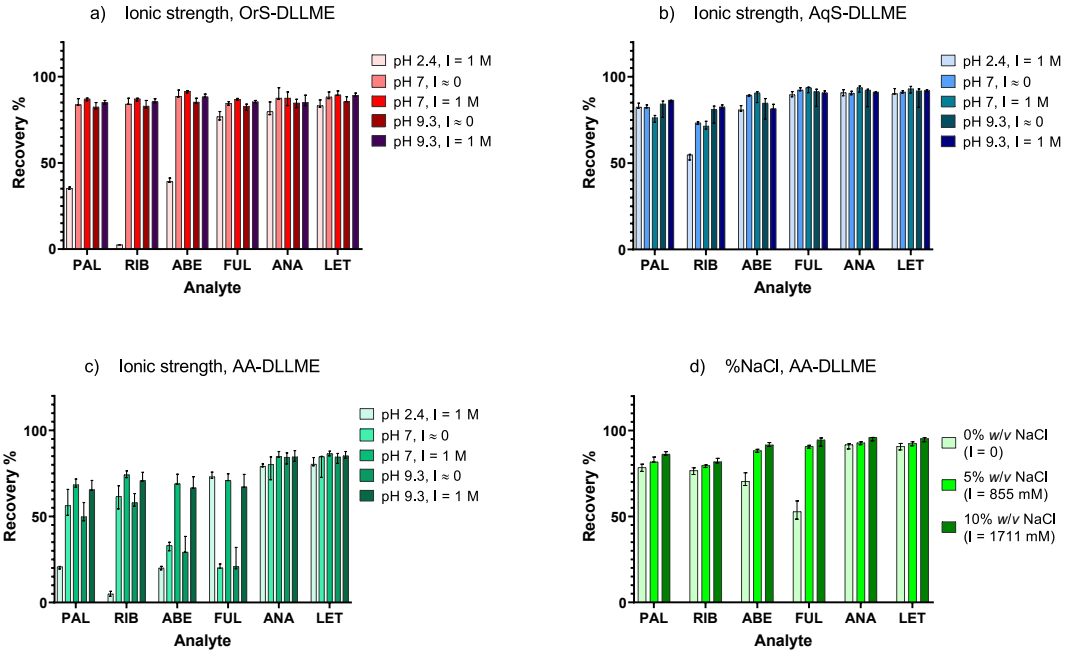

Fig. 5. The effect of ionic strength and pH on extraction recovery. a) OrS-DLLME; b) AqS-DLLME; c) AA-DLLME; d) different concentrations of salt in AA-DLLME.

3.4. Volume of the extracting phase

After establishing the key conditions necessary for successful extraction, the volumes of the extracting phase were finally fine-tuned for each of the developed DLLME modes, in order to reduce hazardous waste while maintaining the efficacy and robustness of the procedure.

In AqS-DLLME, iPrOH was reconsidered as a potential disperser, due to its lower toxicity compared to ACN and the promising results obtained in the initial experiments. It was assumed that it might show optimal dispersing properties at a different volume than ACN, therefore the volumes of 50 and 200 μL were tested. The volume of CLF accompanying the selected disperser was fine-tuned as well. The results are shown in Fig. 6a and b.

In the case of AA-DLLME, the optimal volume of CLF was chosen in the presence of 10% *w/v* NaCl (Fig. 6c).

OrS-DLLME was performed without additional ACN other than the volume leftover from PPT (160 μL), while the volume of CLF was varied (Fig. 6d).

Volumes of CLF lower than 50 μL were not considered, since it was determined already in the preliminary experiments that low obtained organic fraction volumes hindered the precision of extract collection. At the same time, volumes higher than 400 μL were dismissed due to their high risk/benefit ratio.

As visible from Figs. 6a and 50 μL of iPrOH and 200 μL of ACN showed favourable results as dispersers in AqS-DLLME, with mutual differences within the margin of experimental error. Therefore, owing to its better alignment with green chemistry principles, iPrOH was selected, in the volume of 50 μL.

The volume of 50 μL of CLF showed slightly lower extraction efficacy, while volumes higher than 100 μL did not prove significantly favourable neither in recoveries nor in precision for any of the tested DLLME modes. Therefore, 100 μL of CLF can be considered optimal for all three tested DLLME modes. These results are in accordance with the results obtained in the preliminary volume ratio experiments described in section 3.2.3.

3.5. Summary of the optimal conditions

Protein precipitation with ACN in the volume ratio to plasma 4:1 was selected as the first step in sample preparation. The following DLLME conditions were optimised:

a) AqS-DLLME: dry sample residue after PPT dissolved in 100 μL of water without additives, extraction with a mixture of 50 μL iPrOH and 100 μL CLF. The achieved median extraction recoveries of all analytes were between 84.63 and 96.61%.
b) AA-DLLME: dry sample residue after PPT dissolved in 100 μL of water with 10% *w/v* NaCl, extraction with 100 μL of CLF. The achieved median extraction recoveries of all analytes were between 82.62 and 93.89.

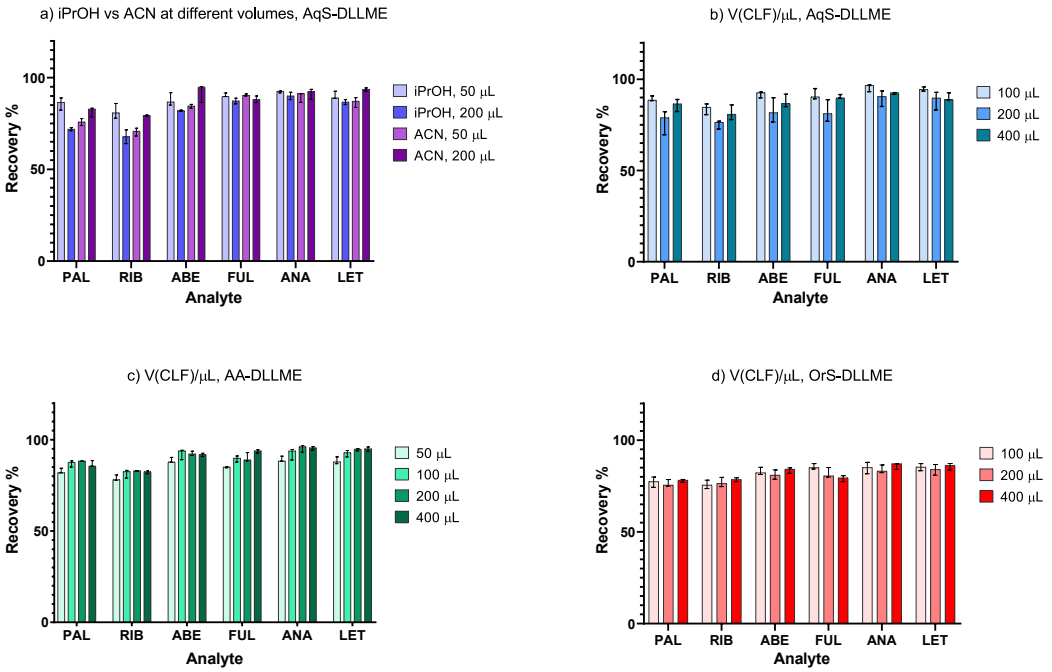

Fig. 6. Fine-tuning of the organic phase: a) type and volume of disperser in AqS-DLLME; b) volume of extractant in AqS-DLLME; c) volume of extractant in AA-DLLME; d) volume of extractant in OrS-DLLME.

c) OrS-DLLME: 200 μL of sample supernatant after PPT, containing approximately 160 μL of ACN, extracted with 100 μL of CLF and 100 μL of water without additives. The achieved median extraction recoveries of all analytes were between 75.52 and 85.26%.

The main advantage of OrS-DLLME is its straightforwardness and speed, since only one short evaporation step is involved. Furthermore, no additional disperser (other than the already present ACN leftover from PPT) is necessary, which improves this method's environmental impact. However, the minimal amount of disperser is limited by the volume of the PPT agent which, in turn, is governed by the volume of the sample. This limits the use of analyte preconcentration from larger sample volumes in OrS-DLLME.

AqS-DLLME requires a lengthy evaporation step after PPT, however this allows for more flexibility in choosing the type and volume of the disperser, as well as the sample volume. Thus, higher recoveries than with OrS-DLLME can be obtained.

High recoveries obtained with AA-DLLME showed that a disperser is not obligatory for DLLME, when using small volumes of sample and solvent that enable adequate stirring of the whole mixture. Similarly to OrS-DLLME, no additional disperser in AA-DLLME also improves the ecological impact.

The achieved extraction recoveries for each of the six diverse breast cancer drugs are exceedingly high (Supplementary Table S3). Notably, extraction recoveries obtained with AqS and AA-DLLME are similar, and slightly better than those of OrS-DLLME, probably due to its high ACN content in the mixture.

3.6. Alignment with green analytical chemistry principles

The developed DLLME procedures were assessed from a green analytical chemistry perspective, using AGREE software [26]. The filled assessment form, including the user-defined weighting, is shown in Supplementary Table S4, and the results are reported in Supplementary Fig. S3. The obtained AGREE scores are higher than 0.50 (0.63 for AqS and AA-DLLME, and 0.66 for OrS-DLLME), indicating that all three modes are in accordance with green analytical chemistry principles. This is mostly thanks to low amounts of sample ($\leq$100 μL), hazardous waste ($<$550 μL), and energy used. The main drawback is in the number of steps in the process, but the techniques require minimal special laboratory equipment, therefore their cost-effectiveness and accessibility outweigh this disadvantage. It is important to note that method greenness in its entirety also encompasses the analytical technique. The proposed DLLME can be applied in sample preparation for both the less environmentally friendly liquid chromatography analyses and the greener techniques, such as capillary electrophoresis, since the dry residue after evaporation can be dissolved in a solvent appropriate for the technique used.

3.7. Method validation and application

For application on patient plasma samples using HPLC-DAD-FLD, the method was modified in order to achieve higher sensitivity, since most of the analyte concentrations in real samples are relatively low. For sample preconcentration, a larger initial sample volume is needed. To this end, the DLLME procedures which include the evaporation of the PPT solvent are more suitable, since large volumes of ACN would hinder the extraction efficacy in OrS-DLLME and call for additional adjustments of the aqueous and extractant volumes.

To illustrate the applicability of DLLME for real samples, an AqS-DLLME procedure was chosen to be validated and used for the determination of the drugs of interest in patient plasma samples. The AqS-DLLME procedure has overlapping properties with both AA and OrS-DLLME, with similar extraction recoveries, therefore it was considered a reasonable representation. The initial sample volume was increased to 100 μL, a larger portion of the supernatant after PPT was collected (95%) and the dry residue was dissolved in a smaller volume (38 μL), to achieve a sample preconcentration factor of 2.5. Finally, the injection volume was increased from 10 to 25 μL. The DLLME step was performed without changes (H_2O:iPrOH:CLF volume ratio 100:50:100 μL).

3.7.1. Robustness

Method robustness was tested prior to validation, in terms of varying volumes of all components in the extraction mixture (V), number of suction-injection cycles (N) and duration of vortex mixing (T). The results are shown in Fig. 7. All the effects were below

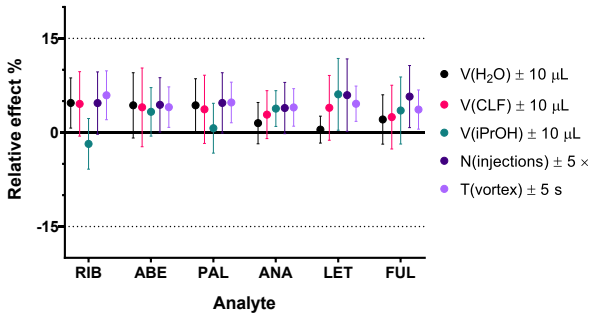

Fig. 7. Results of the robustness tests: the effects of different conditions on peak areas. Error bars are calculated as the standard deviation between the six corresponding samples (positive and negative at each condition).

15%, which is deemed acceptable for bioanalytical application [25].

3.7.2. Method validation

Representative chromatograms of a standard sample, blank solvent, blank plasma samples and a plasma sample spiked at LLOQ level are shown in Supplementary Figs. S4–S7. No matrix interferences were observed at the retention times of the analytes at the selected detection wavelengths. The method was successfully validated in terms of linearity, precision, accuracy, and extraction recoveries. More emphasis was placed on the validation of parameters depending on the proposed sample preparation procedure (precision, extraction recovery), than on those affected by the instrumental properties (calibration range, linearity, accuracy), which are not in the primary focus of this work and are subject to change depending on the analytical technique used. The results are summarised in Table 2. The linearity over the calibration range was confirmed with curves constructed on three separate days, with low between-day slope variability. The precision (%RSD) and accuracy (%bias) within one day and between three days were both below 15% at all tested concentration levels, as prescribed by the guidelines [25].

3.7.3. Method application

Finally, samples from four patients treated with the drugs of interest in different combinations were analysed. A representative chromatogram of a patient sample is shown in Supplementary Fig. S8. The samples were collected during the steady state. Identification was carried out by comparing the retention times and UV-spectra of the peaks with a chromatogram of a spiked plasma sample, and the analytes were quantitated using a freshly prepared calibration curve. PAL, RIB, ABE and LET were detected in the samples, in the following concentrations: RIB 1152.2 ng/mL, LET 130.7 ng/mL (Patient 1), RIB 884.4 ng/mL, LET 150.4 ng/mL (Patient 2), ABE 234.9 ng/mL, LET 96.7 ng/mL (Patient 3), and PAL 98.9 ng/mL (Patient 4). These measured concentrations were within the linear range of the method and correspond to the steady state concentrations expected in real patient samples [5,32].

4. Conclusions

In this work three simple, ecologically friendly, economically favourable, robust, precise, and accurate DLLME sample preparation procedures for the extraction and analysis of palbociclib, ribociclib, abemaciclib, anastrozole, letrozole and fulvestrant in human plasma are developed.

DLLME is successfully optimised to maximally reduce the necessary sample and solvent volumes, as well as to achieve excellent extraction recoveries of all six diverse drugs of interest. All three suggested DLLME modes comply with the green analytical chemistry principles.

To the best of our knowledge, this is the first report of DLLME applied to plasma samples using only μL levels of samples without excessive dilution. Novel extraction conditions and procedures adapted for plasma-specific requirements are proposed. Furthermore, this is the first bioanalytical DLLME method for the simultaneous extraction of CDK 4/6 inhibitors in therapeutic combinations with endocrine therapy from human plasma.

In relation to the previously published literature, the DLLME modes explored in this work employ different sample volumes that are more suitable for the preparation of complex and scarce plasma samples, combined with volume ratios of all components adjusted for obtaining increased extraction yields. Pitfalls experienced due to matrix components are addressed, such as the extensive foaming due

Table 2
Summarised results of the method validation.

Analyte	Linear range, LLOQ –ULOQ (μg/mL)	Weighted calibration curve equation (±SD), n = 3	R	Mean % extraction recovery (±SD), n = 8	Concentration level (μg/mL)	Precision (% RSD)		Accuracy (% bias)	
						Intra-day, n = 15	Inter-day, n = 27	Intra-day, n = 15	Inter-day, n = 27
PAL	0.08–1.92	$y = 319.77x$ (±8.22) − 12.63 (±0.46)	0.9967	81.65 (±2.85)	0.08	5.8	1.8	−1.9	−4.6
					0.16	2.8	13.2	1.1	3.3
					1.08	7.3	3.6	0.5	−2.6
RIB	0.25–5.95	$y = 111.77x$ (±3.61) − 17.19 (±0.61)	0.9935	85.79 (±6.47)	0.25	4.2	11.4	−2.0	−11.9
					0.50	3.4	13.8	−0.8	13.1
					3.35	6.7	3.9	0.4	−5.5
ABE	0.11–2.61	$y = 263.63x$ (±4.99) − 8.02 (±1.35)	0.9989	94.44 (±5.77)	0.11	10.0	5.3	0.2	−3.3
					0.22	3.2	12.0	2.8	2.2
					1.47	7.0	2.4	0.5	0.3
FUL	0.50–12.04	$y = 439.03x$ (±1.70) + 65.42 (±25.32)	0.9971	95.58 (±3.59)	0.50	6.6	9.6	−0.1	8.4
					1.00	4.2	13.8	1.7	−13.1
					6.77	5.9	2.0	−0.6	3.5
ANA	2.51–60.30	$y = 18.21x$ (±0.13) +7.72 (±0.91)	0.9987	90.74 (±2.83)	2.51	3.2	1.0	−0.4	5.2
					5.03	3.7	7.3	2.2	−9.1
					33.92	6.2	1.4	1.0	2.3
LET	0.04–1.01	$y = 582.90x$ (±17.77) + 2.85 (±1.45)	0.9975	91.08 (±6.21)	0.04	5.4	7.1	4.2	8.4
					0.08	3.4	12.1	5.6	−1.7
					0.57	5.5	2.3	−0.4	1.5

Abbreviations: LLOQ – lower limit of quantitation, ULOQ – upper limit of quantitation, SD – standard deviation, RSD – relative standard deviation.

to leftover plasma phospholipids. It is found that the volume ratios of water, disperser and extractant, type of the extractant solvent and the presence of either a disperser or concentrated salt are the most significant factors for feasible extraction. High extraction recoveries (75.52–96.61%) of all six breast cancer drugs are achieved using DLLME, contrary to previously reported works [21,22].

The successful simultaneous extraction of all six drugs of interest, as well as the clinical application of the method to patient samples, demonstrate the potential of DLLME as a versatile technique for plasma sample preparation.

Authorship contribution statement

Lu Turković: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Wrote the paper. **Natan Koraj**: Performed the experiments; Wrote the paper. **Zvonimir Mlinarić**: Analysed and interpreted the data. **Tajana Silovski, Slaven Crnković**: Contributed reagents, materials, analysis tools or data. **Miranda Sertić**: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University of Zagreb Faculty of Pharmacy (approval number 251-62-03-19-30, date of approval April 23, 2019) and by the Ethics Committee of University Hospital Centre Zagreb (approval number 02/21-JG, date of approval August 20, 2019).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Miranda Sertic reports financial support was provided by Croatian Science Foundation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18880.

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