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Determination of Doxorubicin in Urine Samples using Syringe to Syringe Dispersive Liquid-Phase Microextraction through Fluorescence Spectrometry

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Abstract

In this research, to identify the trace amount of doxorubicin, a syringe-to-syringe dispersive liquid-phase microextraction (SS-DLPME) procedure combined with fluorescence spectrometry was applied. The syringe-to-syringe process was used to speed up the formation of the acceptable cloudy solution by a low volume of extraction solvent, which reduced the equilibrium time and increased the extraction efficiency. To optimize the parameters affecting the procedure, pH, type and volume of extraction solvent, the effect of salt, the number of injections, and the centrifuge speed were investigated. Under optimal conditions, the linear range of doxorubicin was found to be 3.0-300.0 ng mL^{-1}. The limit of detection, the limit of quantification, inter-day, and intra-day precision (RSD%) were 0.76, 2.55 ng mL^{-1}, 0.82-2.11% and 1.20- 2.37%, respectively. The proposed method was successfully applied for pre-concentration and determination of the doxorubicin in urine samples.

Keywords: Doxorubicin, Syringe to syringe dispersive liquid-phase microextraction, Fluorescence spectrometry, Urine sample.

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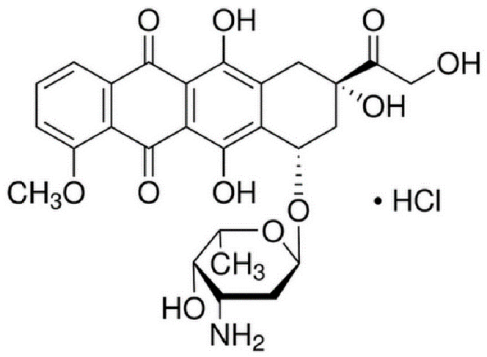
Introduction

Doxorubicin (DOX), Scheme 1, is a vital element of the anthracylinic group. It is known as an effective and crucial anticancer (antineoplastic) chemotherapy drug to cure some cancers. DOX comprises a tetrahydroxy-anthraquinone, which is a six-member duanosamine sugar with a hanging glycosyl moiety, fundamentally demonstrating anthracycline antibiotics structure [1]. The hydrochloride form of DOX is widely used because it is soluble in water [2, 3]. DOX contains intrinsic fluorescence serving as a useful tool in investigations and imaging. Excitation and emission spectra of DOX are 480 and 558, respectively. It interferes with DNA like all anthracyclines [4].Despite the prevalent consumption of this drug in curing different cancers, the attachment of DOX to the cell can create reactive oxygen kinds that decrease mitochondrial oxidative phosphorylation.Hence, it results in some side effects such as bone marrow suppression, heart attack, and gastrointestinal disorders [3, 5]. Therefore, it is necessary to detect a negligible amount of DOX by a highly selective and sensitive technique in biological specimens.



Scheme 1. Chemical structure of Doxorubicin.

The sample preparations -extraction methods- are used to pre-concentrate the target analyte and eliminate matrix effect in solution [6]. The dispersive liquid-liquid microextraction (DLLME) is a kind ofmodified liquid phase microextraction (LPME) [7, 8] which works based on the creationof a cloudy state through a dispersivesolvent, aqueous sample, and small value of extraction solvent (mostly severalmicroliters). The DLLME is a simple, low cost, and rapid method [9, 10]. This method is highly toxic due to large amounts of dispersive solvent [11]. To control these problems, Asadi et al. created a new technique based on a syringe to syringe dispersive liquid-phase microextraction(SS-DLPME) for the first time[12]. In SS-DLPME, the sample solution is pulled in syringe 1 (S1), and the extraction solvent is injected into it. Then, syringe 2 (S2) is connected to

syringe 1 by a silicone connector, which makes a closed extraction container. The sequence of reciprocal injections causes the extraction solvent to disperse into the aqueous sample medium. The homogenous cloudy solution is moved to an appropriate container. Finally, the two phases (aqueous and organic) are separated by centrifugation.

By far, limited number of studies have been reported using extraction methods for the pre-condensation of DOX. Martins et al. [13] developed a liquid-liquid extraction (LLE) combined with a high-performance liquid chromatography method to determine four antineoplastic drugs (cyclophosphamide, doxorubicin, 5-fluorouracil, and ifosfamide) in plasma. Using solid-phase extraction (SPE) in which molecularly imprinted polymer-coated magnetite nanospheres are employed as nano adsorbent has been reported by Ahmadi and coworkers for DOX extraction in urine samples [2]. Martins et al. have applied a chromatographic method based on DLLME and SPE to determine doxorubicin, daunorubicin, epirubicin, and irinotecan in hospital sewage. This study showed that DLLME requires smaller volumes of solvents and samples than SPE [14].So far, several techniques have been reported based on UV-vis spectrophotometry [15, 16], liquid chromatography [9, 13, 17], fluorimetry [2], Raman spectroscopy [18], and electroanalytical measurement techniques [19, 20]to determine DOX in different specimens. However, long analysis time, high costs, and lack of sensitivity and selectivity are some disadvantages to these methods. To overcome these drawbacks, we can consider spectrofluorimetry as an alternative methoddue to its high sensitivity, specificity, fast and rapid diagnosis ability.

In the present study, a rapid, sensitive, accurate and precise method was developed to determine DOX using a SS-DLPME. Lastly, fluorescence spectroscopy was used to determine the trace amount of DOX in human urine. Different extraction factors, including pH, the type and volume of extractant, ionic strength, and number of injections, were examined and optimized. To the knowledge of the researchers, no papers have been published on the determination of DOX in human urine samples using SS-DLPME coupled with fluorescence spectroscopy.

Experimental

Materials and instruments

All the chemicals used in this study were of analytical reagent grade and needed no further purification. Doxorubicin hydrochloride was purchased from Sigma-Aldrich company. All solvents, such as 1-dodecanol, 1-decanol, 1-octanol, 1-undecanol, cyclohexane and acetonitrile were provided by Merck Company (Darmstadt, Germany). All the solutions were prepared using double distilled water (DDW). The hydrochloride salt of DOX stock solution was prepared in DDW, and working standard solutions of different DOX concentrations were prepared daily by diluting the

stock solution. Universal buffer solutions were prepared by mixing phosphoric, acetic and boric acid [21]. A Perkin Elmer (LS45) Fluorescence spectrometer was used to determine DOX spectrofluorometric concentration. A Metrohm 744 pH meter combined with a glass–calomel electrode was employed to measure pH selections. The separation phase was conducted with a Behdad Universal centrifuge.

SS-DLPME procedure

The pH of 20 mL of a sample solution containing the target analyte (150 ng mL^{-1}) was adjusted to 8.0 using the required volume buffer solution. The solution was placed in syringe 1 (S1), and 250 µL 1-dodecanol (as an extraction solvent) was dispersed into it by using Hamiltonian syringe. Then, syringe 1 was connected to syringe 2 (S2) through a silicone connector. Now, the mixture in syringe 1 was easily dispersed into the syringe 2 through the back and forth sequential injections (7 injections). At the end of extraction, the homogenous cloudy solution was poured in a homemade vessel with a narrow-necked tube and was centrifuged for 5 min at 4000 rpm. After centrifuging, the supernatant phase was removed by Hamilton syringe and was transferred into a quartz cell. Finally, its fluorescence intensity was measured by setting the excitation wavelength at 480 nm and recording in the emission at 558 nm with the slit widths of (excitation and emission)10 nm. The schematic diagram of the proposed method is shown in Figure 1.

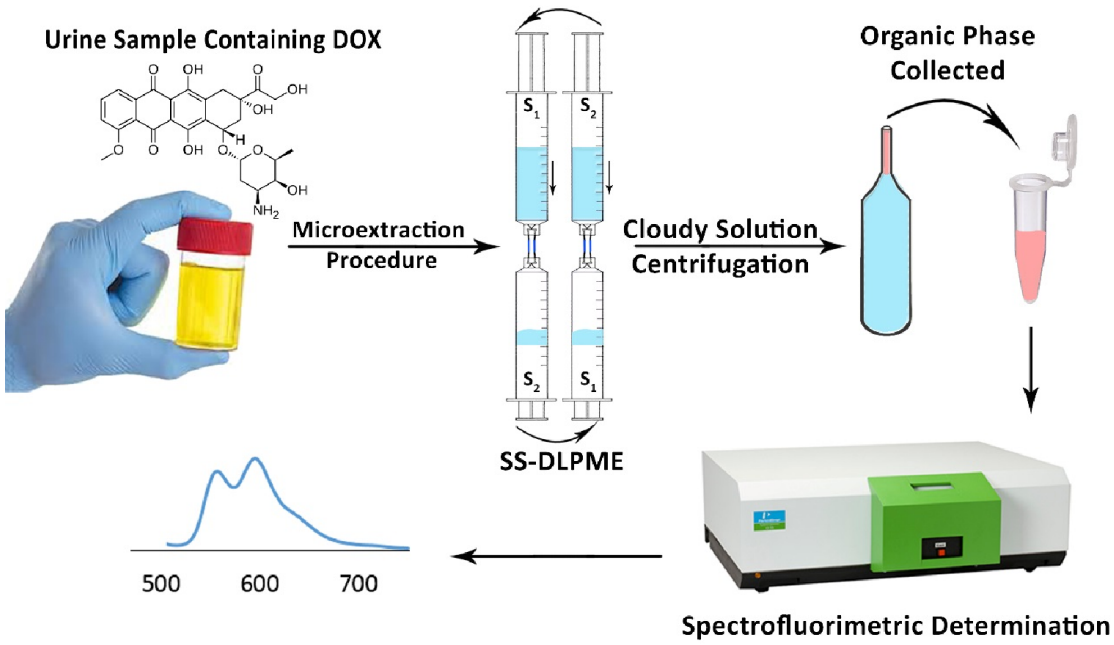


Figure 1. Schematic procedure of the syringe-to-syringe dispersive liquid-phase microextraction.

Urine sample preparation

Drug-free urine samples were collected from healthy donors with their consent, and all experiments were performed in compliance with the relevant laws and institutional guidelines. All urine samples were stored at -20^{0}C. The frozen urine samples were thawed at room temperature and transferred to a conical test tube. To precipitate the protein, 10% acetonitrile was added and centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and passed through a 0.45 µm filter. This solution was used to perform the extraction process with SS-DLPME.

Results and discussion

Effect of pH

The pH value is significant since it influences the ionization condition and the solubility of the analyte. In general, organic compounds may be extracted from the aqueous phase in a non-ionized form. To gain the intended analytes in their unionized forms, sample solution pH should be lower than the pK_a of the analytes so that they acquire a higher tendency to move into the organic phase [22]. The relation between pH and fluorescent intensity is complicated relatively since there are many probable protonation equilibria in the DOX solution. In this article, the relationship of the fluorescent intensity with pH value was examined in the range of 5.0–10.0. Figure 2 illustrates that fluorescent intensity increased by rising the pH up to 8.0 and then decreased. Therefore, the pH value of 8.0 was selected for the extraction of DOX. This is logical since the only present types in pH<7 are in form of monocation MC-DOX, which is the only charged type having a positive charge at the amino sugar group. Nonetheless, at a pH higher than 8.0, MC-DOX may lose a proton either from a phenolic group or from the amino sugar group to create a zwitterion (Z-DOX) or the neutral species (N-DOX) respectively. This form of medicine is interested in moving to the organic phase. Z-DOX or N-DOX may then lose a proton to form the monoanions (MA-DOX) [19].

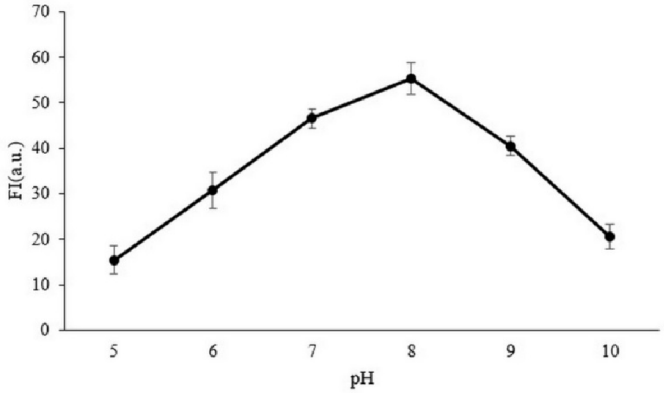


Figure 2. The relationship of fluorescent intensity of DOX solution with pH value. Conditions: 20 mL of aqueous sample, 150 ng mL^{-1} DOX, 250 µL of 1-dodecanol (extraction solvent), rate of centrifugation of 4000rpm.

Extraction solvent type and volume

The selection of organic extraction solvent is based on the following items: a different density relative to water, low solubility in water, extraction ability of intended compounds, and compatibility with final analytical instruments as well[9, 23]. Owing to the above-stated features, five organic solvents such as 1-dodecanol, 1-decanol, 1-octanol, 1-undecanol, and cyclohexane were investigated as the extraction solvent, and three repeatable tests were conducted for each solvent. The results indicated that in the same extraction condition, the DOX fluorescence intensity for 1-dodecanol provided the best outcome (Figure 3). This result can be due to lower polarity and higher viscosity of 1-dodecanol in comparison with others.

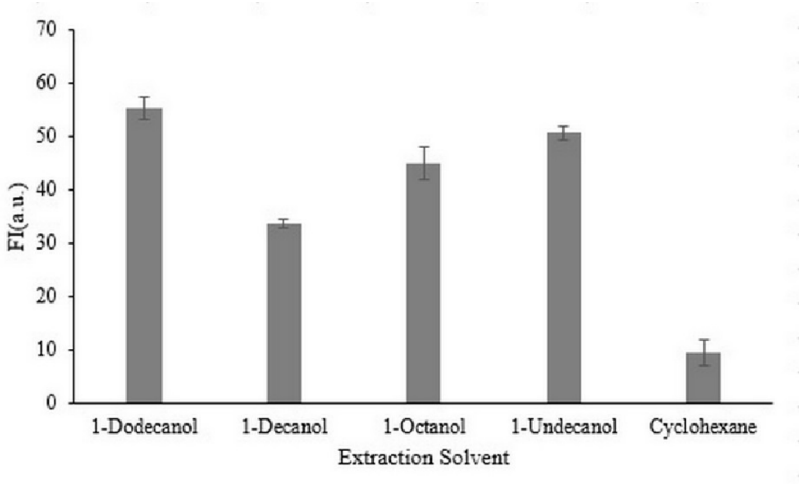


Figure 3. Effect of the type of the extraction solvent on fluorescent intensity of DOX**.** Conditions: 20 mL of aqueous sample, 150 ng mL^{-1} DOX, pH 8, the number of injections 7, rate of centrifugation of 4000 rpm.

After selecting the extractant, its volume was optimized in a similar extraction situation. For the selection of the optimum volume, different amounts of 1-dodecanol (50-450 µL) were added to 20 ml of the sample containing 150 ng mL^{-1} of DOX. The maximum fluorescence intensity was achieved when the volume was 250 µL (Figure 4). Some problems may occur when the volume of 1-dodecanol is low such as the 1-dodecanol might not be dispersed successfully in the sample solution, and also the upper organic phase might not be separated easily. On the other hand, when the volume of 1-dodecanol is higher than 250 µL, the amount of the collected organic phase increases,which in turn results in a decrease in the enrichment factor [24]. In addition, applying a high volume of 1-dodecanol causes an unsteady cloudy solution in which the surface area between the aqueous phase and extractant decreases. Therefore, volume 250 µL was selected as the optimum amount of extraction solvent.

The analyte solubility can decrease in the aqueous phase by adding salt and can increase its transfer to the organic phase due to the salting-out effect, thus it can improve the extraction efficiency. The

solubility of the target analyte and organic extraction solvent in aqueous phase usually decrease with increasing ionic strength, which is favorable for reaching high recovery. However, at the same time, the obtained volume of organic phase increases, resulting in a decrease of both the target analyte concentration and the enrichment factor [24].In this study, the influence of ionic strength was verified by adding different amounts of NaCl (0–10.0 %, w/v) into the sample solutions. The results showed that salt concentration had no significant effect on the recovery and fluorescence intensity of the DOX. Hence, NaCl was not used in this method.

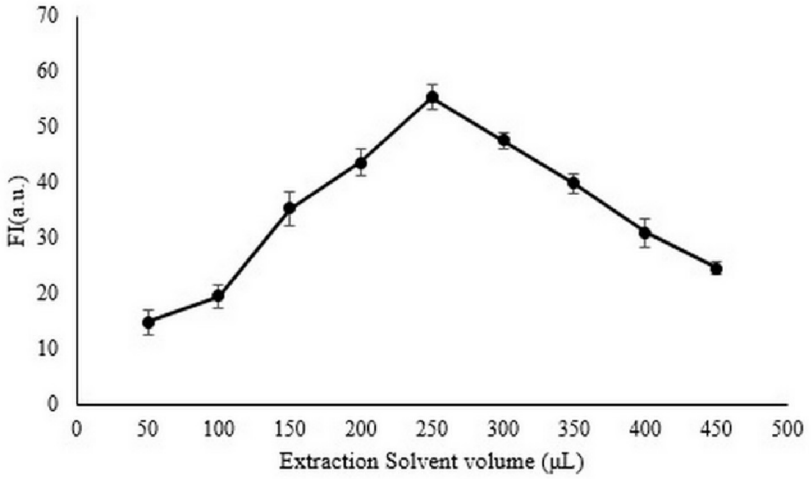


Figure 4. Effect of extraction solvent volume on fluorescent intensity of DOX**.** Conditions: 20 mL of aqueous sample, 150 ng mL^{-1} DOX, pH 8, the number of injections 7, rate of centrifugation of 4000 rpm.

Effect of the number of injections

To achieve high efficiency in a short time, the number of injections should be optimized in SS-DLPME. The effect of the number of reciprocal injections was tested from 1 to 10 injections under the same experimental conditions. Figure 5 illustrates that the analytical responses of DOX reached a peak and then stayed constant after seven injections, and this shows that the system has reached a steady-state. When the number of injections is low, the fluorescence intensity is lower because the extraction solvent is not able to disperse in aqueous sample properly. When it is more than 7, the efficiency of extraction decreases due to the dissolution of the extracted solvent in the water, so the fluorescence intensity decreases, and may be change to a possible reduction of the solvent volume due to its evaporation. Hence, a seventh injection was chosen by means of the optimum injection for extraction.

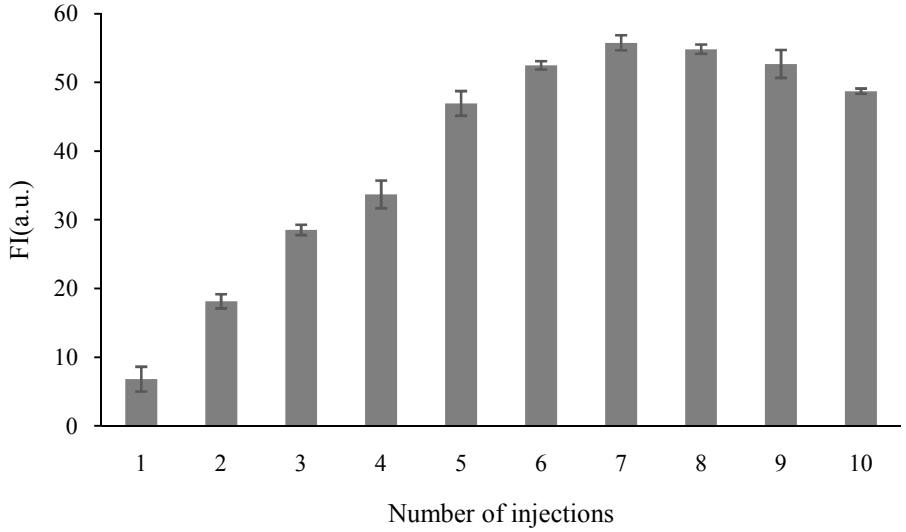


Figure 5. Effect of the number of injections on fluorescent intensity of DOX**.** Conditions: 20 mL of aqueous sample, 150 ng mL^{-1} DOX, pH 8, rate of centrifugation of 4000 rpm.

Effect of centrifuge speed

To achieve the highest fluorescence intensity, the speed was verified from 2000 to 4000 rpm. The results showed that by increasing the centrifuge speed, up to 4000 rpm, the fluorescence intensity of DOX increased. Hence, 4000 rpm was selected as the optimum speedof the centrifuge. In this study, a higher speedof centrifuge (>4000 rpm) was not investigated.

Analytical performance

The analytical characteristic data for the SS-DLPME system are shown in Table 1. Under the optimized experimental circumstances, a calibration curve was achieved with a linear dynamic range of 3.0-300.0 ng mL^{-1} with the regression equation FI = 0.3474C+ 1.6891 (r^2 = 0.998 for n = 8), in which FI is the fluorescence intensity at I_{em} = 558 nm (I_{ex} = 480 nm), and C is the concentration of the drug in ng mL^{-1}. The limit of detection (LOD) and the limit of quantitation (LOQ) values were calculated based on the following equations:

$$LOD = 3 \times \text{Standard Deviation (lowest concentration)/Slope} \quad (1)$$

$$LOQ = 10 \times \text{Standard Deviation (lowest concentration)/Slope} \quad (2)$$

The limit of detection and quantitation were found 0.76 and 2.55 ng mL^{-1}, respectively. The precision stated as the inter-day (n = 3) and intra-day (n = 3) RSD of the SS-DLPME method. For this purpose, spiked samples at three different concentrations (10, 100, and 200 ng mL^{-1}) were

applied. The enrichment factor was found by the slope ratio of the calibration curve after (0.3474) and before (0.0034) the extraction, which was about 102.18.

Table 1. Analytical characteristics of the proposed method.

Parameters	Analytical feature
Linear range (ng mL^{-1})	3-300
Linear regression equation	Y=0.3474C + 1.6891
Correlation coefficient (r^2)	0.998
Limit of detection (ng mL^{-1})	0.76
Limit of quantification (ng mL^{-1})	2.55
Intra-day precision (%)	0.82-2.11
Inter-day precision (%)	1.20-2.37
Enrichment factor	102.17

Accuracy

The analytical performance of the SS-DLPME method was verified by determining the various amounts of DOX-spiked in specimens of healthy human urine. The results showed a significant recovery of DOX-spiked to urine samples (Table 2). The proposed method can be used for DOX determination in urine samples successfully. During the initial 48 hours, only 5.9% of DOX gets rid of the human body [25]. Therefore, the concentration of the DOX in the urine is much lower than in the plasma. A low detection limit in this technique makes DOX determination in urine samples possible. Table 3 shows a comparison between the results obtained by the present method and some other methods. As it is shown, SPE/HPLC/MS (HPLC, in combination with mass spectrometry with the SPE) has the lowest LOD. In comparison with SPE/HPLC/MS, which is an expensive and complicated method, the proposed method is cheaper, simpler to determine the natural fluorescence of the drug, and the low consumption of toxic organic solvents. Other advantages of the SS-DLPME are wide linear range, low LOD, and high enhancement factor.

Table 2. Determination of DOX in in human urine samples by SS-DLPME (n=3).

	Sample 1 50 ng mL^{-1}	Sample 2 90 ng mL^{-1}	Sample 3 140 ng mL^{-1}	Sample 4 200 ng mL^{-1}
Found (ng mL^{-1})	49.62 (±0.68)	88.45 (±1.06)	143.10 (±1.10)	202.90 (±1.42)
Recovery %	99.25 (±1.36)	98.28 (±1.17)	102.21 (±0.79)	101.45 (±0.71)

Table 3. Comparison of the SS-DLPME method with other reported methods for DOX determination.

Sample	Method	LDR (ng mL^{-1})	LOD (ng mL^{-1})	RSD%	Ref.
Human urine	MIP/SPE/FL[a]	2.0-150.0	1.3	1.41	[4]
Water	MSPE[b]	10.0-100000.0	1.8	1.6	[20]
Hospital effluent	DLLME	0.8-1000	0.3	<15	[32]
Human urine	SPE/HPLC/MS[c]	0.1-2.0	0.04	<9.1	[33]
Human urine	SS-DLPME	3.0-300.0	0.76	0.82-2.11	This work

[a] Molecularly imprinted polymer/Solid phase extraction/spectrofluorometric; [b] Magnetic solid-phase extraction; [c] Solid phase extraction/high-performance liquid chromatography/tandem mass spectrometry

Conclusion

In this research, in order to determine DOX in urine samples, a low cost, simple, environmentally friendly, and the fast method combined with fluorescence spectroscopy was applied. The effective factors for the extraction were verified. The method has a low limit of detection and a wide linear dynamic range. Although the sample preparation time and the amount of organic solvent used are low, the method is highly sensitive. Great recovery and acceptable enrichment factor express the applicability and efficiency of this procedure for different samples. The results state that SS-DLPME is an influential method to quantify DOX in comparison with formerly reported methods.

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