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EXPLORATION OF NATURAL DEEP EUTECTIC SOLVENT AS THE ALTERNATIVE DISPERSIVE SOLVENT IN DLLME FOR THE EXTRACTION OF ANABOLIC STEROID DRUGS IN WATER

(Penerokaan Pelarut Eutektik Semulajadi Sebagai Alternatif Pelarut Serakan dalam DLLME untuk Pengekstrakan Ubat Steroid Anabolik dalam Air)

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Abstract

By combining hydrogen bond donors such as D-sorbitol, glucose, and sucrose with hydrogen bond acceptors like L-proline and lactic acid, a successful preparation of six new types of eco-friendly solvent known as natural deep eutectic solvents (NADES) was achieved. Their capability as dispersive solvent substituting hazardous and non-biodegradability organic solvents was explored in vortex-assisted dispersive liquid-liquid microextraction (VADLLME) method for determination of anabolic steroid drugs in aqueous samples. Optimization combination of hydrogen bond donor-hydrogen bond acceptor and their molar ratio were investigated, and the result showed high viscosity of lactic acid: sorbitol at a molar ratio of 2:1, whereby recorded the highest extraction efficiency towards the determination of nandrolone and testosterone. This optimum NADES combination was then performed in three significant VADLLME procedures, including the volume of NADES as the dispersive solvent, type, and volume of extraction solvent. The VADLLME optimal conditions are as follows: NADES dispersive solvent volume of 500 µL, chloroform as an extraction solvent and extraction solvent volume at 200 µL. Under the optimum conditions, good linearity was achieved (0.5 - 10 mg/L) with the coefficient of determination (R^2) of 0.9990 and 0.9996 for the nandrolone and testosterone, respectively. The LOD values were recorded in the range of 0.0020 - 0.0117 mg/L, while LOQ values were recorded in the range of 0.0067 - 0.0392 mg/L, respectively. The developed NADES-VADLLME method was applied for the determination of nandrolone and testosterone in tap water samples, with relative recovery values ranging from 88.63 - 97.23%. Based on the results obtained, the developed method demonstrated excellent sensitivity for the extraction of nandrolone and testosterone in water samples. The prepared NADES showed great potential as an alternative green dispersive solvent for the extraction of anabolic steroids active contaminants in the aquatic system.

Keywords: natural deep eutectic solvents, dispersive liquid-liquid microextraction, anabolic steroid drugs, dispersive solvent, green analytical chemistry

Abstrak

Dengan menggabungkan penderma ikatan hidrogen seperti D-sorbitol, glukosa, dan sukrosa dengan penerima ikatan hidrogen seperti L-prolin dan asid laktik, penyediaan enam jenis pelarut mesra alam baharu dikenali sebagai pelarut eutektik dalam semulajadi (NADES) telah berjaya disediakan. Keupayaan mereka sebagai pelarut serakan menggantikan pelarut organik berbahaya dan tidak boleh terbiodegradasi telah diterokai dalam kaedah pengekstrakan mikro cecair-cecair serakan dibantu vorteks (VADLLME) untuk penentuan ubat steroid anabolik di dalam sampel akueus. Pengoptimuman gabungan penderma ikatan hidrogen dan nisbah molarnya telah disiasat dan hasilnya menunjukkan kelikatan asid latik:sorbitol yang tinggi pada nisbah molar 2:1 menunjukkan kecekapan pengkestrakan tertinggi terhadap penentuan nandrolon dan testosteron. Gabungan optimum NADES ini kemudiannya dilakukan dalam tiga prosedur utama VADLLME termasuk isipadu NADES sebagai pelarut serakan, jenis dan isipadu pelarut pengekstrakan. Keadaan optimum VADLLME seperti berikut: isipadu pelarut dispersif NADES 500 µL, kloroform sebagai pelarut pengekstrakan dan isipadu pelarut pengekstrakan pada 200 µL. Pada keadaan optimum, kelinearan yang baik telah dicapai (0.5 - 10 mg/L) dengan pekali penentuan (R^2) masing-masing 0.9990 dan 0.9996 untuk nandrolon dan testosteron. Nilai LOD direkodkan dalam julat 0.0020 - 0.0117 mg/L manakala nilai LOQ masing-masing direkodkan dalam julat 0.0067 - 0.0392 mg/L. Kaedah NADES-VADLLME yang dibangunkan telah digunakan untuk penentuan nandrolon dan testosteron di dalam sampel air paip, dengan nilai pemulihan antara 88.63 - 97.23%. Berdasarkan keputusan yang diperoleh, kaedah yang dibangunkan menunjukkan sensitiviti yang sangat baik untuk pengestrakan nandrolon dan testosteron dalam sampel air. NADES yang disediakan menunjukkan potensi besar sebagai pelarut dispersif hijau alternatif untuk pengestrakan bahan cemar aktif steroid anabolik dalam sistem akuatik.

Kata kunci: pelarut eutektik dalam semulajadi, pengekstrakan mikro cecair-cecair serakan, ubat steroid anabolik, pelarut serakan, kimia analitikal hijau

Introduction

The misuse of anabolic drugs has been a crisis way before the pandemic, especially during international sports events. This drug is controlled and typically used to treat problems of hormone in males and Autoimmune Deficiency Syndrome (AIDS) patients that causes muscle loss [1]. Anabolic steroids, including testosterone, nandrolone, methandrostenolone, and others, were strictly used by professional bodybuilders for several decades. However, in recent times, recreational power athletes have regularly started using these drugs due to their impact on muscle growth and performance enhancement in sports [2-3]. The drug test was performed as a qualitative measurement in which it is a presumptive test that detects the drugs qualitatively, which includes opiates, methamphetamine, cocaine, amphetamines, marijuana, ecstasy, oxycodone, methadone, benzodiazepines, a tricyclic antidepressant, and PCP [4]. This urine test may provide a false-positive result that shows the presence of drugs even though a person has not used it, and this has raised some issues due to inaccurate results of the test, especially in forensic chemistry [5]. In order to identify the number of drugs present, the sample must be sent to the laboratory for further analysis and undergoes several steps prior to analysis. Nonetheless, drug testing has been improved in recent years, whereby the ability to identify long-term steroid metabolites, as well as more technologies, have enhanced that ability to detect metabolites at lower thresholds [6]. Nevertheless, these drugs are regularly being improvised to become accessible, which puts one step ahead of athletes to cheat. Each steroid tested in laboratories should store the baseline standards for reference in future testing and thus reducing the likelihood of a person to be tested positive only because the individual has higher levels of testosterone naturally compared to the general population. However, the laboratory test is time-consuming due to the multiple steps of analysis as well as the high labor and cost. Sample preparation takes up to 80% of the total analysis time and is critical in preparing the sample for analysis since it has a significant effect on analyte detection and quantification. These may include extraction, pre-concentration, clean-up, and derivatization steps before the analysis [7]. Therefore, the common conventional extraction methods such as liquid-liquid extraction (LLE) have been evolving into green extraction liquid-liquid microextraction (LLME) to determine drugs in

biological samples by miniaturizing the volume of the solvent and reducing the laborious steps.

Deep eutectic solvents (DESs) are a new class of green and inexpensive solvents. NADES has a variety of physiochemical properties, which gain its recognition as an alternative to ionic liquids and conventional organic solvents with biodegradability, non-flammability, and the potential as customizable for particular purposes [8]. Deep eutectic solvents (DES) consist of two non-toxic components that are mixed and form a new composition of solvent with a low melting point, low vapor pressure, low or negligible toxicity and biodegradable. The incredibly low melting point characteristic of this solvent is due to the formation of intramolecular hydrogen bonds within their molecular structure [9]. The hydrogen bonding between NADES effect varies in chemical properties such as polarity, viscosity, and hydrophilicity or hydrophobicity. In recent studies, DES has been applied as an extractant to substitute hazardous, costly, and non-environmentally friendly organic solvents in the extraction of organic pollutants [10]. For instance, Santana-Mayor et *al*. have successfully utilized DES as an extractant of phthalates and adipate from environmental water samples, with recoveries ranging from 70% to 127% [11]. Apart from that, Khezeli et al. employed DES as an extraction solvent of phenolic acids based on an ultrasonicated-assisted liquid-liquid microextraction method with high recoveries ranging from 94.7% to 104.6% in various vegetable oils samples [12]. Another type of deep eutectic solvent that is made up of primary metabolites components in living cells such as urea, amino acids, sugars, choline, and organic acids is called natural deep eutectic solvent (NADES) [13]. NADES is an alternative to DES due to continued safety concerns regarding DES. As a result, researchers have turned to natural sources and ingredients. A biodegradability in wastewater using microorganisms with closed bottle test, phytotoxicity test on wheat, and vitro toxicity test using fish and human cell lines were evaluated on choline chloride-based with glycerol, oxalic acid, and glucose and found that low toxicity in all tests, making them a great potential of green solvents in the applications of cosmetics, pharmaceutical, and food industry [14].

In this research, the utilization of NADES as a dispersive solvent is studied to substitute the toxic organic solvent in the DLLME method assisted by vortex agitation, abiding by the green chemistry principles. A dispersive solvent must be soluble in both organic solvent and aqueous phase [15]. NADES act as a disperser to assist the interaction of analytes in sample and extractant as well as favor the separation of two phases of sample solution and extraction solvent by allowing the formation of the cloudy solution containing tiny droplets, which speeds up the mass transfer process due to large contact surface area. The most common disperser solvents applied in DLLME are methanol, acetonitrile, and acetone [16]. Therefore, this study will focus on the vortex-assisted dispersive liquid-liquid microextraction (VADLLME) technique with the aid of DES as a dispersive solvent. The goal of this study was to develop a simple, rapid, inexpensive, and efficient sample preparation strategy for the measurement of testosterone and nandrolone in water samples prior to HPLC-DAD analysis. The technique is much simpler and eliminates the need for converting derivatization into a volatile phase during sample preparation. Additionally, it has the capability to simultaneously analyze multiple target analytes within a single run [17, 18]. Besides, this technique is trustworthy, particularly in clinical application fields such as toxicological diagnostic, therapeutic drug monitoring, and newborn screening. Various composition of NADES was studied from different hydrogen bond donor (D-sorbitol, glucose and sucrose) and hydrogen bond acceptor (proline and lactic acid), and molar ratio optimization was evaluated to obtain selective extraction on targeted analytes. The optimum NADES combination was then performed in three significant VADLLME procedures, including the volume of NADES as the dispersive solvent, type, and volume of extraction solvent.

Materials and Methods

Materials

Testosterone ($C_{19}H_{28}O_2$, ≥98%), nandrolone ($C_{18}H_{26}O_2$, ≥98%), lactic acid ($C_3H_6O_3$, 85%) solution ACS reagent, L-proline ($C_5H_9NO_2$, 99.5%) reagent standard, glucose ($C_6H_{12}O_6$, 99.5%) reagent standard, sucrose ($C_{12}H_{22}O_{12}$, 99.5%) reagent standard, D-sorbitol ($C_6H_{14}O_6$, 98%) reagent standard. Chloroform ($CHCl_3$, 98%), ethanol

(C_2H_6, 99.9%), acetonitrile (C_2H_3N, 99.9%) HPLC grade, 1-dodecanol ($C_{12}H_{26}O$, 98%), ethyl acetate ($C_4H_8O_2$, 98%), and dichloromethane (CH_2Cl_2, ≥ 99.8%) were all purchased from Sigma Aldrich.

HPLC instrumentation

The HPLC separations were carried out on a Dionex Bonded Silica C_{18} column (4.6 x 150 mm, 5 µm) (Acclaim™ Polar Advantage II) equipped with the ultraviolet detector and a 20 µL sample loop. The chromatographic condition was optimized by varying the mobile phase composition with acetonitrile-water, and the flow rate was set at 1.0 mL min^{-1} as well as the detection wavelength at 242 - 254 nm for all the analytes and recorded using Chromeleon software [19].

FTIR analysis

Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) Spectroscopy (PerkinElmer, USA) was applied in this study to determine the functional groups and possible interactions between the NADES. The type of crystal used as zinc selenide. All the samples were scanned in the range of 650 to 4000 cm^{-1} for 16 times.

Preparation and optimization of NADES

NADES was prepared by varying a few parameters, such as the type of NADES and the molar ratio of NADES. Firstly, the optimization of different groups of hydrogen bond acceptor (HBA) (organic acid – lactic acid and amino acids – L-proline) with sugars (monosaccharide – glucose, disaccharide – sucrose, and alcohol sugar – D-sorbitol) as hydrogen bond donor (HBD) by mixing and heating the components at 80°C in sonicator until a homogenous solution is formed [20]. These selected NADES were proved to act as good dispersers in DLLME due to their properties of being highly soluble and denser than water which led the targeted analyte easily diffuse in extractant solvent and increased their recovery from environmental water samples [21]. The combinations of NADES studied were as follows: Proline: Sorbitol (Pro: Sor), Proline: Glucose (Pro: Glu), Proline: Lactic Acid (Pro: LA), Lactic Acid: Glucose (LA: Glu), Lactic Acid: Sucrose (LA: Suc), and Lactic Acid: Sorbitol (LA: Sor) followed by identifying the optimum HBA: HBD mixture as disperser solvent by using the different molar ratio as follow: HBA: HBD, 1:1, 1:2, 1:3, 2:1, 3:1. Figure 1 presenting the molecular structure of HBA components together with HBD components.

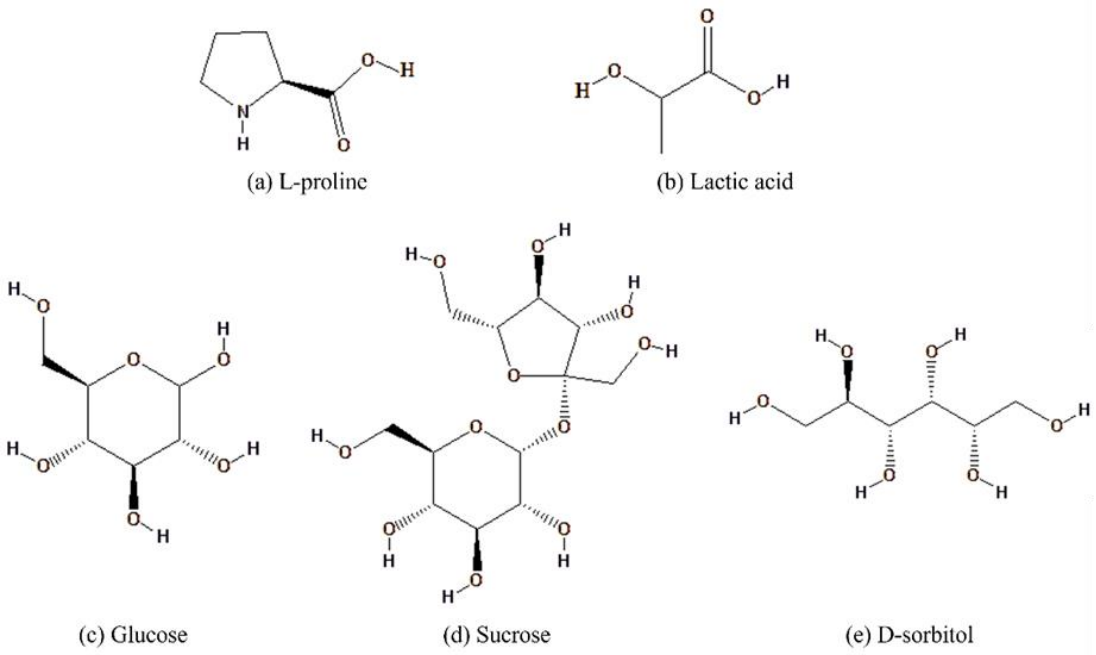

Figure 1. Molecular structure of HBA components: (a)-(b) and HBD components: (c)-(e)

Vortex-assisted dispersive liquid-liquid microextraction (VADLLME) procedure

The procedure began by adding extraction (chloroform) and dispersive (NADES) solvent into 5 mL of distilled water spiked with 1 mg/L of target analytes. The mixture was vortexed for 15 s until the formation of a cloudy solution and centrifuged for 10 min at 4000 rpm to form the two aqueous layers of phase. The bottom layer (organic phase containing analytes) was taken out and blown with nitrogen gas until dried. The dried organic phase was reconstituted in 50 µL of methanol and 50 µL of ultrapure water since chloroform cannot be directly injected into the instrument [19]. Then, the solution was injected into the HPLC system. Figure 1 shows the schematic diagram of VADLLME.

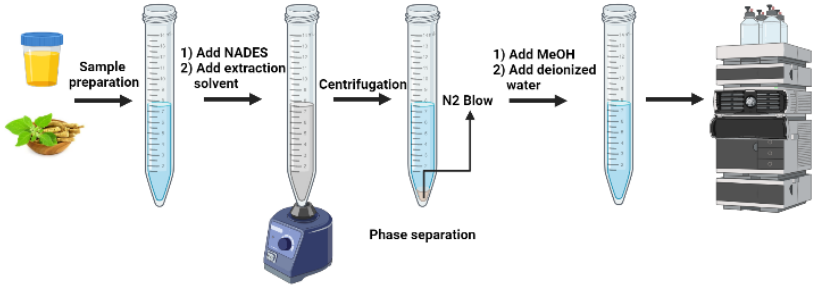

Figure 2. VADLLME process

Results and Discussion

FTIR analysis

FTIR analysis is commonly used to characterize a sample by determining the functional groups of the bonds and atoms within the molecules [22]. Thus, the hydrogen bonding formed in developed NADES was studied, including their functional groups. The wavenumbers for all synthesized NADES are recorded in Table 1. From the table, all the synthesized NADES showed a broad band at 3200 - 3600 cm^{-1}, indicating the formation of hydrogen bonds between the two components of HBA and HBD, as portrayed in Figure 3 (i). Apart from that, only Pro: Sor, Pro: Glu, and Pro: LA provided N-H bending at the range of 1609.3 - 1612.0 $cm^{-1,}$ which came from the functional group of L-proline and all synthesized NADES portrayed C-H stretching at the range of 2929.6 - 2992.1 cm^{-1}. The functional groups of all NADES except Pro: Sor and Pro: Glu displayed C=O of the carboxyl group between 1712.8 - 1723.7 cm^{-1}. Other medium-intense bands were observed at 1027.7 - 1222.9 cm^{-1} in which, indicating the C-O stretching of alcohol in their structure, as shown in Table 1. Besides, the C=O bond at 1719.8 cm^{-1} of lactic acid was shifted to the higher wavelength at 1721.7 cm^{-1} LA: Sor (1:1), as displayed in Figure 3 (ii), implying the formation of a hydrogen bond [23]. These prove that all the NADES were prepared successfully.

Table 1. Wavenumbers of IR for synthesized NADES

Type of NADES and Molar Ratio	Wavenumber (cm^{-1})				
	O-H Stretching	C-H Stretching	C=O Stretching	N-H Bending	C-O Stretching
Pro: Sor (1:1)	3288.2	2943.6	-	1612.0	1039.5
Pro: Glu (1:1)	3255.8	2929.6	-	1611.9	1027.7
Pro: LA (1:1)	3366.3	2992.1	1712.8	1609.3	1128.0
LA: Glu (1:1)	3335.9	2930.5	1721.2	-	1029.6
LA: Suc (1:1)	3320.5	2933.9	1723.7	-	1032.7
LA: Sor (1:1)	3336.2	2940.7	1721.7	-	1222.9
LA: Sor (2:1)	3352.2	2940.6	1721.5	-	1221.3

Figure 3 (ii) shows the correlation between lactic acid, sorbitol and LA: Sor mixture at molar ratios (1:1) and (2:1). In comparison between the molar ratio of LA: Sor (1:1 to 2:1), a few bands that significantly differed in intensity from each other was observed on Figure 3 (ii). In spectra for LA: Sor at molar ratio 1:1, the absorption band of OH at 3336.2 cm^{-1} was shifted to a higher wavenumber at 3352.2 cm^{-1} (molar ratio 2:1), proving the increasing number of hydrogen bonds formed in the NADES when the lactic acid molecule was increased as recorded in Table 1 [24-25]. Apart from that, the intensity at 1721.5 cm^{-1} and 1221.3 cm^{-1} of LA: Sor (2:1) were greater due to the greater number of the carboxyl group and C-O band, correspondingly because of the increasing number of the molecule of lactic acid [26]. Other than that, both spectra for LA: Sor molar ratio show identical absorption bands as parent lactic acid and sorbitol.

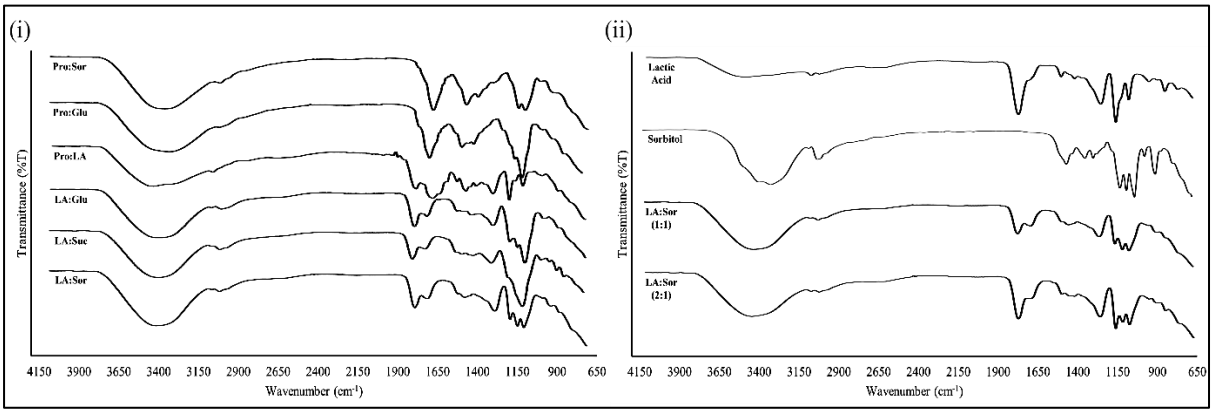

Figure 3. FTIR spectra of (i) synthesized NADES and (ii) lactic acid, sorbitol and optimized NADES (LA: Sor) at two different molar ratios (1:1 and 2:1)

Effect type of NADES (Different mixture of HBA: HBD) and it is the molar ratio

Disperser in DLLME is responsible for assisting the interaction of analyte towards extraction solvent. A tiny droplet of disperser properties contributes to the large surface area that enhances the mass transfer between two phases [27]. In order to determine the most appropriate NADES as the dispersive solvent, proline and lactic acid as HBA were prepared with various types of sugars (glucose, sucrose, and D-sorbitol) as HBD at the molar ratio of (1:1). The analytical results in Figure 4(i) showed the combination of NADES increasing as follows: (Pro: Sor < Pro: Glu < Pro: LA < LA: Glu < LA: Suc < LA: Sor). This indicates that the combination of organic acids with sugar alcohol as NADES prone to enhance the extraction efficiency of testosterone and nandrolone. Compared to all sugar in this study, sorbitol is a sugar alcohol which has a hydroxyl group with a straight chain structure compared to glucose and sucrose, which has a cyclic structure. This improves the solubility and viscosity of the sorbitol, which enhances the efficiency of lactic acid: sorbitol is a dispersive solvent [9, 28]. Therefore, LA: Sor NADES was selected for subsequent optimization of the molar ratio of NADES.

Herein, the most optimal molar ratio of HBA to HBD was determined to allow the appropriate number of hydrogen bonding formed in order to obtain higher extraction efficiency. It is explained that the more the hydrogen bonding, the more viscous the NADES resulting in a low capability to disperse in the sample solution [28]. Nonetheless, less hydrogen bonding formed means less viscous, and it could not provide the maximum efficiency as a disperser. Therefore, pertinent viscosity from the hydrogen bonding should be optimized from the molar ratio of the HBA and HBD. In this study, NADES with different molar ratios (1:1, 1:2, 2:1, 1:3, and 3:1) of lactic acid (HBA): sorbitol (HBD) were prepared accordingly, and their capability affecting the extraction of steroid drugs were determined. As demonstrated in Figure 4 (ii), the extraction efficiency has improved with an increasing number of moles up to two moles of lactic acid. This is explained by how the

structure of lactic acid consists of two HBA groups and by occupying those groups with one mole of sorbitol that might be existed as a stable compound [29]. When the molar ratio is 1:3 (LA: Sor) and 3:1, the analytical response declined slightly subsequently due to the high viscosity of sorbitol and lactic acid, respectively [30]. Hence, the desired molar ratio of lactic acid to sorbitol was chosen to be 2:1.

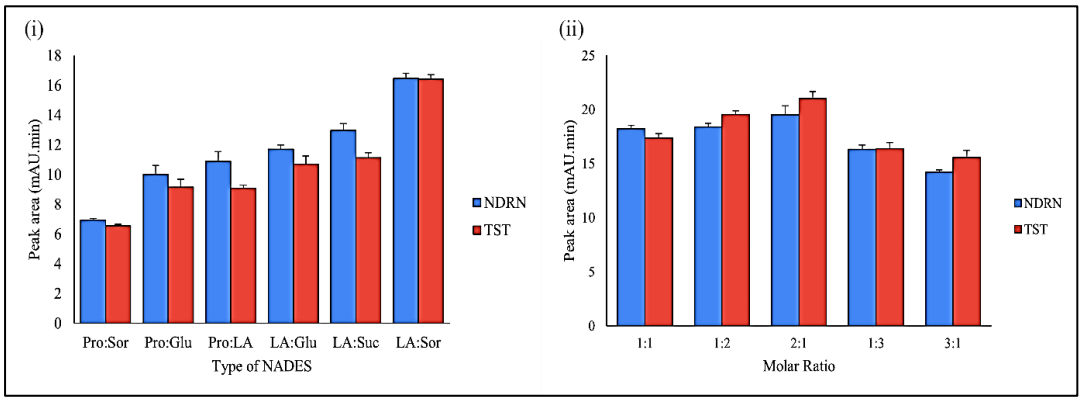

Figure 4. (i) Optimization of NADES combination and the (ii) molar ratio of optimized NADES (LA: Sor)

Effect volume of NADES

The volume of the disperser solvent causes changes in the sedimented phase volume (extraction solvent) and thus affects the extraction efficiency. Therefore, it is crucial to vary the disperser solvent volume and the extraction solvent volume simultaneously to achieve a constant volume of the sedimented phase containing analytes [16]. In the meantime, the ratio between the extraction solvent volume and the disperser solvent volume is also important, and it should be adjusted accordingly to form a cloudy solution. Hence, the impact of the volume of dispersion solvent on the extraction efficiency was investigated in the range of 200 - 600 µL. The volume of NADES that is lower than 200 µL was not evaluated as the volume of NADES must be the same or larger than the extractant to assist the partition coefficient of the analytes onto the extractant. During this optimization, one factor at a time approach was applied in the study, and thus, the extraction solvent was fixed at 200 µL. As depicted in Figure 5, the peak areas were gradually increased until 500 µL and declined slightly afterwards due to the dissolution of target analytes in the aqueous sample [31]. Therefore, the volume of NADES at 500 µL was optimized for further optimization.

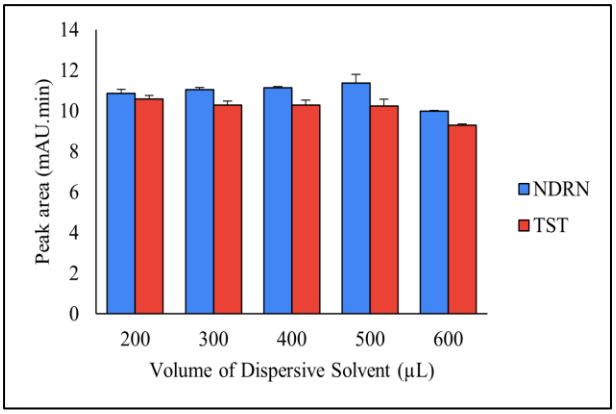

Figure 5. Optimization volume of dispersive solvent

Effect type and volume of extraction solvent

Choosing an appropriate organic solvent is significant to establish a successful microextraction method because its physicochemical properties should be suitable with the analytes for the extraction to occur. Various factors should be taken into consideration when selecting the appropriate extractant. For instance, the extraction solvent should be insoluble in water while having decent solubility of the target analytes, having a higher or lower density than the aqueous solution with high affinity for the analyte of interest and excellent chromatographic resolution. Furthermore, the extractant also must be soluble in the NADES prepared. For the extraction of steroid drugs, the selected solvent was investigated based on a previous study by [19, 32], including 1-dodecanol, ethyl acetate, hexane, dichloromethane and chloroform was experimented with as the potential extractant. However, only dichloromethane and chloroform formed a clear separation phase with an aqueous solution, while the other organic solvents did not form a clear separation phase at all or a very ambiguous phase separation formed. Based on the results tabulated in Figure 6 (i), chloroform provided the highest peak area for all the studied steroid drugs because chloroform is more non-polar and has high density than dichloromethane. This non-polar nature of chloroform significantly facilitates the isolation of non-polar anabolic steroid drugs resulting in good analytical response [29]. Thus, chloroform which provides high extraction capability for steroids, was selected as the extraction solvent for further optimization.

The volume of extraction solvent is key in extraction techniques. The amount of extraction solvent is required to be as minimal as possible for the purpose of environmental protection and to obtain an excellent analytical signal. Nonetheless, the amount should be large enough to ensure a sufficient amount of organic phase prior to chromatographic analysis and maximize the isolation of target compounds in the extractant [33]. Therefore, the impact of the volume of extractant on the extraction efficiency was studied in the range of 100 - 500 µL. The volume of chloroform that is lower than 100 µL was not evaluated as the amount of the extraction solvent left after extraction is insufficient, hindering the collection process. As outlined in Figure 6 (ii), 200 µL was observed to have a higher peak area, and there was an achievement in the equilibrium state of the extraction. At higher volume, the peaks of the analyte started to split and consequently, 200 µL of chloroform was employed in this study despite the volume of the sedimented phase [34].

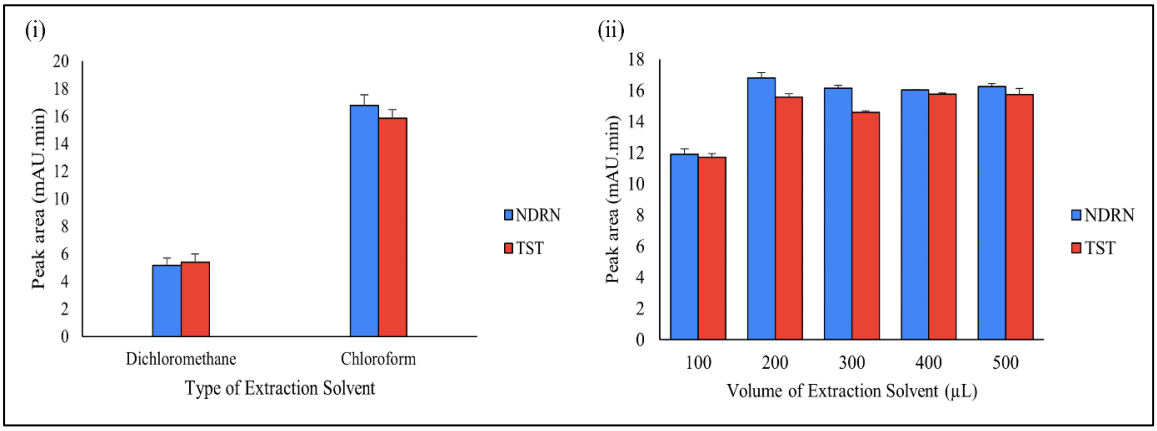

Figure 6. (i) Optimization type and (ii) volume of extraction solvent

Method validation

The capability of NADES (LA: Sor) as a disperser solvent was studied in a real sample solution to investigate the matrix effect on the extraction of anabolic steroids. Tap water samples were sonicated and filtered before being spiked with standard analyte concentrations ranging from 0.5 - 10 mg/L to construct a calibration curve. The analytical characteristics of the target steroid under optimized parameters are presented in Table 2. Both nandrolone and testosterone provide

dynamic linearity with an outstanding coefficient (R^2), 0.9996 and 0.9990, respectively. The value of LOD and LOQ was determined by using blank determination with the formula LOD = 3 *s/m,* while LOQ = 10 *s/m* is the standard deviation of blank signals, while *m* is the slope of the calibration curve. The values of LODs and LOQs showed to be in the range of 0.0020 - 0.0117 mg/L and 0.0067 - 0.0392 mg/L, respectively. The relative recoveries (RR) of both analytes have been found at 97% for nandrolone and 89% for testosterone at a spiking level of 1 mg/L with relative standard deviation (RSD), 0.79% and 0.42%, respectively. The precision of the method was determined in terms of intra-day and inter-day by spiking two analytes concentration (1 and 3 mg/L) into sample solution as listed in Table 3. Three replicates were employed to measure the intra-day on the same day and inter-day throughout 3 consecutive days. As shown in Table 3, the relative recovery of inter-precision for testosterone is considerably low, which can be explained by the degradation of the stock solution due to the nature of testosterone that is affected by light while conducting the experiment [35]. It is advised to keep the standard and stock solution short and out of light. The RSD values representing the precision of intra-day and inter-day appeared to be in the range of 0.05 - 0.20% and 0.07 - 0.35%, respectively. Figure 7 illustrates HPLC-DAD chromatograms of (i) unspiked, (ii) spiked with 1 mg/L and (iii) 3 mg/L of anabolic drugs of real samples. All peaks in spiked water sample extracts were finely separated with good resolution in less than 6 mins. Thus, the overall results suggest that this method could be satisfactorily applied for anabolic drug analysis and monitoring.

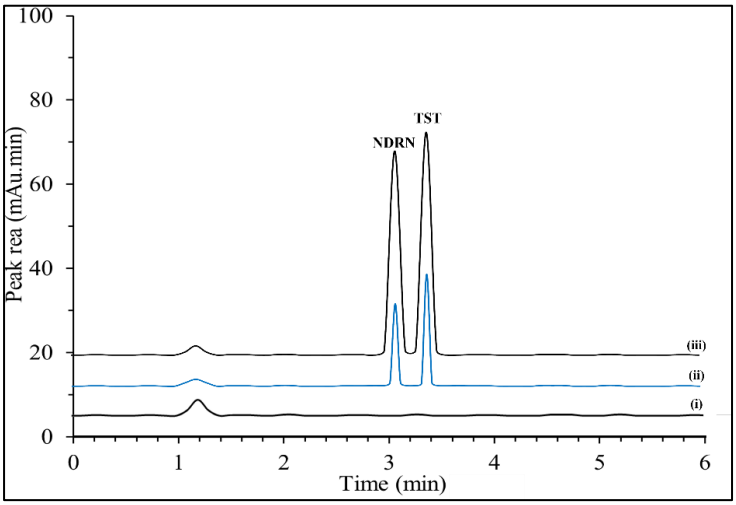

Figure 7. Chromatograms of HPLC-DAD of (i) unspiked, (ii) spiked with 1 mg/L and (iii) 3 mg/L of anabolic drugs in tap water under optimal conditions

Conclusion

The newly proposed NADES as a dispersive solvent in the DLLME technique provided remarkable linearity, acceptable LOD and LOQ, as well as outstanding precision values and satisfactory relative recoveries for the two target analytes under optimized conditions in which 97.23% for nandrolone and 88.63% for testosterone. The DLLME method in this research was employed successfully when extraction solvent was highly dispersed in the sample solution with the assistance of NADES as disperser by utilizing vigorous agitation of the vortex. Moreover, the newly prepared natural source of amino acid and organic acid-based NADES exhibited no toxicity to normal cell lines, affirming that the NADES used in this study is safe and green for the environment. Briefly, the proposed NADES in DLLME approach is environmentally favorable and practical for the extraction of drugs. NADES, in this study, is also expected to display great potential for the analysis of various organic contaminants, heavy metals, and other types of drugs.

Table 2. Analytical characteristics of the proposed procedure and recoveries of nandrolone and testosterone in tap water

Analyte	LOD (mg/L)	LOQ (mg/L)	Linear Range (mg/L)	R^2	RR (%)	RSD (%)
Nandrolone	0.0117	0.0392	0.5 - 10	0.9996	97.23	0.79
Testosterone	0.0020	0.0067	0.5 - 10	0.9990	88.63	0.42

Table 3. Precision (intra-day and inter-day) of the proposed method

Analyte	Concentration (mg/L)	Intra-Precision (n=3)		Inter-Precision (n=3)	
		RR (%)	RSD (%)	RR (%)	RSD (%)
Nandrolone	1	109.37	0.07	77.95	0.35
	3	94.12	0.05	93.59	0.07
Testosterone	1	116.41	0.20	61.09	0.25
	3	89.95	0.06	40.88	0.09

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