Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2413

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **4 (6):** 27-46 (2016)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Simultaneous Detection of Tricyclic Antidepressants using Higher Performance Liquid Chromatography

Jamiu Mustapha Sulayman[1*] and Bashir Abdulkadir[2]

[1]Department of Anatomy, Faculty of Health Sciences University of Ilorin, Nigeria

[2]Department of Microbiology, Umaru Musa Yar'adua University Katsina, Nigeria

*Corresponding Author E-mail: jamiu.sulayman@yahoo.com

Received: 29.11.2016 | Revised: 14.12.2016 | Accepted: 18.12.2016

ABSTRACT

Despite the introduction of newer antidepressants, tricyclic antidepressants are still widely used for the treatment of major depression, chronic pain and anxiety. And is the most leading cause of drug-induced death in many patients. Monitoring their blood/plasma concentration is therefore a priority in forensic toxicology. In this study, a quick and rapid method for simultaneous detection and quantification of three TCAs (desipramine, nortriptyline and amitriptyline) in whole blood was developed and validated on HPLC-UV under a gradient elution, using a C_{18} column and a mobile phase mixture of ACN (35% v/v) and a pH 3.5, 50mM phosphate buffer (65% v/v). The evaluated validation parameters were satisfactorily accepted in accordance with ICH and FDA guidelines. The determination coefficients were more than 0.99 within the tested concentration range (0.1–1mgL^{-1}), LOD and LOQ were between 0.0027-0.0051 and 0.27-0.051 respectively, Accuracy expressed as % Er was found to be between 0.1-7% and precision expressed as %CoV was found to be between 2-10%. All compounds were satisfactorily determined in one single injection within 20 min. sample pre-treatment was optimised on three sets of cartridges (C_2,C_{18} and Strata-X) on a solid phase extractions, with C_{18} giving the best recovery for all analytes ranging from 60-105%. The developed method proved to be suitable for routine work and it was used to successfully analyse forensic blood samples.

Key words: *Antidepressants, HPLC, tricyclic, toxicology and simultaneous.*

INTRODUCTION

Depression is a mental disorder that is characterised by low self-worth, low mood, fatigue and loss of appetite[35]. Pharmacological treatment has immensely advanced since the development of the tricyclic antidepressants between the period of 1960 and 1980[31]. Although it gained clinical recognition in the 1950s[12,15] the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors were originally adopted. However, when compared with monoamine oxidase inhibitors. The TCAs offer advantages over monoamine oxidase inhibitor (MAOI) antidepressants because they have less drug to drug interaction and with a lower therapeutic index are much more effective in the treatment of depression.

Cite this article: Sulayman, J.M. and Abdulkadir, B., Simultaneous Detection of Tricyclic Antidepressants using Higher Performance Liquid Chromatography, *Int. J. Pure App. Biosci.* **4(6):** 27-46 (2016). doi: http://dx.doi.org/10.18782/2320-7051.2413

The TCA's also appear to be reducing the suicidal risk compared to the MAOI's[10,25]. This therefore prompted the development of newer and safer antidepressants. However, TCA's are still widely used despite the development of new classes of antidepressants, such as the selective serotonin reuptake inhibitors (SSRIs), serotonin–norepinephrine re-uptake inhibitors (SNRIs), norepinephrine–dopamine reuptake inhibitors (NDRIs), and norepinephrine reuptake inhibitors (NRIs)[17,27] due to their effectiveness and cost[25,31]. TCAs are also regularly prescribed for treatment of a variety of other disorders including for example anxiety, eating disorders, attention deficit hyperactivity disorder, and enuresis in children. It has also been used as a treatment for neuropathic pain which explains why they are still widely in use[9,29].

In a study were TCAs and SSRI were compared in terms of tolerability and potency, TCAs appeared more effective inpatients than SSRI, and however, SSRIs are better tolerated, due to their milder adverse effects than TCAs[1]. This may be because TCAs act on numerous neurons, including serotonergic, dopaminergic and adrenergic neurons while SSRIs act specifically on serotonergic neurons, therefore monitoring plasma concentration is not required with SSRIs as in the case of TCAs[28].

The prevalence use of TCAs on suicide attempt keep increasing as it remains the leading cause of drug-death in many countries[13]. Between the period of1993-2004 England and Wales recorded 5,602 deaths caused by antidepressants and this accounted for about 15% of all deaths due to poisonings. Fatality typically occurs with ingestion of 20 mg/kg body weight however it is not possible to relate a dose to a post mortem blood drug concentration therefore the dose even if confirmed is a poor predictor of the resultant effect. This is in part due to the variation in metabolism and ethnicity[30] as previously indicated but also due to the phenomenon of post mortem drug redistribution[16,21].

The development of High Performance Liquid Chromatography (HPLC) began in the late 1960s and early 1970s, it is a unique form of chromatography commonly used in analysis, to identify, separate, and quantification of simple and complex compounds. It is a conventional method applied in several areas which include but not limited to forensic toxicology, pharmaceuticals, biotechnology, environmental, polymer and food industries[3,34]. HPLC provides a means for isolating analytes from potential interfering impurities to allow detection, identification and quantification. This made HPLC more a prevalent technique in the analysis of TCAs either in combination with UV or MS detection since most drugs will breakdown at high temperature therefore gas chromatography (GC) tends not to be used[11,31]. It remains the most widely used techniques to analyse TCA drugs despite other analytical methods being available[14]. Both normal and reversed-phase HPLC (RP-HPLC) methods are used in the analysis of TCAs[31].

We aimed to develop a method on HPLC-UV for the simultaneous detection of TCA's (Amitriptyline, Nortriptyline and Desipramine) and validate the developed method as well as to optimise and apply the method developed using SPE on whole blood.

MATERIAL AND METHODS

Study Design

This research study take in cognisance with the risk assessment and the ethical approval was granted.

SPE manifold, vacuum pump, SPE cartridges (C2, C18 by Varian and strata X by Phenomenex), Ammonium, Acetonitrile, Methanol, Nitrogen and collecting tubes. 1ml of whole blood spiked with 1mgL-1 of TCAs was transferred to Eppendorf and either centrifuged at 4000rpm for 10mins or sonicated for 15mins depending on the pre-treatment type.

The SPE procedure was evaluated according to the methods described by Bielicka-Daszkiewicz et al[4]., and Camel[7]. This consisted of evaluating the influence of parameters in each of the standard four steps thus, (i) conditioning of the sorbent; (ii) isolation of the analytes; (iii) washing of sorbent (iv) elution step.

Method Application

Three types of SPE cartridges (C2, C18, and Strata-X) were tested. The first two columns belong to the group of reverse-phase sorbent. The Strata-X column is a hydrophilic polymeric sorbents (mixed-mode) bonded silica containing both reversed phased and cation ion exchange sorbent, where the starting methods for SPE methods are similar. However, the results obtained on the C2 cartridge did not allow the use of this column to isolate TCAs from spiked blood samples and this cartridge was discarded. This might be due to fact that C18 contains octadecyl (higher alkyl), while C2 contains ethyl (lower alkyl chains) as reactive site. More so, the lower the alkyl chain the lesser the retention. The remaining two cartridges were further optimised from the starting method, however during the optimization process, sample pre-treatment, loading conditions, washing reagents, and volume of eluent were considered. Whole blood was selected, as this is mostly encountered in most forensic toxicological analysis.

RESULTS AND DISCUSSION

To improve the recovery of the TCAs, the washing reagents, elution volume/type were changed (See Table 1).

Table1. Methods 1-5, showing SPE optimization steps involving sample pre-treatment, washing reagents and elution solvents. The elution volume were sequentially applied 4 times in the order; 1ml, 0.5ml, 0.5ml and 0.5ml and the eluent were collected 4 times for each cartridge used on each method.

EXPT 1	1	2	3	4	5
STEPS	C18	C18	STRATAX	STRATAX	STRATAX
CONDITIO	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH30H
	1ml H20	1ml H20	1ml H20	1ml H20	1ml H20
LOADING	sonicate	sonicate	centrifuge	sonicate	sonicate
WASHING	1ml H20	1ml H20	1ml H20	1ml H20	1ml H20
	1ml CH3CN	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH3CN
	1ml CH3CN	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH3CN
VACDRY (5MINS)					
ELUTION	(CH3CN:NH40H)	(CH30H:NH40H)	(CH30H:NH40H)	(CH3CN:NH40H)	(CH3CN:NH40H)
	(98:2)	(98:2)	(98:2)	(98:2)	(98:2)
	0.5ml*(2)	0.5ml*(2)	0.5ml*(2)	0.5ml*(2)	0.5ml*(2)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
RECONSTI	Iml CH3CN	Iml CH3CN	Iml CH3CN	Iml CH3CN	1ml mobile phase
EVAPORATE TO DRYNESS UNDER NITROGEN AT 40 DEGREE CELCIUS					
1ml of TCAs containing amitriptyline, nortriptyline and desipramine were spiked into 1ml of blood before					
subjecting to either sonication for 15 mins or centrifugation at 4000rpm for 10mins					

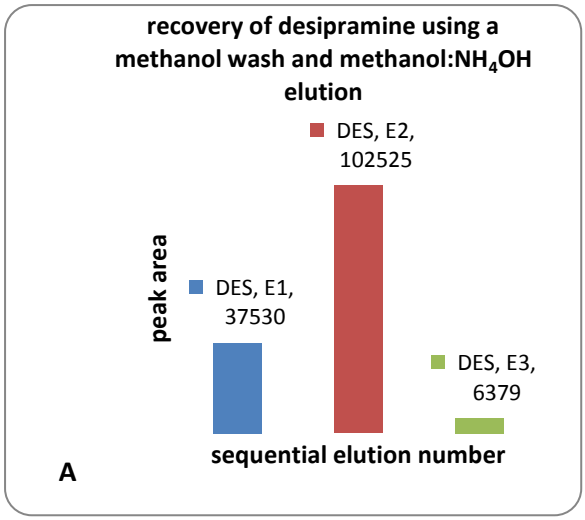
Moreover, the first method was not sufficient to yield good result but it was not surprising i.e. eluting with Acetonitrile: Ammonium hydroxide (CH_3CN:NH_4OH) ratio (98:2). Since the retention of basic drugs in aqueous sample on reversed-phase SPE sorbents is not only due to weak hydrophobic interactions between analyte and apolar group of silica modified surface, but also strong ionic interaction between the drug in its charged form and with charged residual silanols[19]. This therefore prove that acetonitrile either alone or combination with ammonium hydroxide is not sufficient to break the hydrophobic interaction. This findings corroborated another findings that discover acetonitrile cannot interact with silanols via hydrogen bonding however dipole–dipole interactions are possible[6]. More so, in another study where acetonitrile and methanol were compared as wash solvent for basic drugs, more recovery was obtained when acetonitrile was used as wash solvent compared to when methanol was used[33]. Since a good eluent is supposed to be strong enough to elute the compounds of interest in a limited volume irrespective of the binding force, acetonitrile was therefore alternated as a washing solvent. This was however in contrast with the claimed that methanol was unable to elute TCAs on C_{18}[36].

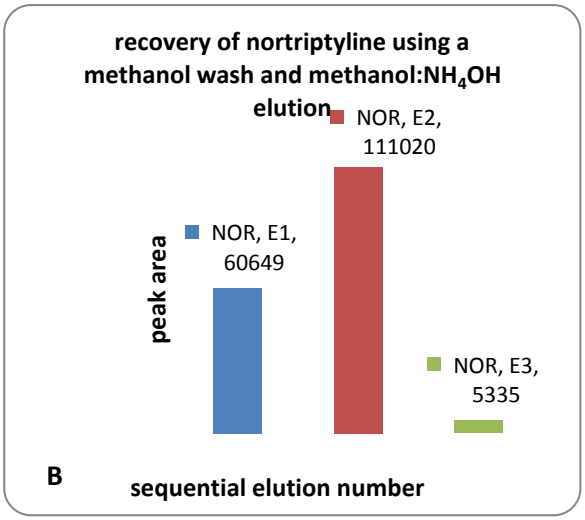
Although, methanol was suggested to be too strong as wash solvent, yet it was used as wash solvent while the eluent solvent was changed (Table 1) to methanol and ammonium hydroxide (NH_4OH) the result of this is shown in Figure 1 (a, b & c). Since the breakthrough volume is a function of the analyte hydrophobicity and the mass of the sorbent used[26].

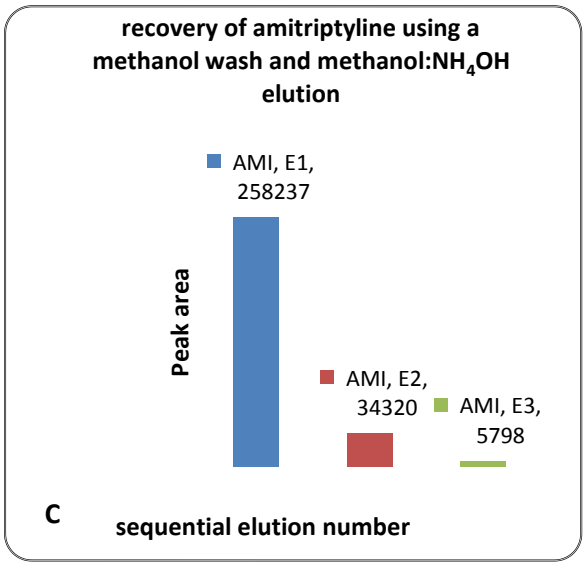
The first elution volume was able to break the van der wall for amitriptyline whereas additional volume was required to elute both desipramine and nortriptyline. This phenomenon might be attributed to the structure of the analytes. Amitriptyline is a tertiary amine with three carbon substitutes located on the terminal nitrogen of it side chains chain, which gives it more affinity to the eluent solvent and at the same time easy for disrupting it binding force whereas desipramine and nortriptyline are demethylated metabolite of imipramine and amitriptyline respectively and are secondary amines; with two carbons on the terminal nitrogen of the side chain.

This therefore supports the claim that retention of analyte is a function of the analyte's structure, rather than, on interactions of it functional groups with the sorbent surface[37]. This procedure is similar to a study where olanizapine (a typical basic analyte) was extracted, methanol was used as wash solvent and methanol/NH_4OH was also used as elution solvent and a higher recovery was observed[19]. Although its recovery was more than the observed recovery on CH_3OH:NH_4OH (98:2). However, the observed recovery on CH_3OH:NH_4OH (98:2) was better than that observed on similar column by Madej et al[23].

Its noteworthy to mention that adding a competing cation (NH_4OH) to the organic elution solvent block the charged silanol groups of the C_{18} sorbent thereby allowing maximum elution of the adsorbed analytes[19]. See percentage recovery in Figure 1d.







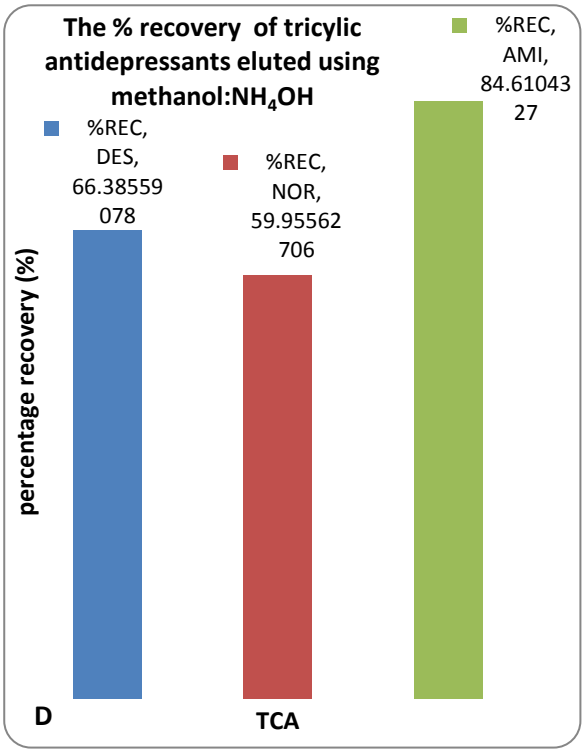


Fig. 1d: The percentage recovery of TCA's following elution with methanol: NH4OH (98:2).Percentage recoveries were calculated to be 66%, 60% and 85% for Desipramine, Nortriptyline & Amitriptyline respectively

Fig. 1 (a-d): The effect of sequential elution for the recovery of the tricylic antidepressants (desipramine (a), nortriptyline (b) and amitriptyline (c).

Note E1 = 1ml, followed by E2=0.5ml and finally E3=0.5ml of methanol: NH_4OH

Furthermore, the effects of sample pre-treatment was assessed between two column types (C18 and Strata X) – methods 2 and 3. Unfortunately, all Strata-X methods (3-5) yielded no good results indicating that the method does not apply to Strata-X **Sample Pre-treatment.**

TCAs are extremely bounded to plasma-proteins, majorly alpha1-glycoprotein, albumin and lipoproteins. During the analysis of these drugs especially at extraction process, the binding reduce the percentage recovery of the drugs because their active site have been occupied by the protein binding resulting in ineffective interaction between the drugs and the sorbent binding site more so, the larger the protein the more it block the sorbent pores thereby leading to clogging and the drug

therefore pass through the sorbent un-retained. However in this study disruption of cell membrane and separation of blood components was tested (since clogging was experienced in the starting method) by two techniques including sonication and centrifugation. But sonication was finally chosen as it simultaneously allows moderate flow of the blood and sufficient retention between TCAs and the non-polar sorbent of the cartridge whereas centrifugation flows were too fast as it does not provide enough retention for the drugs to adsorb to the sorbent. Choosing sonication was consistent with previous studies[33], although it was not compared with any other techniques.

A study claimed that reconstitution plays a role in recovery[22] thus reconstitution was therefore tried using mobile phase on Strata-X (CH_3CN:NH_4OH) but unfortunately the peaks was very poor suggesting that reconstitution is independent on recovery provided that reconstitution is done with appropriate polar solvent. Although the method was not effective on Strata-X, thus observing reconstitution effect on Strata-X does not give a conclusive remark on effect of reconstitution.

Optimisation of Strata-X on loading pH, and elution solvents.

Table 2. The SPE procedure for methods 1-6 on the Strata-X column. The methods show changes in pH conditions of the columns before loading the pre-treated samples and elution solvents on each column. The elution volume was increased from 1ml to 1.5ml in the 1stelution and 0.5ml in the subsequent elution for a total of 4 times.

Figure 1: The effect of sequential elution for the recovery of the tricylic antidepressants (desipramine (a), nortriptyline (b) and amitriptyline (c)). Note E1 = 1ml, followed by E2=0.5ml and finally E3=0.5ml of methanol: NH_4OH.

EXPT 2	1	2	3	4	5	6
STEPS	STRATAX	STRATAX	STRATAX	STRATAX	STRATAX	STRATAX
CONDITIO	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH30H
	1ml H20	2ml Buffer	1ml H20	1ml H20	2ml Buffer	1ml H20
LOADING	Sonicate	Sonicate	Sonicate	Sonicate	Sonicate	Sonicate
WASHING	2ml H20	2ml H20	2ml H20	2ml H20	2ml H20	2ml H20
	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN
	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN
VACDRY (5MINS)						
ELUTION	(CH30H:NH40H)	(CH30H:NH40H)	(CH3CN:NH40H)	(ACN:MeOH)	(ACN:MeOH)	(ACN:MeOH)
	(98:2)	(98:2)	(98:2)	(60:40)	(60:40)	(98:4)
	0.5ml*(3)	0.5ml*(3)	0.5ml*(3)	0.5ml*(3)	0.5ml*(3)	0.5ml*(3)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
RECONSTITUTION IN 1ml CH3CN						
1ml of TCAs containing amitriptyline, nortriptyline and desipramine were spiked into 1ml of blood before						
subjecting the sample to sonication for 15 mins						
EVAPORATE TO DRYNESS UNDER NITROGEN AT 40 DEGREE CELCIUS						

Strata-X is a surface-modified styrene skeleton with a pyrrolidone group, whose retention mechanisms exploithydrogen-bonding, aromatic and hydrophobic interaction with the analytes for the extraction of basic, acidic, amphoteric and neutral analytes[26]. However, the extraction of basic drugs requires the analyte to be ionized at pH either two units/more lower than the p*K*a of the compounds. At this pH, approximately 99% of the group is charged[36]. TCAs are expected to be ionised under the experimental conditions. Unfortunately, no appreciable recovery was achieved in all methods but one (method 5) where the sample was buffered with 50mM phosphate (pH 3.5) before loading.

The recovery is shown in Figure 2 (a,b,&c). Although the rationale for this is unclear, but it has previously been noted that Strata-X exploits both reversed-phase and hydrogen bonding for retention, as such retention of analyte increases with increasing hydrophobicity[5].Thus, the analytes held too tight/strong to the sorbent which might have led to no recovery on other experiments. The percentages recovery for this experiment is shown in Figure 2 (d). With the recovery of this method, the Strata-X is still not as good as the C_{18} cartridge (Figure 1d). TCAs were strongly retained on Strata-X, which can be justified by the strong interaction of NH_2 (amine) group with the carboxylic acid moiety present in the sorbent, thus this account for it low recovery of TCAs[2].

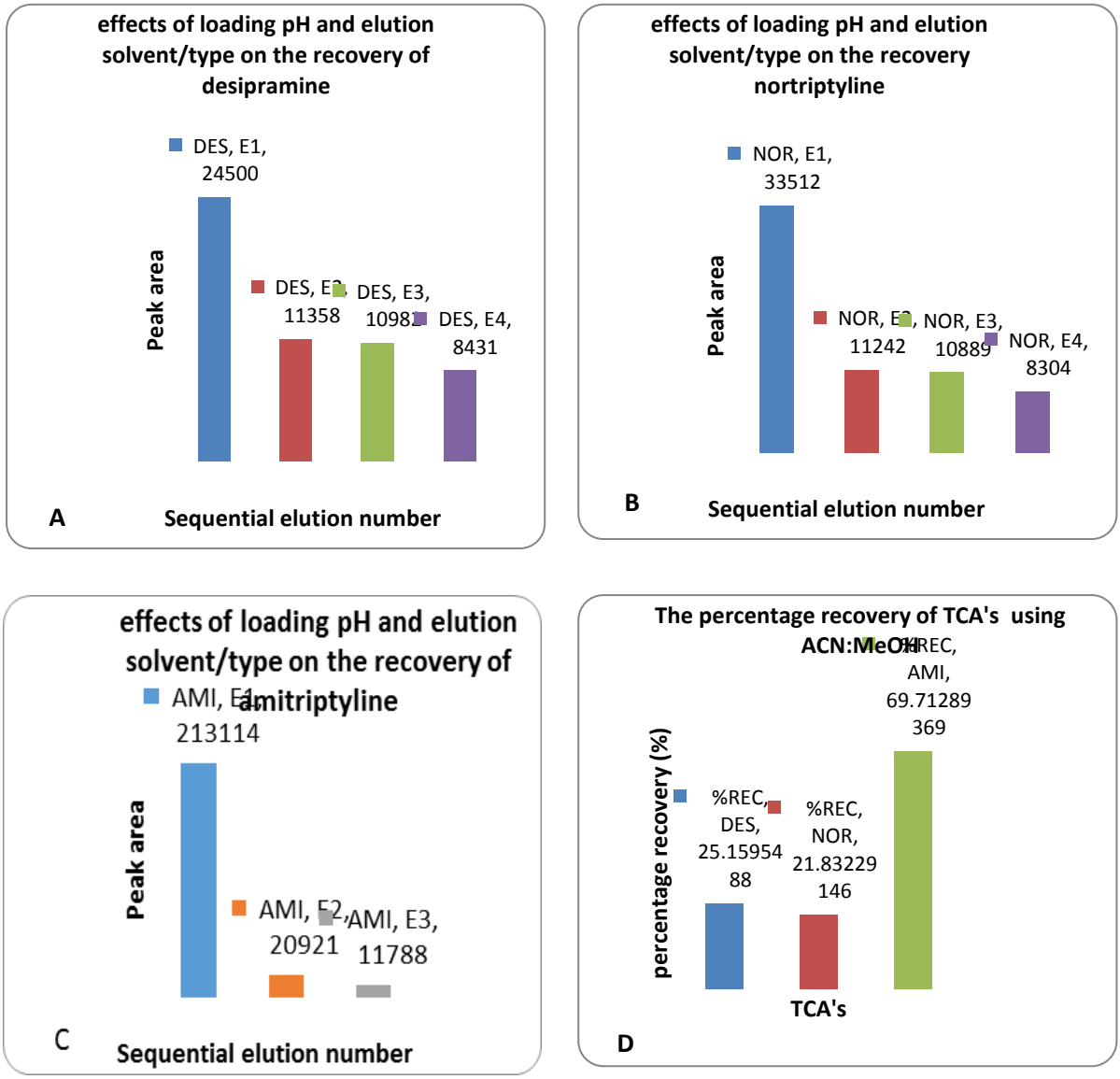


Fig. 2 (d): The percentage recovery of TCAs following elution with Acetonitrile:Methanol in the ratio (60:40). Percentage recoveries were calculated to be 25%, 22%, and 70% for Desipramine, Nortriptyline and Amitriptyline respectively.

Fig. 2: The effect of sequential elution for the recovery of the tricyclic antidepressants (desipramine (a), nortriptyline (b) and amitriptyline (c)). Note E1 = 1.5ml, followed by E2=0.5ml, E3=0.5ml and finally E4=0.5ml of acetonitrile: methanol (60:40).

In each case it can be seen that the 1st 1.5ml elutes the majority of the TCA, but some still remains on the cartridge. Subsequent elution volumes do appear to contain the TCA's as well indicating a larger volume would be required.

Further optimisation of loading pH on Strata-X

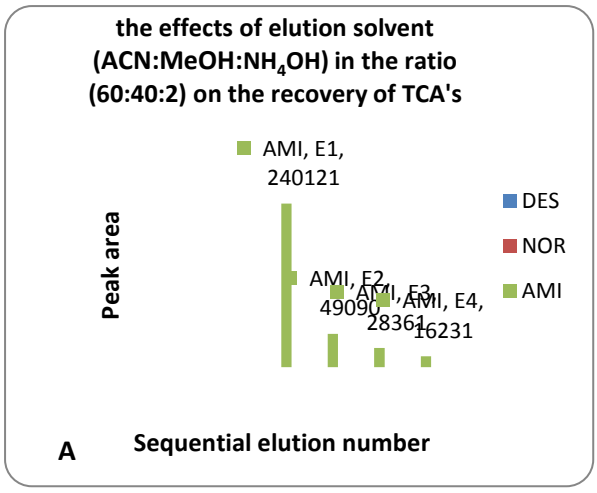
It is expected that pH is important to improve recovery; therefore the pH was adjusted using ammonium hydroxide.

Table 3.The SPE procedure for experiment 3, method 1-3, the effect of adding NH_4OH to the elution solvents

EXPT 3	1	2	3
STEPS	STRATAX	STRATAX	STRATAX
CONDITIO	1ml CH30H	1ml CH30H	1ml CH30H
	2ml Buffer	2ml Buffer	2ml Buffer
LOADING	Sonicate	Sonicate	Sonicate
WASHING	2ml H20	2ml H20	2ml H20
	1ml CH3CN	1ml CH3CN	1ml CH3CN
	1ml CH3CN	1ml CH3CN	1ml CH3CN
VACDRY (5MINS)			
ELUTION	(ACN:MeOH:NH40H)	(ACN:MeOH:NH40H)	(CH30H:NH40H)
	(60:40:2)	(60:40:4)	(96:4)
	0.5ml *(3)	0.5ml *(3)	0.5ml *(3)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
RECONSTITUTION IN 1ml CH3CN			
1ml of TCAs containing amitriptyline, nortriptyline and desipramine were spiked into 1ml of blood before			
subjecting the sample to sonication for 15 mins			
EVAPORATE TO DRYNESS UNDER NITROGEN AT 40 DEGREE CELCIUS			

Since the combination of both ACN:MeOH in ratio 60:40 as eluent yielded recovery of all anaylytes, although at unsatisfactory amount.

This might be due to the claim that methanol possess the ability to break hydrogen-bonding whereas acetonitrile only have the ability to break dipole–dipole interaction[6]. Thus ammonium hydroxide was then added to the elution solvent; unfortunately only amitriptyline was recovered ACN:MeOH:NH_4OH ratio (60:40:2), see figure 3(A&B).Increasing the percentage of $NH_4OH$by 4%did not still improve the recovery of analytes, but did improve the recovery of amitriptyline ACN:MeOH:NH_4OH ratio (60:40:4) Figure 4 (A&B) nor did the combination of methanol:NH_4OH ratio (94:4). This result was in contrary to the observed result where NH_4OH in methanol was claimed to give a higher recovery when used as an elution solvent in the extraction basic of drugs on Strata-X[5]. This therefore suggested that the neutral polymers (Strata-X) is definitely not a primary extraction mechanism for TCAs under the prescribed conditions and this might probably be due to it aromatic structure which allow interaction with aromatic analytes via π-π interactions[20].



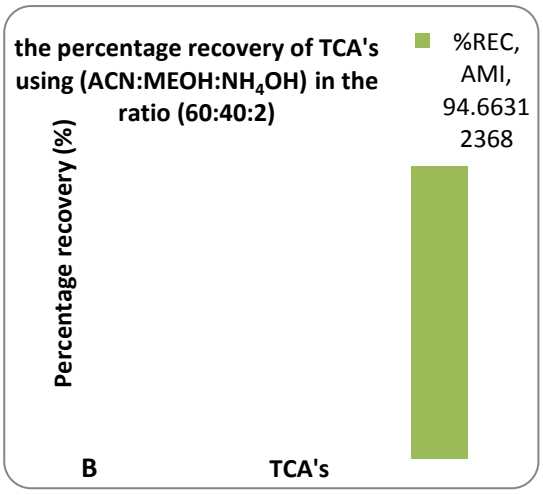
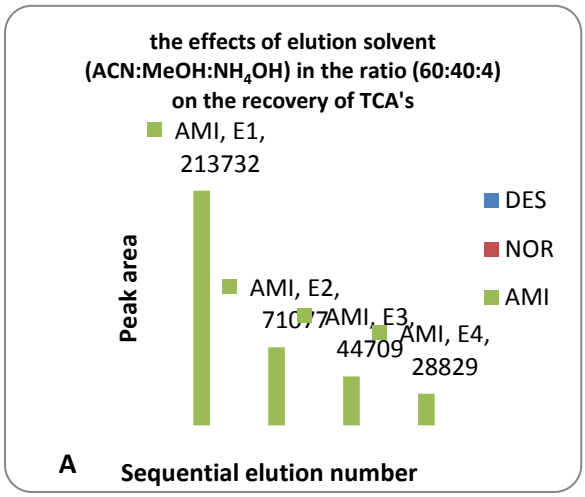


Fig. 3A: The recovery of TCA following sequential elution using ACN:MeOH:NH_4OH in the ratio of (60:40:2). Note E1 = 1.5ml, E2=0.5ml,E3=0.5ml and E4=0.5ml. Amitriptyline was detected in all sequential elution volumes with a percentage recovery calculated to be 95% (Figure 31B). Desipramine and Nortriptyline were not detected.



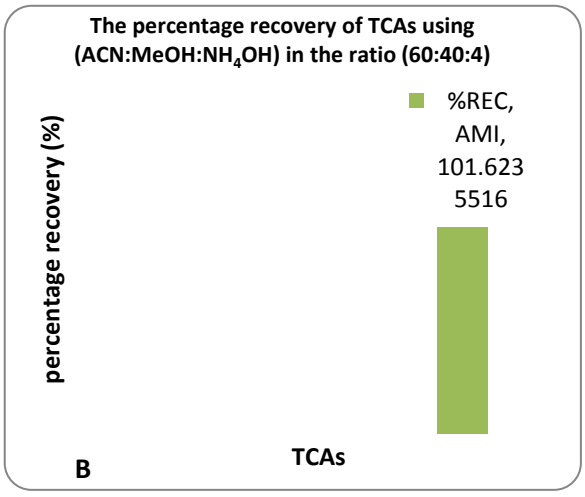


Fig. 4A: The recovery of TCA following sequential elution using ACN:MeOH:NH_4OH in the ratio of (60:40:4). Note E1 = 1.5ml, E2=0.5ml, E3=0.5ml and E4=0.5ml. Amitriptyline was detected in all sequential elution volumes with a percent recovery calculated to be 102% (Figure 4B). Desipramine and Nortriptyline were not detected.

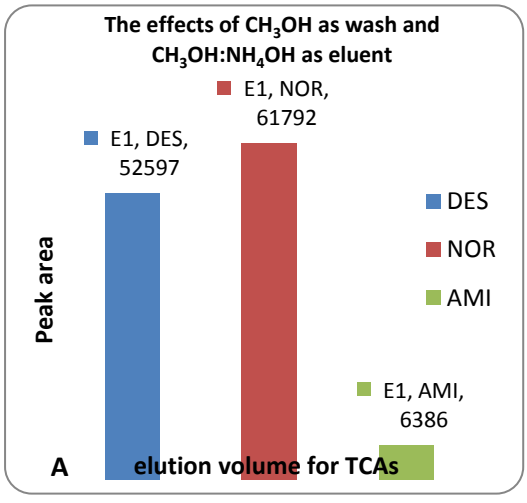
Optimisation of the C_{18} SPE method

Method 1 in this sequence has already been shown to produce good recoveries of the TCAs and was used as the starting point for the development of this application method.

Table 4. The SPE procedure for experiment4 method 1-7.Changes to the method includes different wash solvents (method 1&2) and different elution solvents (method3-7)

EXPT 4	M1	M2	M3	M4	M5	M6	M7
STEPS	C18(1)	C18(2)	C18 (3)	C18(4)	C18(5)	C18(6)	C18(7)
CONDITIONING	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH30H	1ml CH30H	1ml MeOH	1ml MeOH
	1ml H20	1ml H20	1ml H20	1ml H20	1ml H20	1ml H20	1ml H20
LOADING	Sonicate	Sonicate	Sonicate	Sonicate	Sonicate	Sonicate	Sonicate
WASHING	2ml H20	2ml H20	2ml H20	2ml H20	2ml H20	1ml H20	1ml H20
	1ml CH30H	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN
	1ml CH30H	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN	1ml CH3CN
ELUTION	(CH30H:NH40H)	(CH30H:NH40H)	(CH30H:NH40H)	(CH30H:NH40H)	(ACN:CH30H)	(ACN:CH30H:NH40H)	(ACN:CH30H
	(98:2)	(98:2)	(98:2)	(96:4)	(60:40)	(60:40:2)	:NH40H)
							(60:40:4)
	0.5ml*(4)	0.5ml*(4)	0.5ml*(3)	0.5ml*(4)	0.5ml*(4)	0.5ml *(3)	0.5ml *(3)
	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)		0.5ml*(1)	0.5ml*(1)	0.5ml*(1)	0.5ml*(1)
RECONSTITUTION IN 1ml CH3CN						0.5ml*(1)	0.5ml*(1)
1ml of TCAs containing amitriptyline, nortriptyline and desipramine were spiked into 1ml of blood before							
subjecting the sample for sonication for 15 mins							
EVAPORATE TO DRYNESS UNDER NITROGEN AT 40 DEGREE CELCIUS							

The elution volume was finally optimised in experiment 4 table17. Thus 2ml of eluent was enough to disrupt any interactive force between the analytes and the sorbent. In addition, the wash solvent was finally optimised between methanol and acetonitrile. Since amitriptyline has proven to possess higher affinity/polarity than other analytes, it was however not properly recovered when methanol was used as wash solvent in figure 5, this suggest that it might have probably been washed away during methanol wash. This findings was also supported in experiment 3 where Strata-X was used, amitriptyline was the only recovered analyte, this probably suggest that amitriptyline is although structurally similar with other observed TCAs but it mechanism of activities and binding may be however different and more so, it belong to a tertiary group of TCAs whereas the other analytes belong to secondary group. Although the percentage recovery figure 33Bwas inconsistent with that obtained in figure 2 d, where similar procedure gave a better recovery. It however evident that, it's better to use a less polar solvent such as acetonitrile as washing solvent, if recovery is to be maximised and maintain consistency of results.



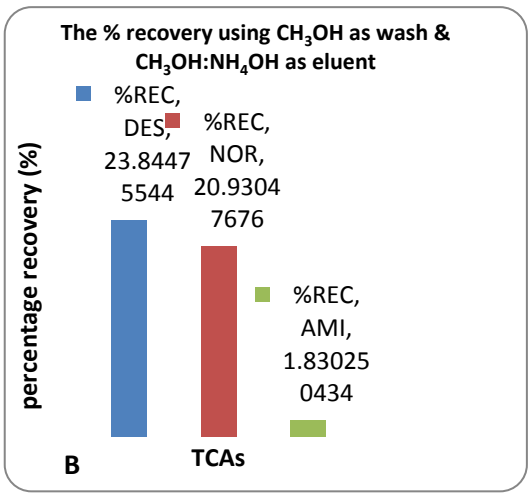


Fig. 5A. The recovery of TCAs following the use of 2ml of ($MeOH:NH_4OH$) in the ratio of (98:2). E1 signifies that all TCAs were eluted during the 1st elution using 2ml of the elution solvent. Percentage recoveries were calculated to be 24%, 21%, and 2% for Desipramine, Nortriptyline and Amitriptyline respectively (Figure5B).

Using acetonitrile as the wash solvent (method 2,3) but at different elution volumes gave a better a better recovery (Figure 6) when compared with the recovery of both figure 1d 4B where methanol was used as wash solvent. Figure A and B in the ratio (98:2).E1 signifies that all analytes were eluted during the 1st elution using

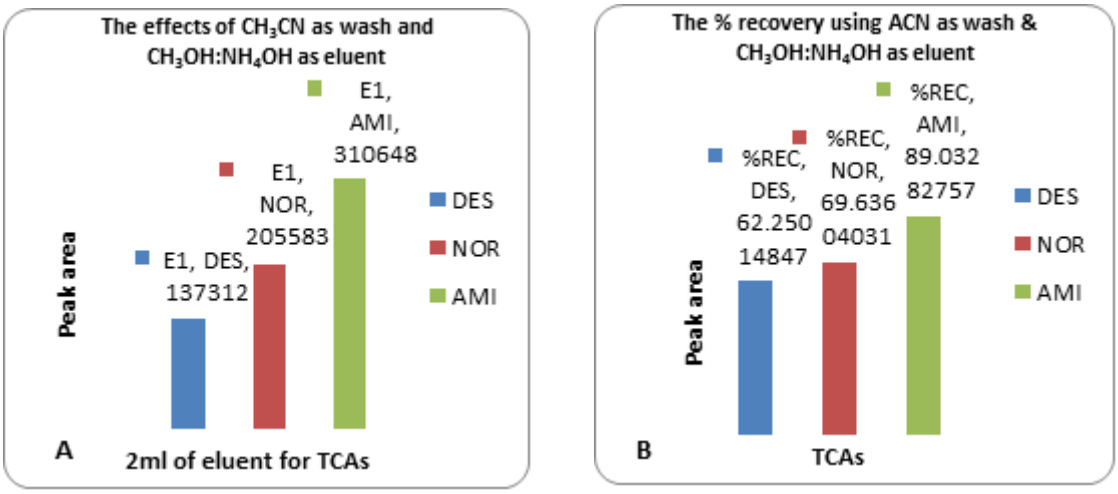


Fig. 5A: The recovery of TCAs following single elution with 2ml of $CH_3OH:NH_4OH$ 2ml of the elution solvent.

The percentage recoveries were calculated to be 62%, 70%, and 89% for Desipramine, Nortriptyline and Amitriptyline respectively (Figure 5B).

Acetonitrile has proven to be a suitable wash solvent in term of consistency in both methods 2 and 3 Figure. In contrast to methanol wash that resulted into inconsistent recovery in both. Thus acetonitrile was therefore maintained in the subsequent analysis. Figure 6.

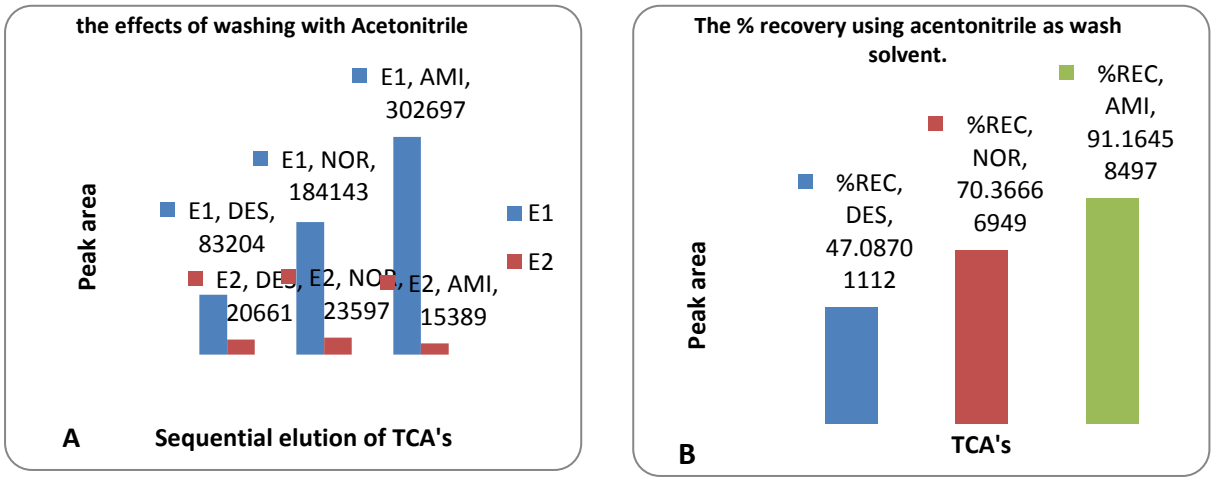


Fig. 6A: The recovery of TCA's following the use of acetonitrile as wash solvent and sequential elution using $CH_3OH:NH_4OH$ in the ratio 98:2. Note E1=1.5ml and E2=0.5ml. The percentage recoveries were calculated to be 47%, 70%, and 91% for desipramine, nortriptyline and amitriptyline respectively (Fig. 6B).

Increasing the percentage of ammonium hydroxide (method 4) although resulted in increased recovery of amitriptyline but reduced desipramine. But better, compared to the recovery in figure 35b, where 2% NH_4OH was used with 98% CH_3OH as eluent.

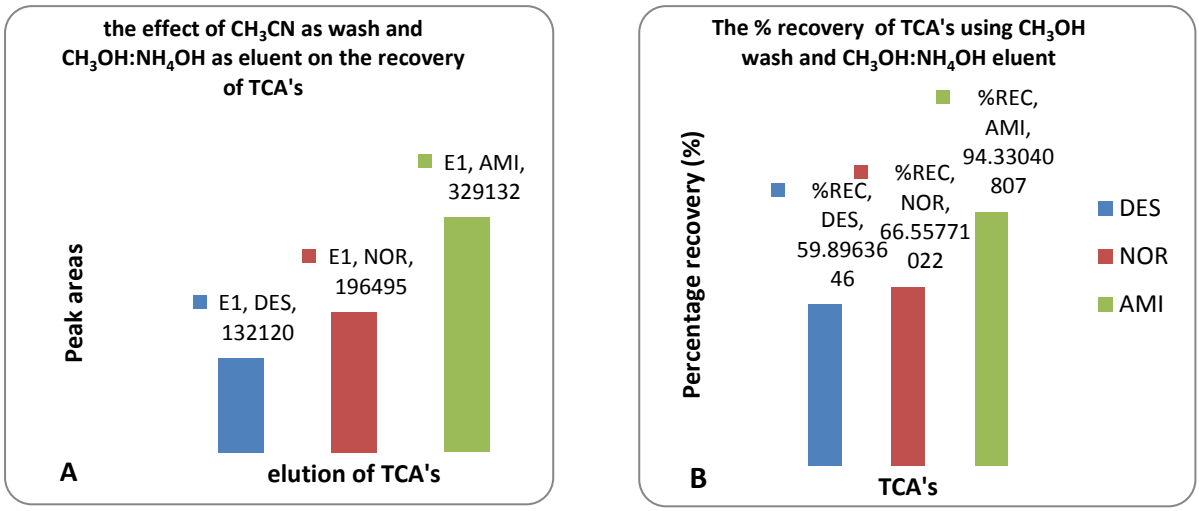


Figure7A.The recovery of TCAs following single elution with 2ml of CH_3OH:NH_4OH in the ratio (96:4).E1 signifies that all analytes were eluted during the 1st elution using 2ml of the elution solvent. The percentage recoveries were calculated to be 60%, 67%, and 94% for Desipramine, Nortriptyline and Amitriptyline respectively (Figure7B).

Using a different eluent (method 5), it was expected that a better recovery would be achieved due to the combination of solvent used, however only amitriptyline was extracted. This was not surprising because the NH_4OH that was claimed to block the charged silanol groups was not in the combination.

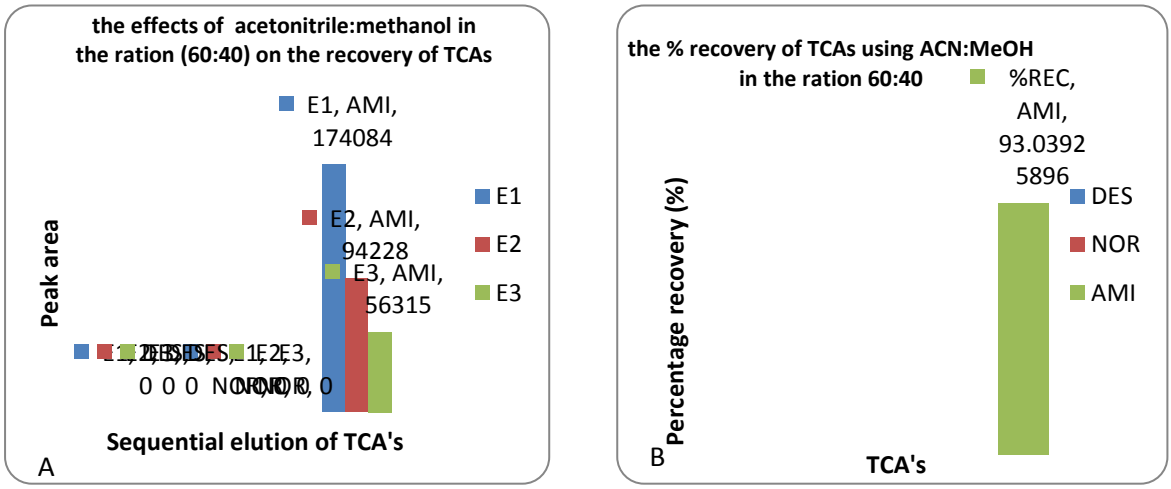


Fig. 8A. The recovery of TCA's following sequential elution using acetonitrile: methanol in the ratio (60:40) E1=2ml, E2=0.5ml and E3=0.5ml. The percentage recovery was calculated to be 93% for Amitriptyline (Fig. 8B). Desipramine and Nortriptyline were not detected.

NH_4OH was therefore added to the subsequent elution solvent (method6) to confirm if NH_4OH must be used in combination with any intended solvent for elution. Figure 9

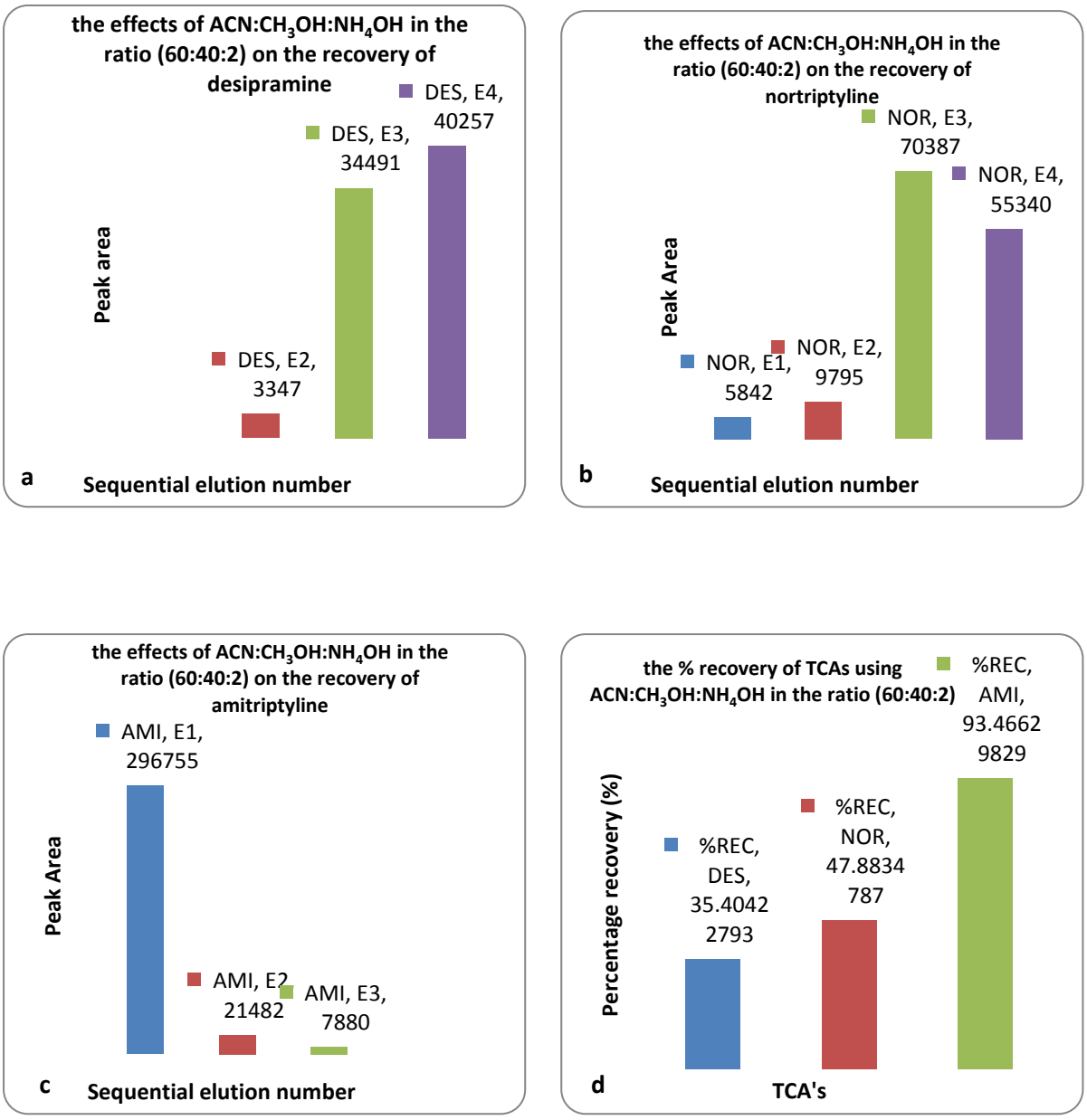
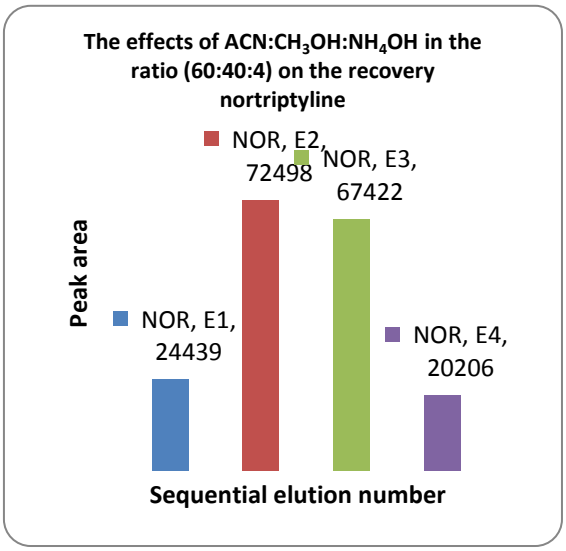
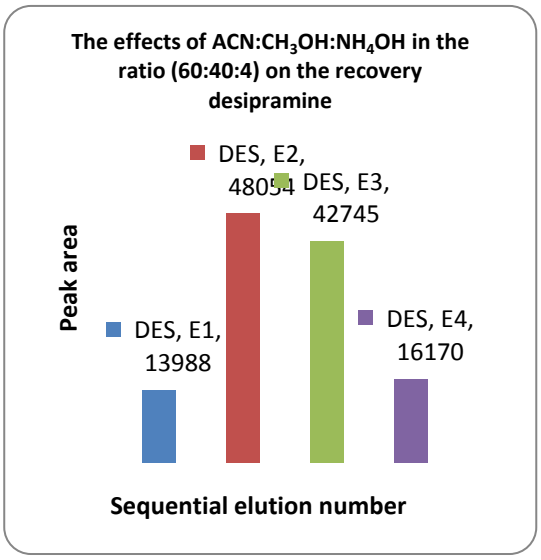


Fig. 9 (a,b&c).The recovery of TCA's following sequential elution using CH_3CN:CH_3OH:NH_4OH in the ratio (60:40:2) E1=1.5ml, E2=0.5ml, E3=0.5ml and E4=0.5ml.The percentage recovery was calculated to be 35%, 48% and 93% for Desipramine, Nortriptyline and Amitriptyline (Fig. 9(d). The recovery obtained was low but was better when compared with (CH_3CN:CH_3OH) in the ratio 60:40 where desipramine and nortriptyline were not detected/recovered.

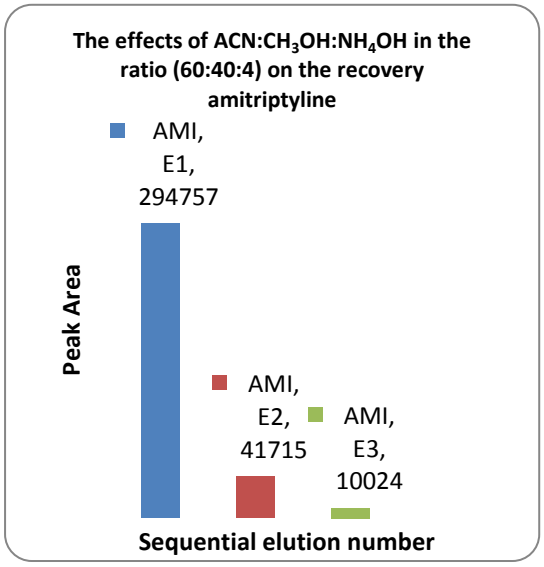
The percentage of NH_4OH was further increased) to 4% (method7) and this although gave a better recovery of TCAs when compared with the 2% NH_4OH.



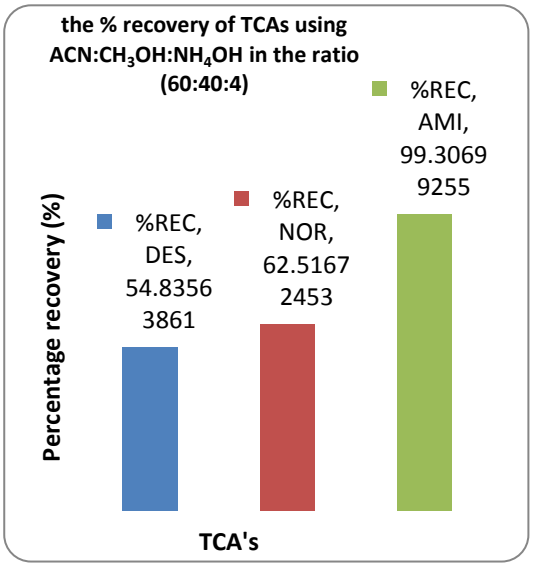
(a)



(b)



(c)



(d)

Fig. 10 (a,b&c). The recovery of TCA's following sequential elution using CH_3CN:CH_3OH:NH_4OH in the ratio (60:40:4) E1=1.5ml, E2=0.5ml, E3=0.5ml and E4=0.5ml. The percentage recovery was calculated to be 55%, 63% and 99% for Desipramine, Nortriptyline and Amitriptyline (Fig. 10d).

When the recovery of this method {CH_3CN:CH_3OH:NH_4OH in the ratio (60:40:4)} was compared with that of method4 {(CH_3OH:NH_4OH) ratio 96:4}used as eluent, the CH_3OH:NH_4OH was observed to be better even when both eluent were at the same proportion of NH_4OH (4%). Hence, NH_4OH must be used with any other polar analyte in the extraction of TCAs. And increasing the level of NH_4OH to at least by (4%) also increases the percentage recovery.

Final optimisation step – influence of loading pH on C_{18}

The final optimisation step was to examine the effects of loading pH of sorbent because in a reversed phase, the pH of the sorbent plays an important role in the retention of the analyte. pH 2unit below the analyte's pKa create an environment where the analyte is uncharged. Thus, resulting in better retention[36]. Another study claimed that addition of water adjust the pH of the sorbent to about 7 and thereby creating a microenvironment for quick and easy[8]. In view of this two, set of pH were achieved by using a 50mM phosphate buffer at pH 3.5 and distilled water (pH about7).

Table 5: The SPE procedure for experiment 5 method 1&2.

EXPT 5	M1	M2
STEPS	C18(1)	C18(2)
CONDITIONING	1ml MeOH	1ml MeOH
	1ml Buffer	1ml H20
LOADING	1ml(Sonicated blooc	1ml(Sonicated blood+TCA)
WASHING	1ml H20	1ml H20
	1ml CH3CN	1ml CH3CN
	1ml CH3CN	1ml CH3CN
VACDRY (5MINS)		
ELUTION	(CH30H:NH40H)	(CH30H:NH40H)
	(96:4)	(96:4)
	0.5ml *(3)	0.5ml *(3)
	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)
	0.5ml*(1)	0.5ml*(1)
EVAPORATE TO DRYNESS UNDER NITROGEN AT 40 DEGREE CELCIUS		
RECONSTITUTION IN 1ml CH	1ml CH3CN	

The effects of loading pH. Since acidic pH was claimed to be better in the extraction of TCAs, method 1 was then buffered with 50mM phosphate buffer at pH 3.5 compared to method 2 which remained loaded with water. The recoveries of TCA's on both methods were compared in figure 40 and 41. These two methods display almost similar/consistent recoveries and are therefore accepted among all other observed procedures.

Method 1 buffered with 50mM phosphate at pH 3.5

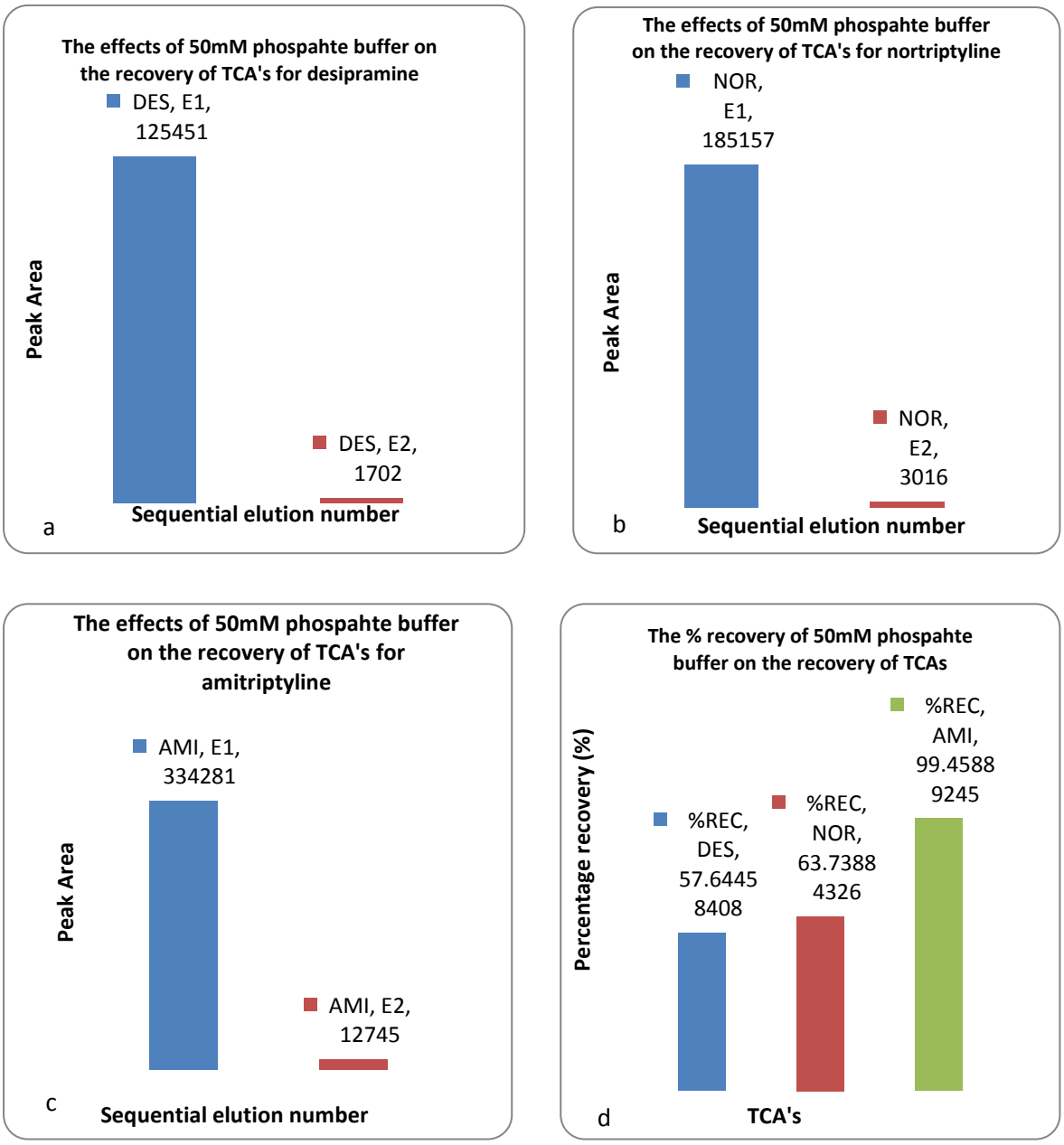


Fig. 11(a,b&c) The recovery of TCA's following loading with phosphate buffer and sequential elution using CH_3OH:NH_4OH in the ratio (96:4) E1=1.5ml, E2=0.5ml, E3=0.5ml and E4=0.5ml. The percentage recovery was calculated to be 58%, 64% and 100% for Desipramine, Nortriptyline and Amitriptyline respectively (Fig. 40d).

Method 2: buffered with distilled water

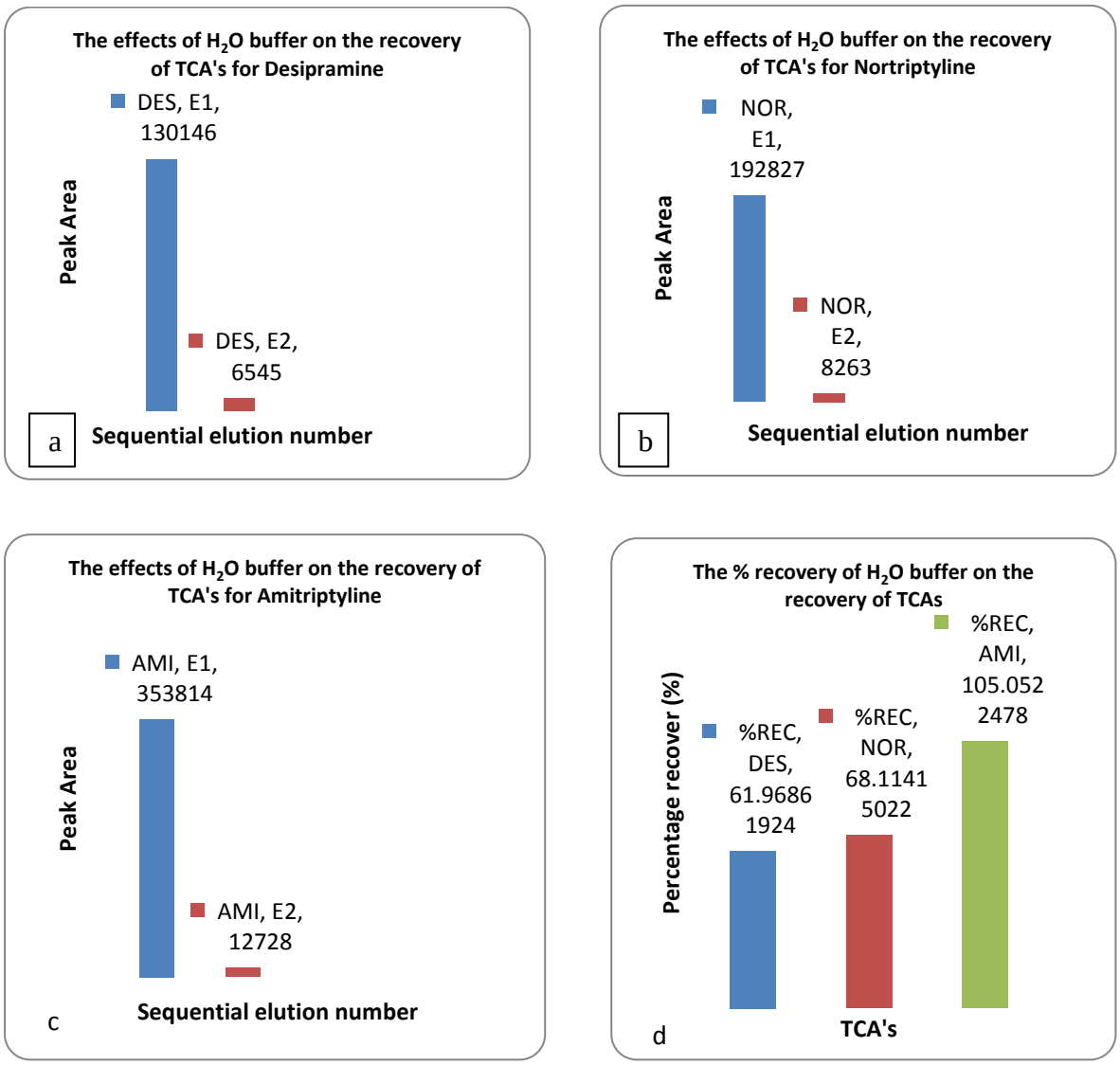


Fig. 12 (a,b&c). The recovery of TCA's following loading distil water and sequential elution using CH_3OH:NH_4OH in the ratio (96:4) E1=1.5ml, E2=0.5ml, E3=0.5ml and E4=0.5ml. The percentage recovery was calculated to be 62%, 68% and 105% for Desipramine, Nortriptyline and Amitriptyline. Of course its recoveries have always been consistent but a little bit change occurs when SPE was performed on different days.

The recovery of amitriptyline in this study was comparable to that observed by Jasinska and Starczewska[18]. In addition the recoveries obtained for this method have shown to be better than those obtained by Martinez et al[24]., and Sanchez de la Torre et al[32].

CONCLUSION

A quick and rapid method for simultaneous detection and quantification of three TCAs (desipramine, nortriptyline and amitriptyline) was developed on HPLC-UV under a gradient elution, using a C18 column and a mobile phase mixture of ACN (35% v/v) and a pH 3.5, 50mM phosphate buffer (65% v/v). The method was successfully validated and the validation parameters were suitably in accordance with ICH and FDA requirement. The validated method was successfully applied

to the analysis and extraction of whole blood with spiked TCAs using SPE technique.

This C18 method gave a satisfactory recovery when compared with all other methods and other cartridges including C2 and Strata-X under the experimental procedures. It has previously been reported using C18 and CH, for the extraction of amitriptyline, imipramine and Chlorprothixene, C18 was claimed to give the best result for amitriptyline (99.5%). It's noteworthy to state one of the significant finding in this research that slight structural differences occur among the TCAs which might account for it binding interactions, retention and recovery.

Limitations of the study

As a result of limited time for the research, some experimental procedures were not subjected to repeats which led to poor reproducibility and consistency of results and finally SPE would be done sequentially rather than simultaneously.

Further work

The eluent pH would be changed since pH of the elution solvent plays a role in the disruption of interactive force, using an acidic methanol such as (98% MeOH:2% HCl) to neutralize the silanol group rather than the basic methanol and other stronger solvents such as methylene chloride, hexane, or ethyl acetate might be tried.

It would be useful to assess other types of cartridges such as the weak cations and hydrophobic due to its ability to increase the retention of basic compounds by strong cationic exchange through its deprotonated carboxylic acid moieties which might therefore increase recovery.

REFERENCES

1. Anderson, I.M., Selective serotonin reuptake inhibitors versus tricyclic antidepressants: a meta-analysis of efficacy and tolerability. *Journal of Affective Disorders,* **58:** 19-36 (2000).
2. Asimakopoulos, A.G., Bletsou, A. A., Wu, Q., Thomaidis, N.S. and Kannan, K., Determination of Benzotriazoles and Benzothiazoles in Human Urine by Liquid Chromatography-Tandem Mass Spectrometry. *Analytical Chemistry,* **85:** 441-448 (2013).
3. Bansal, V.M.R., Pal O.P. and Sharma, P.K., High Perfromance Liquid Chromatography: A short Review. *Journal of Global Pharma Technology***,** 22-26 (2010).
4. Bielicka-Daszkiewicz, K., Hadzicka, M. and Voelkel, A., Optimization of SPE/GC/HPLC Analytical Procedure for Determination of Phenol, Quinones, and Carboxylic Acids in Water Samples. *International Scholarly Research Notices,* (2012).
5. Bones, J., Thomas, K.V. and Paull, B., Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals. *Journal of Environmental Monitoring,* 9**,** 701-707 (2007).
6. Buszewski, B., Bocian, S. and Rychlicki, G.,. Investigation of silanol activity on the modified silica surfaces using microcalorimetric measurements. *Journal of Separation Science,* **34:** 773-779 (2011).
7. Camel, V., Solid phase extraction of trace elements. *Spectrochimica Acta Part B: Atomic Spectroscopy,* **58:** 1177-1233 (2003).
8. Carlsson, B. and Norlander, B., Solid-phase extraction with end-capped C2 columns for the routine measurement of racemic citalopram and metabolites in plasma by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, **702:** 234-239 (1997).
9. Chambers, E.E., Woodcock, M.J., Wheaton, J.P., Pekol, T.M. and Diehl, D.M.,. Systematic development of an UPLC–MS/MS method for the determination of tricyclic antidepressants in human urine. *Journal of Pharmaceutical and Biomedical Analysis,* **88:** 660-665 (2014).

10. Ciraulo, D.A., Shader, R.I. and Greenblatt, D.J., Clinical Pharmacology and Therapeuticsof Antidepressants. *Pharmacotherapy of Depression.* Springer (2011).
11. Duff, K.J., Gas and Liquid Chromatography, Column Selection for, in Drug Analysis. *Encyclopedia of Analytical Chemistry*. John Wiley & Sons, Ltd (2006).
12. Dulkadir, Z., Chaturvedi, A.K., Craft, K.J., Hickerson, J.S. and Cliburn, K.D., Tricyclic Antidepressants Found in Pilots Fatally Injured in Civil Aviation Accidents (2014).
13. Eizadi-Mood, N., Akouchekian, S., Yaraghi, A., Hakamian, M., Soltani, R. and Sabzghabaee, A.M., Memory Impairment following Acute Tricyclic Antidepressants Overdose. *Depression research and treatment*, (2015).
14. Elhamili, A., Samuelsson, J., Bergquist, J. and Wetterhall, M., Optimizing the extraction, separation and quantification of tricyclic antidepressant drugs in human plasma with CE-ESI-TOF-MS using cationic-coated capillaries. *Electrophoresis*, **32:** 647-658 (2011).
15. Eugene Ramsay, R. and Wilder, B.J., Metabolism of tricyclic anticonvulsant drugs. *Epilepsy & Behavior*, **3:** S2-S6 (2002).
16. Giaginis, C., Tsantili-Kakoulidou, A. and Theocharis, S., Applying Quantitative Structure–Activity Relationship (QSAR) Methodology for Modeling Postmortem Redistribution of Benzodiazepines and Tricyclic Antidepressants. *Journal of Analytical Toxicology*, **38:** 242-248 (2014).
17. Horstmann, S. and Binder, E.B., Pharmacogenomics of antidepressant drugs. *Pharmacology & Therapeutics*, **124:** 57-73 (2009).
18. Jasińska, A. and Starczewska, B., The use of the new SPE methods for isolation of some tricyclic antidepressant drugs from human serum. *Journal of pharmaceutical and biomedical analysis*, **31:** 795-799 (2003).
19. Jasinska, A., Starczewska, B., Polkowska, M. and Puzanowska-Tarasiewicz, H.,. Solid phase extraction of olanzapine with reverse phase sorbents prior to UV and HPLC analysis. *Analytical letters*, **38:** 815-824 (2005).
20. Krystyna, P., *Solid-phase extraction for enrichment and separation of herbicides*, INTECH Open Access Publisher. (2011).
21. Langford, A.M., Bolton, J.R., Carlin, M.G. and Palmer, R., Post-mortem toxicology: A pilot study to evaluate the use of a Bayesian network to assess the likelihood of fatality. *Journal of Forensic and Legal Medicine*, **33:** 82-90 (2015).
22. Logan, B., Stafford, D., Tebbett, I. and Moore, C., Rapid screening for 100 basic drugs and metabolites in urine using cation exchange solid-phase extraction and high-performance liquid chromatography with diode array detection. *Journal of analytical toxicology*, 14**,** 154-159 (1990).
23. Madej, K., Kochana, J. and Woźniakiewicz, M., Study of SPE conditions for CE determination of tricyclic antidepressants in body fluids. *Journal of liquid chromatography & related technologies*, **30:** 185-198 (2007).
24. Martínez, M.A., De La Torre, C.S. and Almarza, E., A Comparative Solid-Phase Extraction Study for the Simultaneous Determination of Fluoxetine, Amitriptyline, Nortriptyline, Trimipramine, Maprotiline, Clomipramine, and Trazodone in Whole Blood by Capillary Gas—Liquid Chromatography with Nitrogen-Phosphorus Detection. *Journal of Analytical Toxicology*, **27:** 353-358 (2003).
25. Mercolini, L., Maridrioli, R., Finizio, G., Boncompagni, G. and Raggi, M.A., Simultaneous HPLC determination of 14 tricyclic antidepressants and metabolites in human plasma. *Journal of Separation Science*, **33:** 23-30 (2010).

26. Mutavdžić Pavlović, D., Babić, S., Dolar, D., Ašperger, D., Košutić, K., Horvat, A. J. and Kaštelan-Macan, M., Development and optimization of the SPE procedure for determination of pharmaceuticals in water samples by HPLC-diode array detection. *Journal of separation science*, 33**,** 258-267 (2010).
27. Nam, Y., Lim, C.H., Lee, H.S., Chung, S.J., Chung, Y.H., Shin, Y.K., Kim, M.G., Sohn, U.D., Kim, H.C. and Jeong, J.H., Single-Dose, Randomized, Open-Label, 2-Way Crossover Study of the Pharmacokinetics of Amitriptyline Hydrochloride 10-and 25-mg Tablet in Healthy Male Korean Volunteers. *Clinical therapeutics*. (2014).
28. Noordam, R., Aarts, N., Tiemeier, H., Hofman, A., Stricker, B.H. and Visser, L.E.,. Sex-Specific Association Of Antidepressant Use And Body Weight In A Population-Based Study In Older Adults. *Pharmaco-Epidemiological Studies On Antidepressant Use In Older Adults***,** 37 (2015).
29. Otasowie, J., Castells, X., Ehimare, U. and Smith, C., Tricyclic antidepressants for attention deficit hyperactivity disor-der (ADHD) in children and adolescents. *status and date: New, published in* (2014).
30. Pierog, J.E., Kane, K.E., Kane, B.G., Donovan, J.W. and Helmick, T., Tricyclic antidepressant toxicity treated with massive sodium bicarbonate. *The American Journal of Emergency Medicine*, **27:** 1168.e3-1168.e7 (2009).
31. Samanidou, V.F., Nika, M.K. and Papadoyannis, I.N., Development of an HPLC method for the monitoring of tricyclic antidepressants in biofluids. *Journal of Separation Science*, **30:** 2391-2400 (2007).
32. Sánchez De La Torre, C., Martínez, M.A. and Almarza, E., Determination of several psychiatric drugs in whole blood using capillary gas–liquid chromatography with nitrogen phosphorus detection: comparison of two solid phase extraction procedures. *Forensic Science International*, **155:** 193-204 (2005).
33. Soriano, T., Jurado, C., Menendez, M. and Repetto, M., Improved solid-phase extraction method for systematic toxicological analysis in biological fluids. *Journal of Analytical Toxicology*, **25:** 137-143 (2001).
34. Sundaram, H., Vijayalakshmi, N. and Srilatha, K., High Performance Liquid Chromatography and its role in identification of Mycobacteriae: An Overview. *NTI Bulletin*, **45:** 1-4 (2009).
35. Sundriyal, R. and Kumar, D.R., Depression and Life Satisfaction among Married & Unmarried Women. *IOSR Journal of Humanities and Social Science*, **16:** 33-36 (2013)
36. Wille, S.M.R., Maudens, K.E., Van Peteghem, C.H. and Lambert, W.E.E. Development of a solid phase extraction for 13 'new' generation antidepressants and their active metabolites for gas chromatographic–mass spectrometric analysis. *Journal of Chromatography A*, **1098:** 19-29 (2005).
37. Żwir-Ferenc, A. and Biziuk, M., Solid phase extraction technique–trends, opportunities and applications. *Polish Journal of Environmental Studies*, **15:** 677-690 (2006).