DEVELOPMENT OF ANALYTICAL METHODS FOR TRACE ANALYSIS OF ORGANOPHOSPHORUS PESTICIDES AND BIOGENIC AMINES IN FOOD SAMPLES

BY

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To

my parents, grandparents and family members

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LIST OF ABBREVIATIONS

OPPs	:	Organophosphorus pesticides
SBME	:	Solvent bar micro-extraction
LC_{50}-LD_{50}	:	Lethal concentration-dose to kill 50% of population
USEPA	:	United States environmental protection agency
RUP	:	Restricted use pesticides
POP	:	Persistent organic pollutant
NOAEL	:	No observed adverse effect level
LOAEL	:	Lowest observed adverse effect level
WHO	:	World health organization
OSHA	:	Occupational safety and health administration
NIOSH	:	National institute of occupational safety and health
PEL	:	Permissible exposure limit
IDLH	:	Immediately dangerous to life and health
ACGIH	:	American Conference of Industrial Hygienists
TLV	:	Threshold Limit
ADI	:	Acceptable daily intake

JMPS	:	Joint Meeting on Pesticides Residues
FAO	:	Food and agriculture organization
LE	:	Liquid extraction
LLE	:	Liquid – liquid extraction
HLLE	:	Homogeneous Liquid–Liquid Extraction
LODs/ LOQs	:	Limit of detections/Limit of quantitations
S/N	:	Signal to noise ratio
SPE	:	Solid phase extraction
SPME	:	Solid phase micro-extraction
GC	:	Gas chromatography
GC/MS	:	Gas chromatography/mass spectrometry
LPME	:	Liquid phase micro-extraction
DLLME	:	Dispersive liquid – liquid micro-extraction
HF-LPME	:	Hollow Fiber Liquid-Phase Micro-Extraction
BAS	:	Biogenic amines
HPLC/PDA	:	High pressure liquid chromatography/Photodiode array
HPLC/UV	:	High pressure liquid chromatography/Ultraviolet

CAD	:	Cadaverine
PUT	:	Putrescine
TYR	:	Tyramine
TRY	:	Tryptamine
PEA	:	2-Phenylethylamine
SPD	:	Spermidine
SFE	:	Supercritical fluid extraction
CE	:	Capillary electrophoresis
MAE	:	Microwave assisted extraction
PLE	:	Pressurized liquid extraction
ASE	:	Accelerated solvent extraction
DNS-CL	:	5-Dimethylaminonaphthalene-l-sulfonyl chloride (Dansyl chloride)
SIM	:	Selective ion monitoring
pH	:	-log the concentration of Hydrogen ions
RSD	:	Relative standard deviation
FID	:	Flame ionization detector
NPD	:	Nitrogen and Phosphorus detector

ABSTRACT

Full Name : Ayman Alawi Saeed Al-Majid

Thesis Title : DEVELOPMENT OF ANALYTICAL METHODS FOR TRACE ANALYSIS OF ORGANOPHOSPHORUS PESTICIDES AND BIOGENIC AMINES IN FOOD SAMPLES

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Analysis of trace level contaminants in complex matrices, such as food, often requires an extensive sample preparation prior to instrumental analysis. The typical steps involved in the sample preparation include sampling/homogenisation, extraction, clean-up and pre-concentration followed by the final analysis. In this context, we developed two simple analytical methods for the determination of organophosphorus pesticides (OPPs) and biogenic amines (BAs) in food matrices using two simple extraction techniques.

For the determination of OPPs in liquid food matrices a single step extraction, Solvent Bar Micro-Extraction (SBME) followed by GC/MS was developed and applied for the determination of OPPs compounds in fruit juices. Eight top priority OPPs namely o,o,o-triethyl phosphorothioate, thionazin, sulfotep, phorate, dimethoate, disulfoton, methyl parathion, and parathion were selected as target analytes. The method showed good linearity over the range (0.05–100 μg/L) with correlation determination (R^2) higher than 0.9992, and the detection limits for the pesticides studied varied from 0.7 to 44 ng/L. Repeatability studies resulted a relative standard deviation lower than 13 % in all cases. The proposed method was used to

determine pesticides levels in fruit juice samples purchased from local market and satisfactory results were obtained.

In the other part is the determination of BAs in common solid food matrices using solid-liquid extraction followed by liquid chromatography (LC) for a group of most common biogenic amines, namely; 2-phenylethylamine (PEA), tyramine (TYR), putrescine (PUT), cadaverine (CAD), tryptamine (TRY), spermidine (SPD). Those BAs are used as quality indices of spoiled food. A major challenge in the determination of BAs is the lack of UV-Vis absorption property. Therefore, to improve the sensitivity of the analyses, chemical derivatization using dansyl chloride is often required prior to analysis. For the first time, an ultrasonicated assisted extraction followed by dansyl chloride derivatization of BAs was applied to the traditional food consumed locally in Saudi Arabia. Analysis was performed using high performance liquid chromatography- ultraviolet detection (HPLC/UV). The BAs were dansylated and separated on a C_{18} column under LC isocratic elution 15 min duration, and detected by UV. This method exhibited excellent linearity for all of the analytes with correlation determination (R^2) higher than 0.9997. The limits of detection (LODs) were 5-14 µg/L. The precision results expressed as relative standard deviations (RSDs), ranged from 0.3 to 4.2 %. The results obtained showed very low amounts of BAs in the traditional food indicating its healthiness and good quality.

ABSTRACT (ARABIC)

ملخص الرسالة

الاسم الكامل: أيمن بن علوي سعيد آل ماجد

عنوان الرسالة: تطوير أساليب تحليلية لتحليل أثر المبيدات الفوسفوعضوية والامينات الأحيائية في عينات الطعام

التخصص:كيمياء

تاريخ الدرجة العلمية: أبريل 2013

تحليل كميات دقيقة من الملوثات في أوساط معقدة كالطعام عادة ما يتطلب طرق تحضير مكثفة وصعبة قبل التحليل الآلي. الخطوات التقليدية المتبعة في تحضير العينة تتضمن عادة: جمع العينات, مجانستها, استخلاصها, تنظيفها, تركيزها, فتحليلها كخطوة أخيرة. في هذا البحث, قد طورنا طريقتين تحليليتين سهلتين لقياس المبيدات الفوفسوعضوية و الامينات الاحيائية في عينات الطعام باستخدام تقنيتي استخلاص بسيطتين.

لتقدير المبيدات الفوسفوعضوية في عينات الطعام السائلة قد تم تطوير طريقة الاستخلاص الدقيق بواسطة انبوب السائل ذو الخطوة المفردة متبوعا بالتقدير بجهاز كروماتوغرافيا الغاز-مطياف الكتلة واستخدامها في تقدير المبيدات الفوسفوعضوية في عصيرات الفواكه. ثمانية من المبيدات الفوسفوعضوية ذات الاهمية القصوى وهي كل من: ثلاثي ايثيل الفوسفوروثيوات, الثيونازين, السلفوتيب, الفورات, ثنائي الميثوايت, ثنائي السلفوتون, ميثيل الباراثيون و الباراثيون اختيرت لتكون المركبات المقاسة بالتحليل. طريقة الاستخلاص المستخدمة اظهرت معامل استقامة ممتاز (0.5 – 100 ميكروجرام/لتر) وحدود كشف للمبيدات المدروسة (0.7 – 44 نانوجرام/لتر). دراسة التكرارية نتجت عن حيود عياري نسبي اقل من 13% في جميع الحالات. الطريقة المقترحة استخدمت لتقدير كمية المبيدات في عينات عصيرات الفواكه المشتراة من السوق المحلي ونتائج التحليل كانت مرضية.

الجزء الثاني هو تقدير الامينات الاحيائية في عينات الطعام الصلبة بواسطة عملية الاستخلاص الصلب – السائل متبوعا بالكروماتوغرافيا الغازية. الامينات الاحيائية مثل: 2-فينيل ايثيل امين, تايرامين, بوتريسين, كادافرين, تريبتامين, و سبيرميدين, وجد انها ذات فائدة كمرجع لمدى فساد الطعام. لأول مرة بالمملكة العربية السعودية يتم تطبيق الاستخلاص المحفز بالموجات الفوق صوتية ويليه الاشتقاق بواسطة كلوريد الدنسيل للامينات الاحيائية على عينات الطعام التقليدي المستهلك في المملكة. تم التحليل بواسطة جهاز كروماتوغرافيا السائل ذات الاداء العالي والكشف بالاشعة فوق البنفسجية. تم اشتقاق (دنسلة) الامينات الاحيائية وفصلها في عمود كروماتوغرافيا (C18) تحت ظروف كروماتوغرافية سائلة مفردة وتم الفصل خلال 15 دقيقة. هذه الطريقة أظهرت معامل استقامة ممتاز لكل الامينات وكذلك معامل تقدير اعلى من 0.9997 و حدود الكشف كانت بين 4-15 ميكروجرام/لتر والدقة كانت عالية بناء على نتائج الحيود العياري النسبي والتي كانت بين 0.3- 4.2 %. نتائج التحليل اظهرت وجود كميات قليلة من الامينات الاحيائية في عينات الاطعمة التقليدية التي تمت دراستها مما يدل على جودتها وعدم تعرضها للفساد.

CHAPTER 1

INTRODUCTION

Recently, organophosphorus pesticides (OPPs) have become the most commonly used pesticides in the world. They are more potent in killing pest and at the same time less persistent in the environment than conventional pesticides such as dichlorodiphenyltrichloroethane (DDT), however, their main drawback is they are much more toxic to humans. More specifically, they are neurotoxin as they act directly on the central nervous system, inhibiting the expression of the acetylcholinesterase enzyme. Considering their wide spread use and non-controlled availability to the public they expose serious threat to individuals. Applications of OPPs by non-professional individuals may lead to a presence of OPPs residues in agricultural products and eventually reaching to human through the food chain. Therefore, it is important to develop simple and sensitive analytical methods to quantitate OPPs in food samples.

Considering the nature of the analytical techniques, they are not directly applicable to complex sample matrices. Thus, we developed, a solvent bar microextraction (SBME) technique using a thick hollow fiber membrane for determination of OPPs from food matrices.

The SBME conditions were optimized for achieving high enrichment of the analytes from fruit juice samples, such as extraction time, sample pH, and sample volume. The optimized method was applied to the determination of OPPs in fresh fruit juices available in the market.

Biogenic Amines (BAs), are nitrogenous low molecular weight organic bases of aliphatic, aromatic or heterocyclic structures that are synthesized and degraded during the cellular metabolism activities in microorganisms, plants and animals [21]. BAs are usually formed by decarboxylation of free amino acids by removal of the alpha-carboxyl group from a proteinogenous amino acid leads to the corresponding biogenic amine. The names of many biogenic amines correspond to the names of their originating amino acids. Diamines, Cadaverine (CAD) and Putrescine (PUT), aromatic amines Tyramine (TYR) and 2-Phenylethylamine (PEA), hetrocyclic amine Tryptamine (TRY) and poly amine Spermidine (SPD), are important BAs. BAs importance raised from their toxicity and their usage as an indicator for the degree of freshness or spoilage of food [6,7]. They can be found in a wide variety of foods at low concentration in non-fermented food (e.g., milk) and with a high concentration in fermented food (e.g., cheese) [8, 9].

BAs are precursors of carcinogenic nitrosamine and can't be removed or even reduced by high temperature treatment [10, 11]. Although consumption of low levels of biogenic amines in food is not considered a serious risk, however, if the amount consumed is high enough or normal routes of amine catabolism are inhibited, then, various physiological side effects may appear such as hypotension, hypertension, and death in very severe cases [12].

Usually BAs are solid compounds and present in complex matrices and they need to be extracted before analysis to avoid any interferences in quantitative or qualitative determinations.

Therefore, in this research we proposed ultrasonic assisted liquid extraction and phase separation if it's feasible for BAs in food samples. After extraction was done it was followed by derivatization to enhance the sensitivity of the detection. High performance liquid chromatography with photodiode array detection system (HPLC/PDA) was used for separation, detection and determination of BAs.

1.1. Literature review and current techniques of determination of OPPs

OPPs are organic derivatives of the phosphoric acid; they are used to combat crop pests, plant diseases, ectoparasites of domestic animals (body parasites), weeds, insects, mites, and ticks [20].

OPPs have become one of the most widely used classes of pesticides in the world [1]. Most organophosphates are insecticides [27]. They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932. Some are very poisonous (they were used in World War II as nerve agents) [27].

OPPs are very toxic to human due to its effect of de-activation of acetyl-cholinesterase [2] in the nervous system with subsequent accumulation of toxic levels of acetylcholine. This class possesses acute toxicity against a wide variety of insects and arthropods. They also present a relatively low persistence in the environment, which represents great progress in comparison to the organochlorine pesticides.

OPPs have low stability in the water, and accumulate in living organisms. Most of them decompose after long period of time, with the formation of nontoxic products such as phosphoric acid, carbon dioxide, and water. Their disadvantages include the relatively high toxicity of many such pesticides for humans and animals, which necessitates the observation of safety precautions during application [20].

Some compounds have also been observed to accumulate in adipose tissue, but they decompose within a few days or weeks [24].

O,O,O- Triethylphosphorothioate, Thionazin, Sulfotep, Phorate, Dimethoate, Disulfoton, Methyl parathion and Parathion are some members from the large family of OPPs and most commonly used pesticides in agriculture. These pesticides if accumulated in the food chain can pose a serious threat to both humans and animals [1].

The incorrect use of these pesticides may lead to a presence of these residues in agricultural products and reaching to human through the food chain. For environmental and drinking water, the maximum allowed concentration of single compound established by European Union (EU) is 0.1 µg/L, and 0.5 µg/L is the maximum allowed for the total concentration of all OPPs [5].

LC_{50} (Lethal Concentration that kills 50% population) for rats by inhaling

O,O,O- Triethyl phosphorothioate is 41 mg/L for 4hours [33].

In the case of Thionazin the LD_{50} (Lethal Dose that kills 50% population) for rats by oral route is 3.5 mg/kg [34].

The acute toxicity of Sulfotep is high, with 4h LC50 values in rats and mice of 40 - 60 mg/m3, oral LD50 values of about 5 - 15 mg/kg body weight (rat), about 25 mg/kg body weight (mouse, rabbit) and dermal LD50 values of 250 - 262 mg/kg body weight (rat, 4h) and is about 25 - 70 mg/kg body weight (rat, substance not removed) [51].

According to U.S. Environmental Protection Agency (USEPA) recommendations Disulfoton contamination in drinking water should not exceed 3 µg/L for children, or 9 µg/L for adults. Over a lifetime, average contamination should not surpass 0.3 µg/L [27]. Pesticide formulas with greater than 2% Disulfoton are classified as Restricted Use Pesticides (RUP), making them available only to certified applicators [27]. Refer to table 1 for some more toxicity data.

Phorate has been canceled and it is labeled as "HIGHLY TOXIC - POISON" chemical and as a RUP which allows only certain applications for use. It is not listed as a UNEP Persistent Organic Pollutant (POP) and is registered for use in around half of the world's countries including the US, Canada, the UK, Australia, New Zealand, and India among others [27, 28]. Table 1 shows No Observed Adverse Effect Level (NOAEL) and Lowest Observed Adverse Effect Level (LOAEL) for acute and chronic exposure of some OPPs in rats.

Table 1 (LOAEL) and (NOAEL)

OPPs Conc.	Phorate	Disulfoton	Methyl parathion	Parathion
LOAEL acute	-	0.75 mg\kg\day	0.53 mg\kg	0.25 mg\kg\day
NOAEL acute	0.25 mg\kg\day	0.25 mg\kg\day	0.11 mg\kg	0.1 mg\kg\day
LOAEL chronic	-	0.013 mg\kg\day	0.21 mg\kg	-
NOAEL chronic	0.05 mg\kg\day	0.094 mg\kg\day	0.02 mg\kg	0.04 mg\kg\day

Parathion is extremely toxic from acute (short-term) inhalation, oral, and dermal exposures. Acute exposure of humans to parathion mainly affects the central nervous system and its human carcinogenic according to World Health Organization (WHO). Chronic (long-term) inhalation and oral exposure of humans and animals to parathion have been observed to result in depressed cholinesterase inhibition, nausea, and headache. A dose of 1.6 mg\kg\day of Parathion has been given to rats for 2 years, caused tumors and reduction in body weight gain [27]. Refer to table 1 for more toxicity data [35].

According to Occupational Safety and Health Administration (OSHA), Permissible Exposure Limit (PEL) = 0.1 mg/m³ (skin) (averaged over an 8-hour work shift) [29] and according to National Institute of Occupational Safety and Health (NIOSH), Immediately Dangerous to Life or Health (IDLH) = 10 mg/m³ for Parathion [30].

According to American Conference of Industrial Hygienists (ACGIH), the Threshold limit value (TLV) for Methyl parathion is 0.2 mg/m³, where TLV is defined as the time-weighted average concentration for a normal 8-hour workday and a 40-hour workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect [31].

For Dimethoate, USEPA has stated the LD_{50} for acute rat poisoning per oral as value of 358 mg\kg and the doses that inhibits 10% of brain cholinesterase in female rats are: 1.5 mg\kg in acute cases and 0.25 mg\kg in chronic cases [27].

Acceptable Daily Intake (ADI) for Dimethoate is 0.01 mg/kg of body weight, which has been decided in the Joint Meeting on Pesticides Residues (JMPR) of Food and Agriculture Organization/World Health Organization (FAO/WHO) in 1987 [32].

All OPPs are subject to degradation by hydrolysis, yielding water-soluble products that are believed to be non-toxic at all practical concentrations. The toxic hazard is therefore essentially short-term in contrast to that of the persistent organochlorine pesticides, although the half-life at neutral pH may vary from a few hours for Dichlorvos to several weeks for Parathion. Degradation in the environment involves both hydrolysis and oxidation to mono- or di- substituted phosphoric or phosphonic acids or their thio analogues.

Analytical instruments are not suitable for the direct analysis of OPPs at trace level in fruit juices samples. Sample preparation techniques are required prior analysis. The most common sample preparation methods for the determination of OPPs in aqueous samples are:

Liquid–Liquid Extraction (LLE), also known as solvent extraction and partitioning, which is commonly used to separate compounds based on their relative solubility in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase, figure 1illustrates LLE (source: http://faculty.ksu.edu.sa).

Homogeneous Liquid–Liquid Extraction (HLLE) is new method in LLE was reported in literature were acetone was used as extraction solvent for the extraction of target OPPs from soil samples. Linearity was obtained in the range of 0.04–50 μg/kg. Coefficients of Correlation (r^2) ranged from 0.9993 to 0.9998. The Limits Of Detection (LODs), based on Signal-to-Noise ratio (S/N) of 3, varied between

0.01 and 0.04 μg/kg. The relative recoveries of three pesticides from soil were in the range of 77.10 and 110.5% [36].

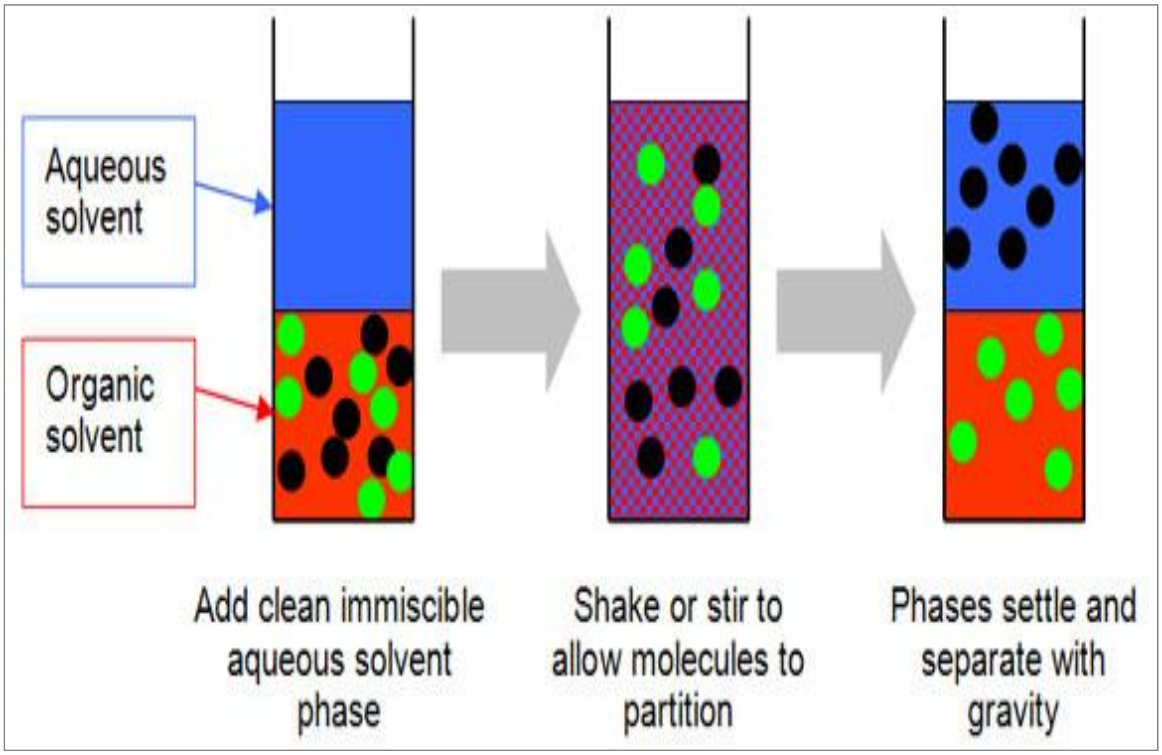

Figure 1 Illustration of simple LLE

Solid Phase Extraction (SPE) which is a broad term used to describe the separation technique in which liquids contact modified solid surfaces and target analytes in the liquid adheres to the solid. Latter, the absorbed analytes were released using organic solvents is in figure 2 (source: www.gerstelus.com).

Recently, continuous SPE with gas chromatographic analysis for the determination of OPPs has been reported in the literature [37]. The continuous system consists of an adsorbent column where pesticides are preconcentrated and subsequently eluted with solvent i.e. ethyl acetate. Various sorbent materials can be used and RP-C_{18} was found to provide the best results, with sorption efficiency close to 100%. LOD were between 50–130 ng/l and with good precision (2.9–4.3%) and recoveries ranging from 93.8 to 104.5% [37].

LLE and SPE techniques need considerable amount of organic solvents for extraction and are time and labor consuming [1, 3].

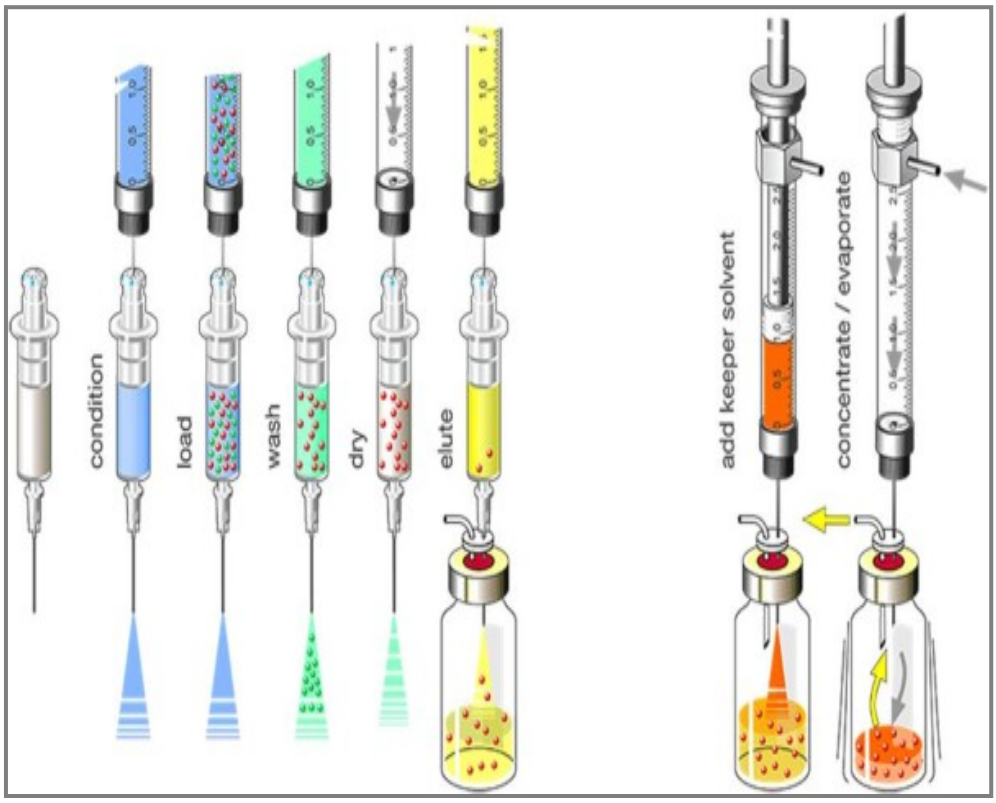

Figure2 SPE process in steps

Solid Phase Micro-Extraction (SPME) is solventless extraction technique suitable alternative for LLE and SPE. In SPME, fused silica or stainless steel fiber coated with a thin film polymer, which acts as the solvent during the extraction of compounds [25]. The schematic diagram is shown in figure 3 (source: www.accessscience.com).

SPME suitable for simultaneously extract and concentrate the analyte from the sample media[1,4]. SPME was reported for the determination of 18 OPPs in textiles. Commercially available SPME fibers, 85 microm polyacrylate (PA) exhibited good performance for the OPPs. The optimized conditions using SPME were: 35 min extraction at 25 °C, 5% NaSO4 content, pH 7.0, and 3.5 min desorption in Gas Chromatography (GC) injector port at 250 °C. The LODs ranged from 0.01 µg/L to 55 µg/L and the RSDs were between 0.66% and 9.22% with recoveries were ranged from 76.7% to 126.8% [38].

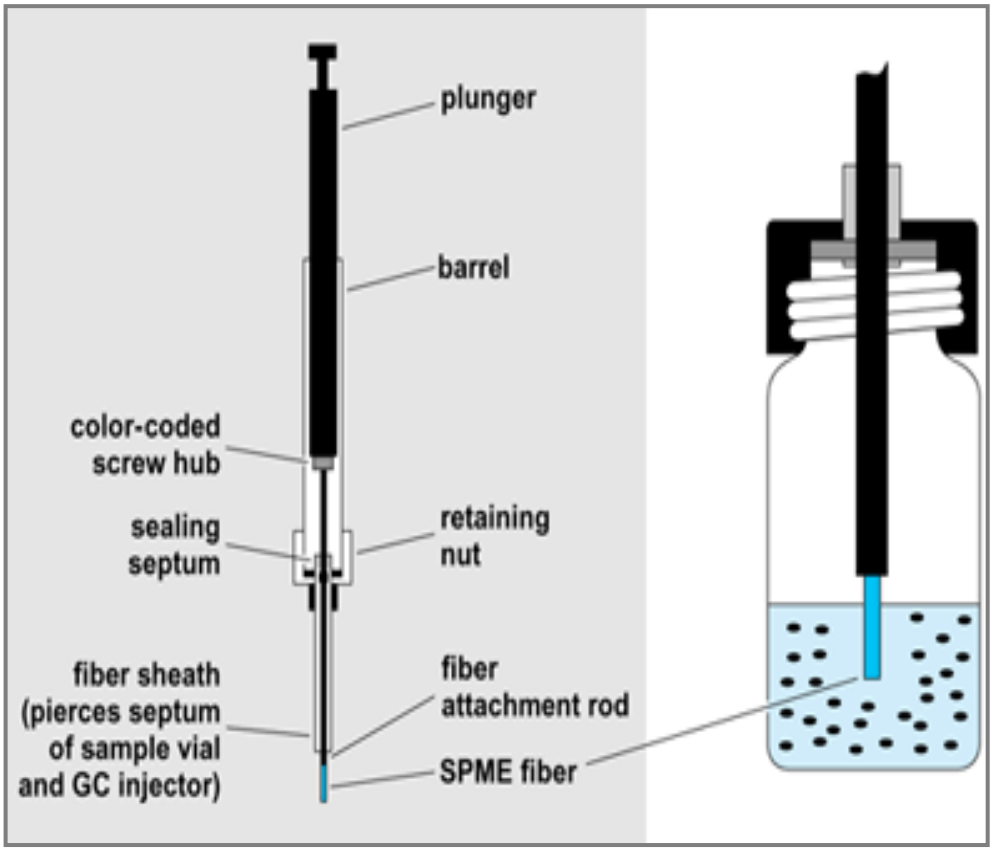

Figure3 SPME device and its components

Liquid Phase Micro-Extraction (LPME), is a solvent minimized technique. It miniaturized implementation of conventional (LLE) in which only microliters of solvents are used instead of several hundred milliliters in LLE [22].

This extraction technique is faster and easy to use, better than SPME approach where we have to use expensive fibers [5].

Various approaches using LPME were reported with different modification to enhance its performance.

Hollow Fiber supported liquid phase micro-extraction (HF-LPME) -figure 4 (source: www.accessscience.com)- technique was reported to be used as a clean-up procedure for the determination of OPPs in fish tissue. Under the optimum conditions, good linearity were observed in the range of 20-500 ng/g, LODs were in the range of 2.1- 4.5 ng/g. The repeatability and recovery of the method also showed satisfactory results.

Compared with traditional sample preparation method for the determination of OPPs in fish tissue, the HF-LPME eliminated the SPE step, simplified the sample preparation procedure and lowered the cost of analysis [40].

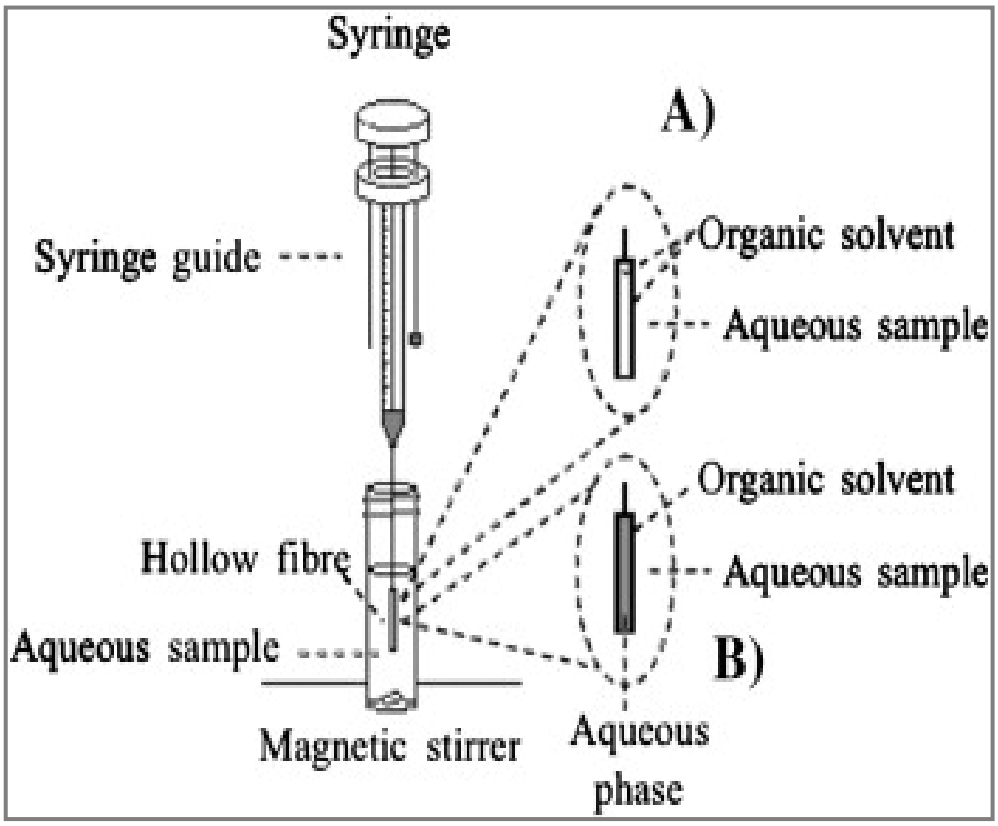

Figure4 Scheme of HF-LPME

Dispersive Liquid–Liquid Micro-Extraction (DLLME), figure 5 shows DLLME mechanism (source: www.accessscience.com). Combined with gas chromatography flame photometric detection was reported to be used for extraction and determination of OPPs in soil samples with such volumes as low as 20 μL Chlorobenzene as the extraction solvent; 1.0 mL Acetonitrile as the disperser solvent. Under the optimum conditions, the linearities for the three target OPPs are obtained by five points in the concentration range of 2.5–1500 μg/kg. The limits of detection, based on a signal-to-noise ratio (S/N) of 3, range from 200 to 500 pg/g [39].

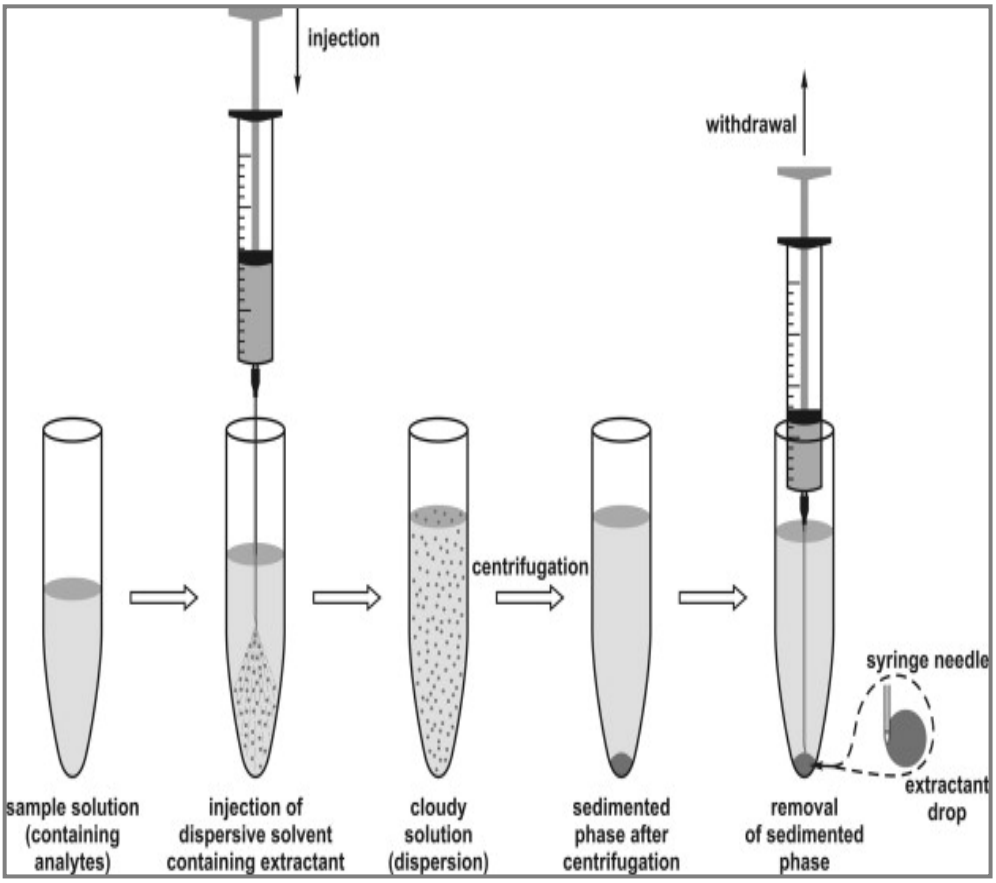

Figure5 Mechanism of DLLME

In LPME and HF-LPME methods extraction solvents were placed into the water samples via 10 μL syringe as shown in figures 4 and 5. In both LPME techniques, samples were extracted one by one and simultaneous extraction of multiple samples is not possible. To increase the throughput of the extraction we introduced technique called solvent bar moicroextraction (SBME). In this method, the organic solvent is confined within a short length of a polypropylene hollow fiber, sealed at both ends. This solvent bar can be directly placed into the sample solution for extraction. Due to the vigorous tumbling of the solvent bar in the agitated sample solution, mass transfer between the organic phase and aqueous phase is facilitated, thus resulting in higher extraction efficiency [23]. Most commonly used membranes in LPME are polypropylene (PP) mostly with 0.2 μm pore size 200 μm thickness and polysulfone with same specifications.

In this study, for the first time in Saudi Arabia we proposed a 500 μm thick membrane for the SBME technique. Thicker membrane prevents the solvent from escaping in long time extraction processes and will serve as a better filter for clean extracted solutions. SBME is an alternative approach in which the extraction solvent tumbled freely in sample solution. The main advantage of SBME is that no extraction syringe required and useful for high throughout analysis. The SBME conditions was optimized for achieving high enrichment of the analytes from fruit juice samples, such as extraction time, sample pH, addition of salt, extracting solvent and sample volume. The optimized method was applied and samples were injected in GC/MS for the determination of OPPs in fresh fruit juices available in the market.

1.2. Literature review and current techniques of determination BAs

BAs naturally occur in food as a result of microbial decarboxylation of corresponding amino acids in plants and animals [7]. BAs importance raised from their toxicity and their usage as an indicator for the degree of freshness or spoilage of food [6,7]. They can be found in a wide variety of foods with low concentration in non-fermented food (e.g., milk, fruits, vegetables) and with a high concentration in fermented food (e.g., cheese and soybeans containing food) [8,9]. BAs are precursors of carcinogenic nitrosamine and can't be removed or even reduced by high temperature treatment [10,11]. Although consumption of low levels of biogenic amines in food is not considered a serious risk, however, if the amount consumed is high enough or normal routes of amine catabolism are inhibited, then, various physiological side effects may appear such as hypotension (in the case of Putrescine and Cadaverine), hypertension (in the case of Tyramine), rash, dizziness and cardiac palpitation and death in very severe cases [12]. Putrescine and Cadaverine are known to enhance histamine toxicity by inhibiting histamine metabolizing enzymes such as monoamine or diamine oxidase and in addition to Spermidine they may react with nitrite to form carcinogenic nitrosoamines [55]. Tryptamine, β-phenylethylamine, and Tyramine are biologically active amines which have important physiological effects in humans, generally either psychoactive or vasoactive. Psychoactive amines affect the nervous system by acting on neural transmitters, while vasoactive amines act on the vascular system [56].

For Tyramine, Putrescine, and Cadaverine; the (NOAEL) is 2000 ppm. Tyramine alone at high levels can be toxic and cause a reaction known as the cheese reaction [52].

For Spermidine the (NOAEL) is 1000 ppm and the approximate LD_{50} is 600 mg/kg in rats [53]. In the case of Tryptamine which stated that it has toxic effects on human beings such as hypertension, however there is no regulation on the maximum amount of Tryptamine consumption in some food such as sausage in some countries[54].30 mg/kg for β-Phenylethylamine have been reported as toxic doses in foods [57].

The following arethe precursors of the studied biogenic amines: Phenylalaninegives β-Phenylethylamine, Tyrosine gives Tyramine, Tryptophane gives Tryptamine, lysine gives Cadaverine, Ornithine gives Putrescine, and Arginine gives Spermidine [56, 57].

Many sample extraction and clean up techniques have been reported in literature which includes solid-liquid extraction followed by SPE cleanup [14, 15]. Supercritical Fluid Extraction (SFE) which is gas based sample preparation method uses liquid such as compressed Carbon Dioxide as an extracting phase that's capable of removing less volatile compounds at ambient temperatures [25]. Figure 6 is a schematic diagram of SFE instrument (source: http://www.scielo.br).

SFE was reported to be used for extraction of a group of non-polar heterocyclic amines in commercial meat samples. The method was combined Capillary Electrophoresis (CE) separation and fluorescence detection.

Linear responses was in the range from 100 to 1000 ng/mL and LODs ranging from 15.9 to 28.1 ng/mL were obtained for different amines in less than 13 min. Laser induced fluorescence detection enhances the sensitivity and avoids interferences coming from non-fluorescent compounds present in the matrices of the sample extracts [41].

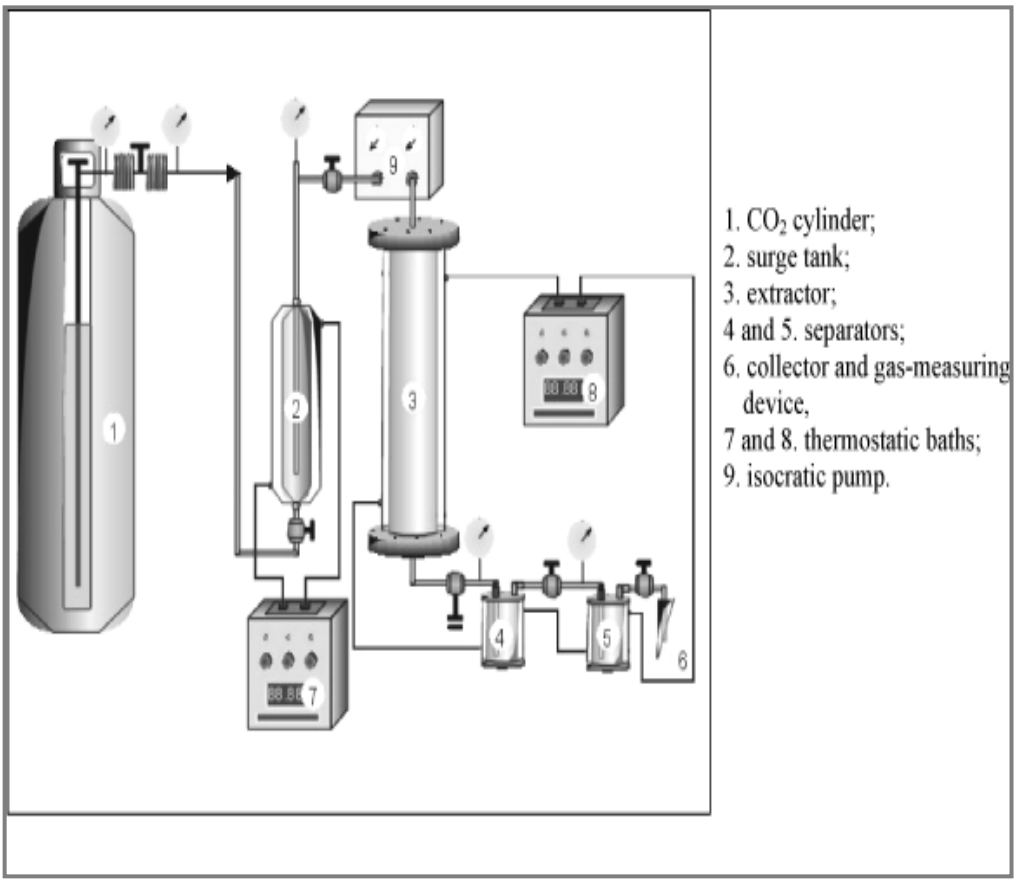

Figure6 Schematic diagram of SFE instrument and process

Microwave-Assisted Extraction (MAE) is another technique used to extract BAs from solid samples. In MAE, sample is held in a confined vessel with the solvent and the microwave is assisting in the extraction process [26]. Figure 7 shows a type of microwave used for this purpose (source: www.labonline.com.au).

A fast microwave-assisted dansylation procedure has been reported for the derivatization of biogenic amines prior to HPLC determination. BAs are quantitatively dansylated in 5 min using a radiation power of 252 W and a maximum pressure of 3.4 bars inside the reactor. As compared to classical longtime dansylation, the microwave-assisted procedure is faster. The method was successfully applied to the determination of the above amines in plant tissues [42].

Figure7 A microwave with reaction vessels inside

Pressurized Liquid Extraction (PLE) is a liquid extraction technique suitable for the application of solid samples. In PLE samples are confined in a closed steel vials and the solvent is forced under pressure (3000 psi) into the vials to extract the targeted analyts. The solvent can be held for predetermined time (static mode) or let freely disperse from the vial (dynamic mode). Heat is usually applied which increases target compound solubility, solvent diffusion rate and mass transfer, while solvent viscosity and surface tension decrease. This method can be automated which then can be called Accelerated Solvent Extraction (ASE) [26] as in figure 8 (source: www.kilu.lu.se).These techniques are usually coupled with analytical methods such as CE [17], HPLC [18], and GC [16] or even the basic Thin Layer Chromatography (TLC) [13] for application of wide range analysis.

Figure8 Pressurized liquid extraction system

Our proposal for BAs analysis was based on separation of the solid BAs from the solid samples. No much work has been done on the extraction of BAs in solid samples using PLE. Extraction of BAs using PLE is a challenging task, due to complexity of food samples and its concentration range. Additionally, BAs have very weak UV absorption characteristics, derivatization must be applied to enhance the UV absorption thus the detection and the sensitivity.

5-Dimethylaminonaphthalene-l-sulfonyl chloride, known as Dansyl chloride (DNS-C1) is one of the most popular agents used for derivatization of BAs [19]. For the derivatization procedure, additional time and temperature are required. Our initial attempt using PLE for simultaneous extraction and derivatazation of BAs was not successful for the following reasons:

Extraction using acidic pH was required, the derivatization step with dansyl required alkaline pH conditions and pressure was exceeding the safe limits in the PLE instrument.

Based on the above discussion and other challenges of instrumental extraction procedure, we decided to use simple solid - liquid extraction assisted by ultrasonication.

This method is very simple, costly instruments not required and suitable for multiple sample extraction at the same time.

CHAPTER 2

OBJECTIVES

The main objective of this research work was to develop simple analytical methods for the determination of OPPs in fruit beverages and BAs from common traditional food samples consumed in gulf area. The research work has been classified as two parts:

Part I was focused on the developing and optimizing porous membrane assisted microextraction procedure namely Solvent Bar Micro-Extraction (SBME) technique combined with GC/MS analysis. After the method evaluation, the method was applied to monitor the concentrations of OPPs in fruit beverage samples. Part II was devoted to the determination of BAs in traditional solid and wet food samples that are consumed in the gulf area. Since the samples are solid, an ultrasonic assisted solid - liquid extraction method was developed. After extraction, the analyts were derivatized with dansyl chloride (DNS-Cl) (to improve the sensitivity toward detection) and analyzed using HPLC-PDA. The analytical performance of the both method was compared with conventional methods reported in the literatures.

CHAPTER 3

EXPERIMENTA DESIGN FOR OPPs

3.1. Chemicals and preparation of solutions

All mentioned chemicals in the research are analytical grade purity unless otherwise stated:

a) M Hydrochloric acid: 86 μL of HCl purchased from J.T. Baker,(New Jersey, USA)were diluted with distilled water to 100 mL used for the pH adjustments.
b) 0.1 M Sodium hydroxide: 0.4 g NaOH purchased from Fluka, (St. Louis, , USA) was dissolved in distilled water and diluted up to 100 mL with recently boiled distilled water for the pH adjustments.
c) Sodium chloride: 3, 5, 10, 20 g NaCl purchased from Fisher scientific, (Pittsburgh, USA) were dissolved in distilled water and diluted up to 100 ml to give 3, 5, 10, and 20 % solution respectively, for salt effect parameter.
d) Analytical grade solvents n-Nonane, Toluene, Xylene mixture of isomers, 1-octanol and Carbon tetrachloride all purchased from Fluka.
e) A stock solution of 200 mg/L of Triethyl phosphorothioate, Thionazin, Sulfotep, Phorate, Dimethoate, Disulfoton, Methyl Parathion and Parathion (Ethyl

Parathion) as the OPPs mixture was prepared by 10 times diluting the ready mixture of 2000 mg/L purchased from Suppelco (Pennsylvania , USA) in 10 mL volumetric flask. Working range standards were prepared at variety concentration by diluting with HPLC grade methanol purchased from fluka and the solutions were sealed tightly and kept refrigerated under 4 °C. Chemical structures of OPPs used in this study are shown in figure 9.

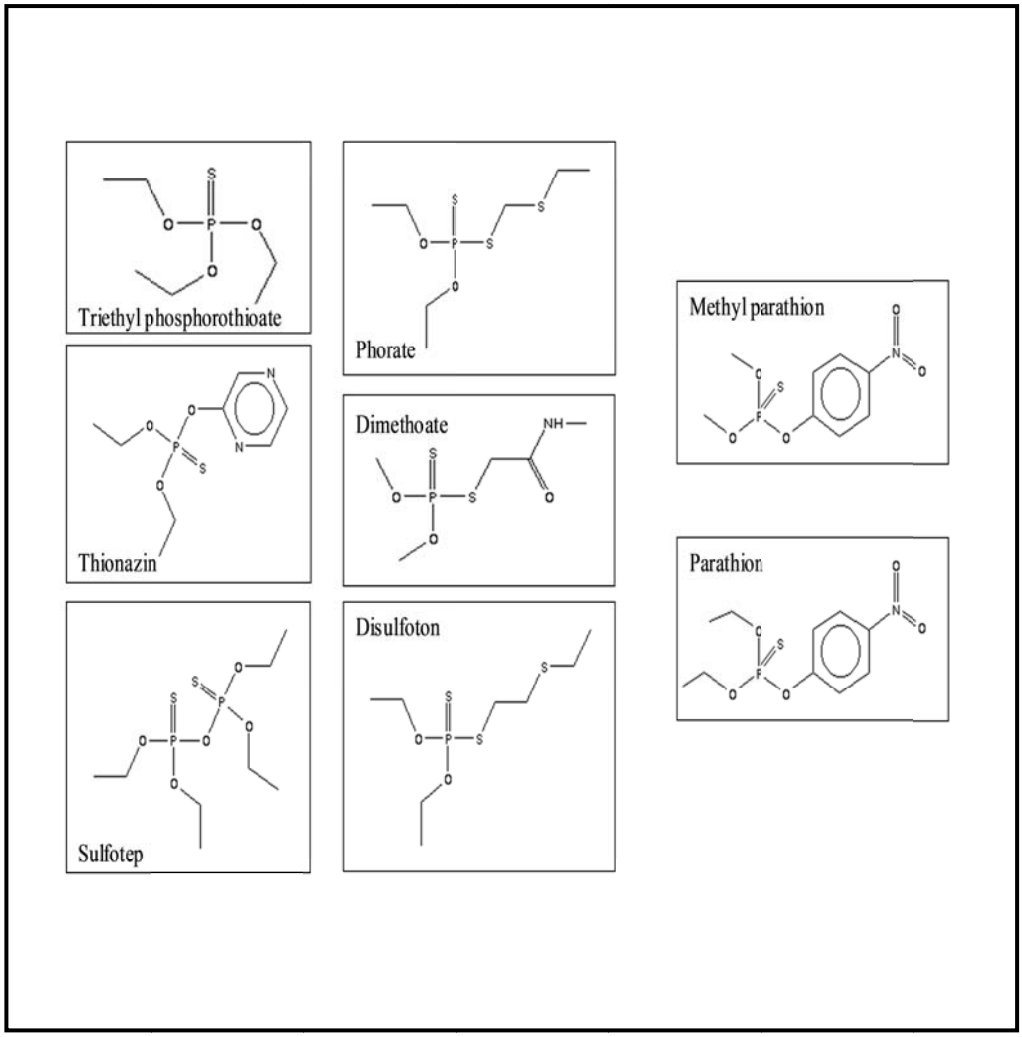

Figure9 Structures of OPPs used in this study

3.2. Properties of membrane

Accurel® hydrophobic Polypropylene capillary membranes capillary membrane were purchased from membrana, (Wuppertal, Germany).

Accurel® PP capillary membranes are also typically used in heavy-duty applications for filtration of chemically or mechanically aggressive media which is the main reason for our selection for the extraction of complex fruit beverage samples.

The fiber was type V8/2 HF, nominal pore size was 0.2 μm, wall thickness was 1550 μm and the inner diameter was 5500 μm.

3.3. Methodology

For the first time SBME technique was developed in Saudi Arabia. Polypropylene hollow fiber membrane was cut into a small pieces (2cm) and sealed from both ends (serves as the solvent bar) for the extraction of OPPs.

Each membrane was cut into length of 2 cm and extra care was taken to make the SBME device into uniform size.

200 μl of the selected solvent was filled inside the fiber and then the fiber was sealed with Gas Chromatograph (GC) injection port septum or with hot forcipes carefully and avoiding it touches the solvent. Then SBME device is ready to be used for extraction.

Various parameters of extraction such as extraction time, pH condition, salt percentage, sample volume and solvent type were optimized. Experimental setup was shown in figure 9.

Calibration graph was constructed using diluted solutions of high purity OPPs standard. Recovery tests were carried out for validating the method accuracy.

Fresh fruit juices were purchased from local market. Sample was be mixed for each batch, randomized and divided for the extraction process and then used for separation and determination of the OPPs by using Gas chromatography/ mass spectrometry (GC/MS). Table 2 shows the GC/MS analysis conditions in the Selective Ion Monitoring (SIM) mode for each compound.

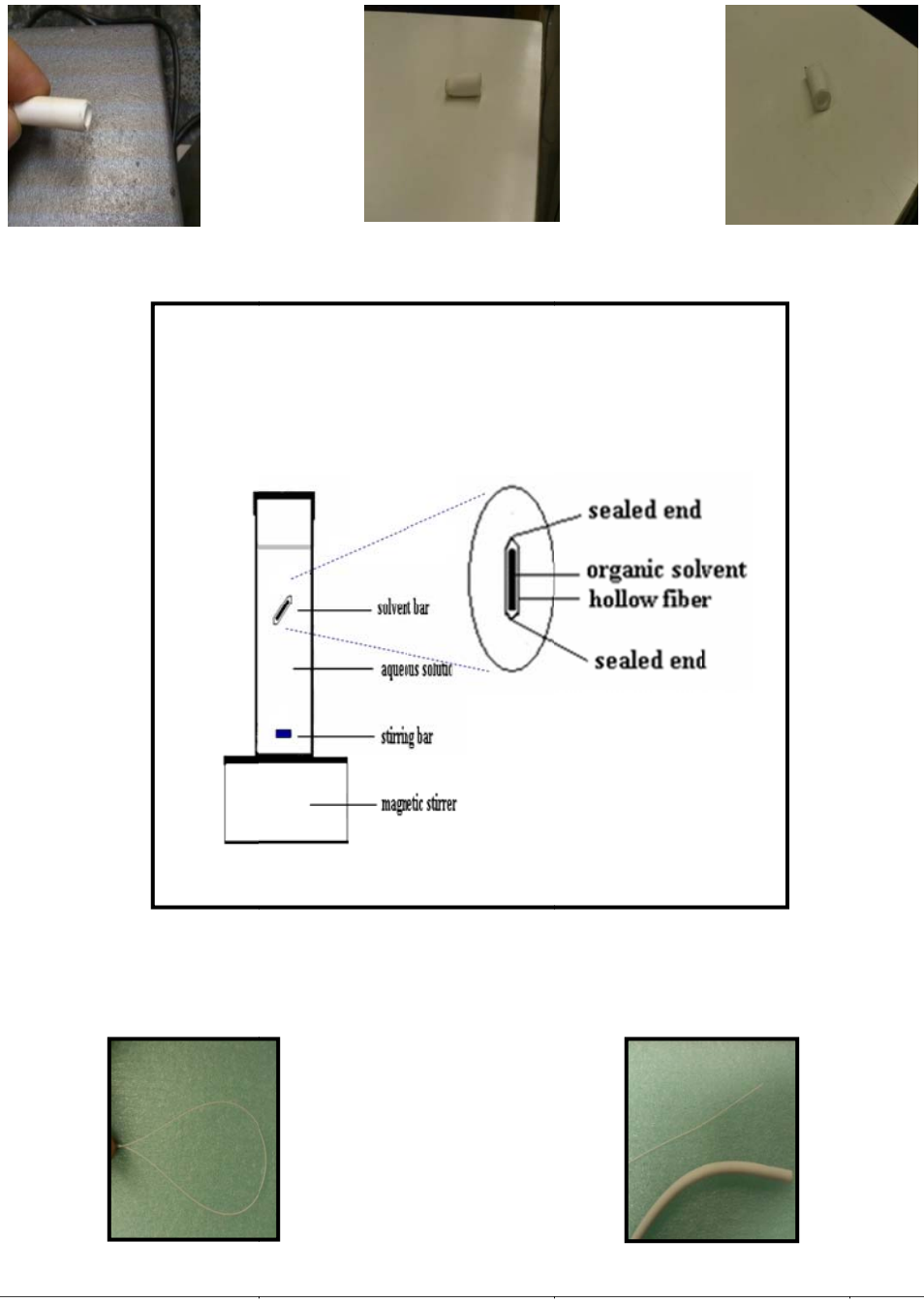

Figure10 SBME setup that shows the polypropylene fiber and its contents

3.4. Instrumentation

The instrument used in the experiment consists of GC system from Agilent technologies 6890N with Autosampler 7683B Series injector equipped with 10 ul syringe. HP-1 fused silica column from Agilent technologies (30 m x 0.32 mm -1 um film- (-60 ºC- 325 ºC)) was used for the separation of OPPs. GC was coupled with5975B MS system with EI/CI operational modes. Software used was Enhanced ChemStation.

The GC/MS conditions were optimized as the inlet temperature was set on 250 ºC and the sample volume injected to the port was 2 µL (direct). Carrier gas used was Helium and its flow rate was 1.5 mL/min. The GC oven temperature was programmed in two ramps, first ramp started at 60 ºC and ended at 250 ºC at rate of 30 ºC min^{-1}, and the second ramp was starting at 250 ºC and ended at 280 ºC at rate of 15 ºC min^{-1} for 2 minutes. Post run was applied for 6 minutes at 280 ºC. Programmed solvent delay was applied for 3.8 minutes to preserve the MS quaderpole. The Aux MSD temperature was 250 ºC and the MSD Quad temperature was 150 ºC and finally the MSD source temperature was 230 ºC. Table 2 shows details for the selective ion monitoring (SIM) mode, the * sign indicates the molecular ion.

Table 2 Details of (SIM) mode with indication of the molecular ion

OPPs	RT (min)	Molecular weight (g/mol)	Primary ion	Secondary ion
Triethyl phosphorothioate	4.532	198.22	198*	121, 154, 170
Thionazin	6.762	248.239	97	143, 192, 248*
Sulfotep	7.061	322.30	322*	294, 266, 202
Phorate	7.103	260.38	75	260*, 231, 153
Dimethoate	7.21	229.28	87	125, 237, 281
Disulfoton	7.677	274.404	88	274*, 186, 142
Methyl Parathion	7.937	263.21	263*	109, 79, 200
Parathion	8.287	291.3	291	139, 155, 235

CHAPTER 4

RESULTS AND DISCUSSION OF THE OPPs

4.1. Optimization

4.1.1. Extraction profile

Extraction time profile was studied in a sample volume of 20 mL which was spiked with 20 µg/L OPPs mixture. The SBME device containing 200 µL of extraction solvent was placed in the sample. After extraction, a GC micro syringe was inserted in the SMBE device and withdraw 2 µL of extraction into the syringe and then injected into the GC/Ms for analysis. SBME device was set to tumble freely in a stirred solution. Various immiscible solvents were tested, however, except 1-Octanol all other solvents; Xylene, Toluene, Carbon tetrachloride and n-Nonane were not successfully retained more than 40 minutes. As its noticed from figure 11, 1-Octanol turned out to have a lot of interferences as an extracting solvent which may be caused by the fragmentation pattern of the solvent and its interference effect on the OPPs fragmentation pattern. Carbon tetrachloride was having the poorest extracting efficiency due to its high density which caused the fiber to drop in the bottom of the sample compartment and not tumble freely. N-Nonane was better than Carbon

tetrachloride, yet is still poor in extracting efficiency which might be from its nature as a normal alkane and poor dissolving ability of OPPs. Toluene and Xylene were having almost same efficiency in extracting with time due to their resemblance though Xylene shows higher extraction capacity than toluene.

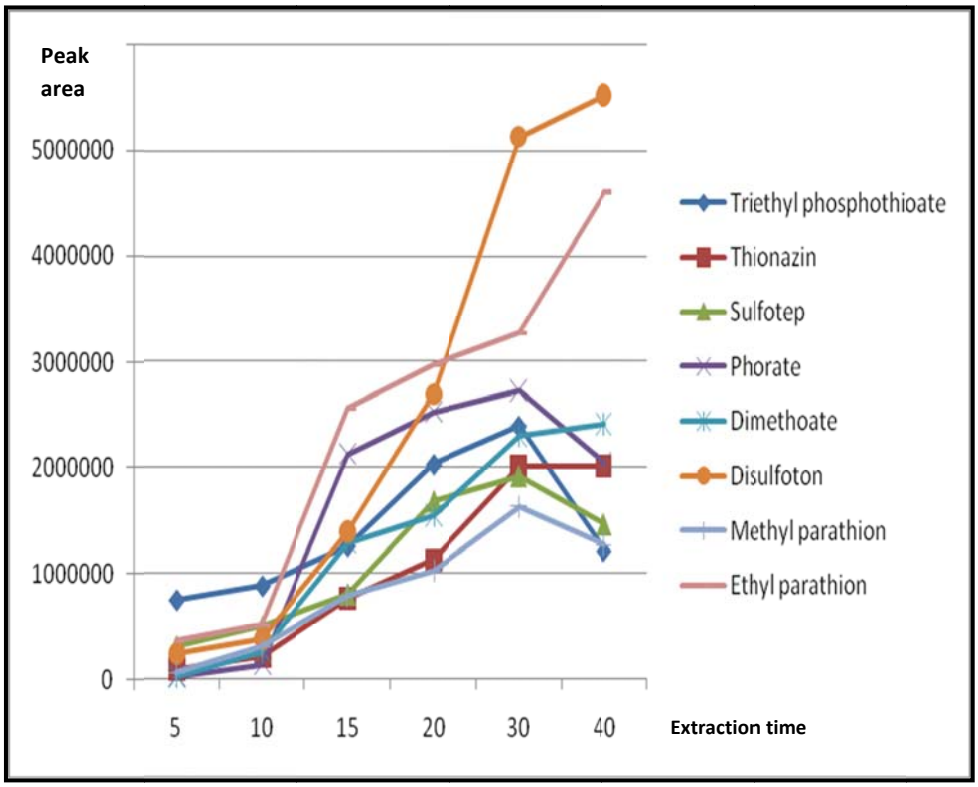

Figure11 Extraction profile

4.1.2. Sample volume

Influence of sample volume on SBME was studied in different volumes of 30, 50 and 100 mL which were spiked with 10 μg/L OPPs mixture. For each of the above sample volume the fiber was sealed from one side and filled with 200 μL of xylene and then used for extraction. The fiber was placed in the sample compartment and the solution was stirred for 30 minutes. After maintaining the other parameters it was found that the volume has an accepted proportional relation with sensitivity of the detection, so the bigger volume of the sample the better result of the extraction was obtained. Therefore the sample volume of 100 mL was selected as the best choice of sample size. See figure 12 for more details.

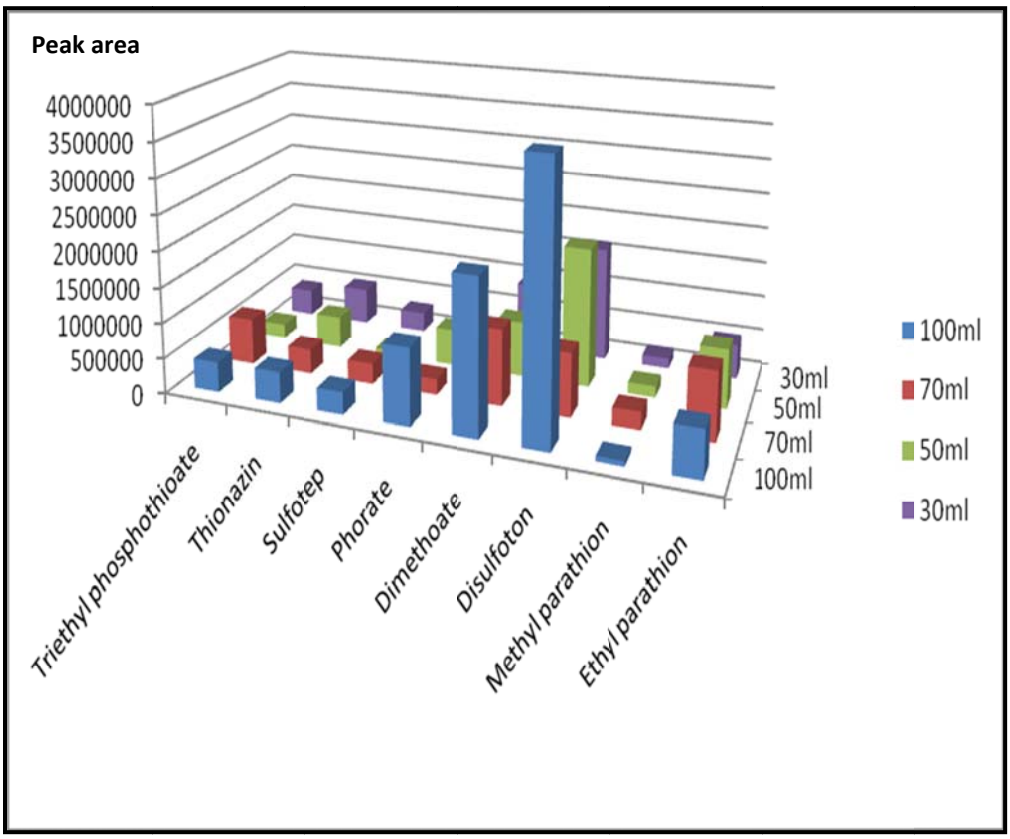

Figure12 Sample volume effect

4.1.3. Solvent selection

The effect of changing the solvent on the extraction process was studied in 100 mL volume sample which was spiked with 20 µg/L OPPs mixture and stirred for 30 minutes to obtain which of 1-Octanol, Carbon tetrachloride, n-Noane, Xylene or Toluene was the best as an extracting solvent. 200 µL of each solvent was confined in fiber with both ends sealed and then transferred to the sample compartment. As figure 13 shows, 1-Octanol turned out to have a lot of interferences as an extracting solvent although it was most volume retained solvent in the fiber with time. Carbon tetrachlotide was having the poorest extracting efficiency since it was always denser than the sample and the fiber drowned in the sample compartment and wasn't tumbling as freely as others. n-Nonae was better than Carbon tetrachloride as it was much less in density and fiber was tumbling more freely, yet was still not as good extracting solvent as Toluene and Xylene, that maybe due to lack of aromaticity. Xylene and Toluene were having almost same efficiency in extracting with all other parameters fixed but Xyelene was selected since it was retained more than Toluene in the fiber which might be a reason of more density in the case of Xylene.

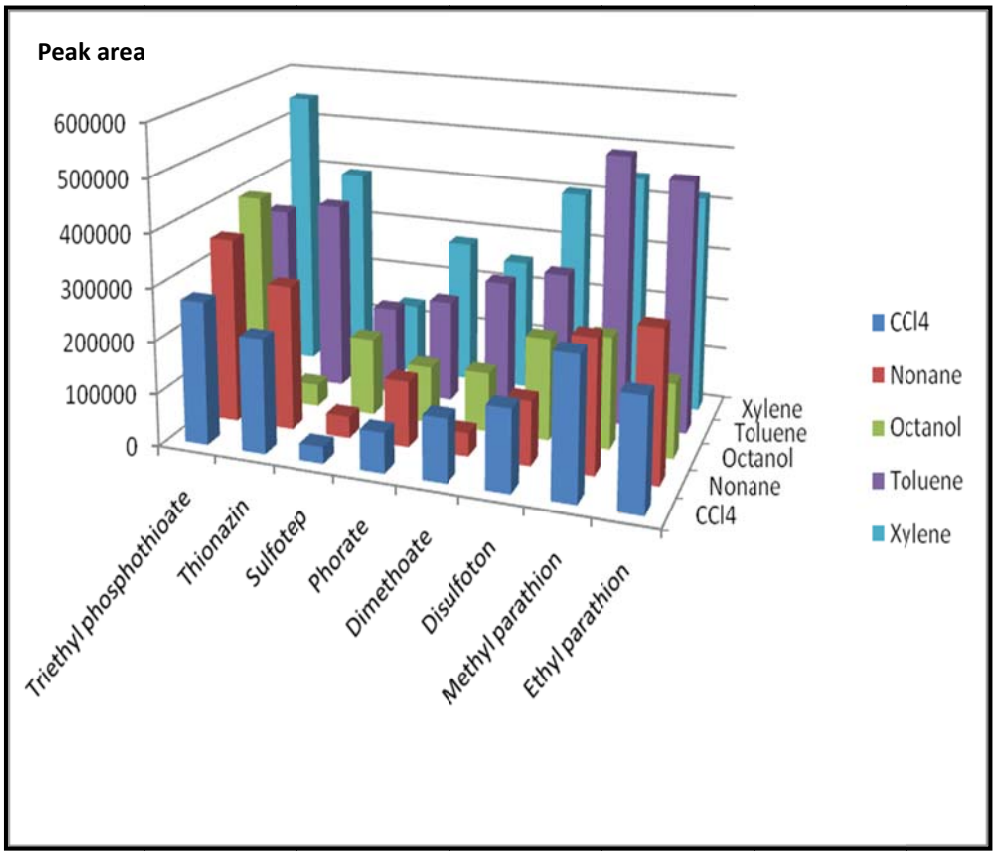

Figure13 Solvent selection

4.1.4. Salt addition effect

The addition of salt, such as NaCl, to an aqueous sample would decrease the solubility of the organic analytes in the aqueous phase which will induce the movement of the analytes into the solvent inside the fiber, and consequently improving the extraction efficiency of the method [43]. This process is called salting out effect.

However, increasing the ionic strength caused a small decrease in the enrichment factor for the target analytes (Phorate, Dimethoate, Disulfoton, Methyl Parathion and Ethyl Parathion) except (O,O,O-Triethylphosphorothiate, Thonazinand Sulfotep,) which might be slightly less polar and therefore would be expected to show an increase in the amount extracted with the increasing amounts of salt (figure 14). Yet the reason is not totally clear for why this result appeared. The extracted amounts of the analytes increase or decrease with the addition of salt depending on the solubility in water and polarity of the OPPs, (table 3) in water [44, 45, 46].

Table 3 Solubility of OPPs under monitoring

OPPs	Formula	Solubility in water (mg/L)
O,O,O-Triethyl phosphorothiate	$C_6H_{15}O_3PS$	845
Thionazin	$C_8H_{15}N_2O_3PS$	1140
Sulfotep	$C_8H_{20}O_5P_2S_2$	25
Phorate	$C_7H_{20}O_2PS_3$	50
Dimethoate	$C_5H_{12}NO_3PS_2$	25000
Disulfoton	$C_8H_{19}O_2PS_3$	25
Methyl parathion	$C_8H_{10}NO_5PS$	55
Ethyl parathion	$C_{10}H_{14}NO_5PS$	24

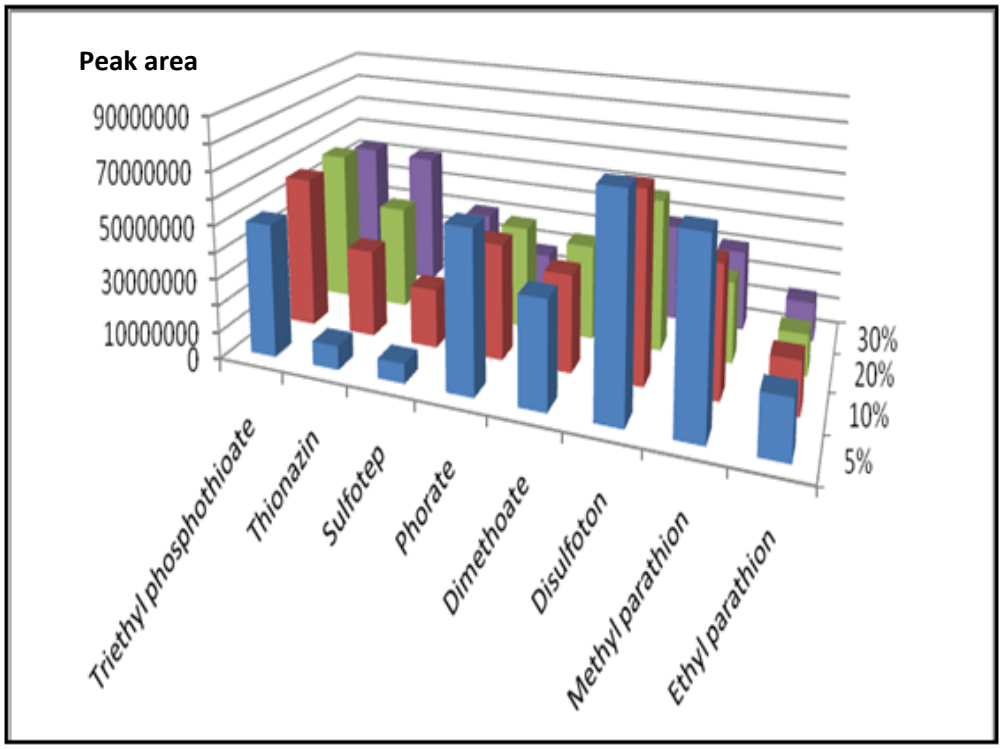

Figure14 Ionic strength modification by the addition of NaC1 and its effect on OPPs extraction

4.1.5. Influence of the pH

pH effect on the extraction process was studied with 100 mL sample spiked with 20 µg/L OPPs mixture. The extraction solvent was 200 µL toluene and sample was stirred for 30 minutes.

pH values from 4 to 10 were having almost similar response. On the other hands more acidic media where pH=2 was negatively affecting the extraction and more basic media pH=12 was having even more negative effect by dramatically reducing the reducing the peak area in the chromatogram when its compared with other pH values as it can be noticed in figure 15. This could be due to hydrolysis of OPPs at high and low sample pH. Therefore no pH modification was needed.

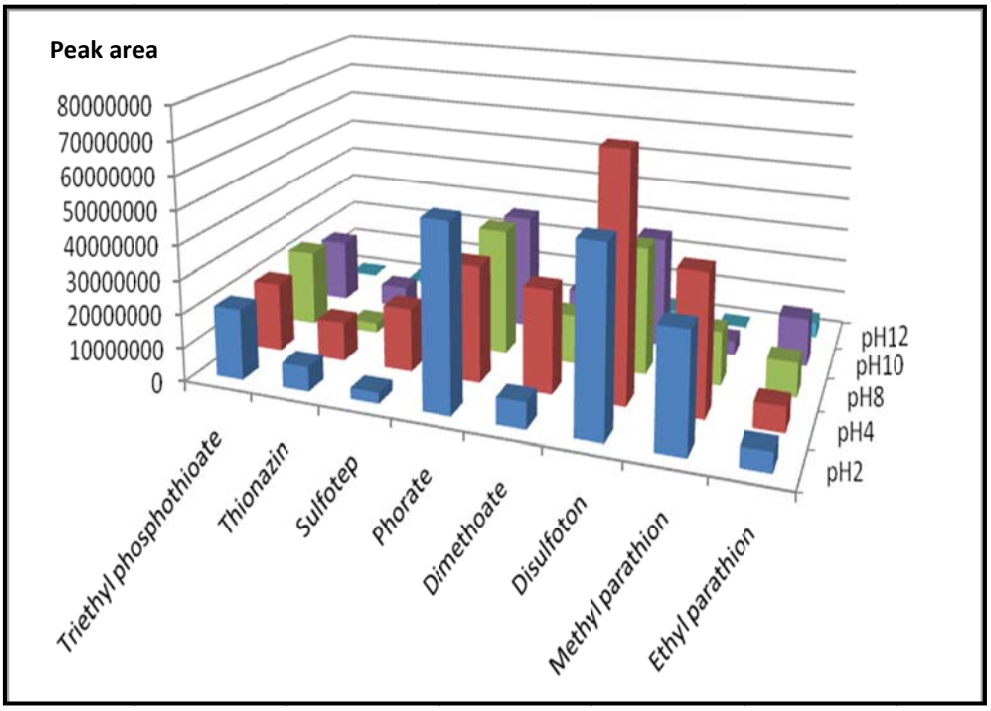

Figure15 Influence of sample pH on the extraction process

4.2. Quantitative information of SBME

After optimizing all experimental conditions, the SBME was evaluated for quantitative parameters such as linearity, reproducibility, limits of detection and limits of quantification. Linearity was obtained for OPPs range of the concentration was from 0.05 µg/L to 100 µg/L with correlation of determination between 0.9992 and 0.9998.Relative standard deviation not exceeding 12.9. The method limits of detection were calculated using signal to noise ratio of 3 and were between 0.7 ng/L and 44 ng/L. The limits of quantification were between 2.3 ng/L and 146.5 ng/L. Refer to table 4 for details. Figures 16-23 are showing the calibration graphs for OPPs and table 4 is showing the quantitative parameters of the calibration.

Figure 24 shows the chromatogram of 10 mg/L OPPs mixture analysed in GC/MS with Selective ion monitoring mode. (SIM).Figure 25 shows blank, Guava juice sample and OPPs 100µg/L standard.

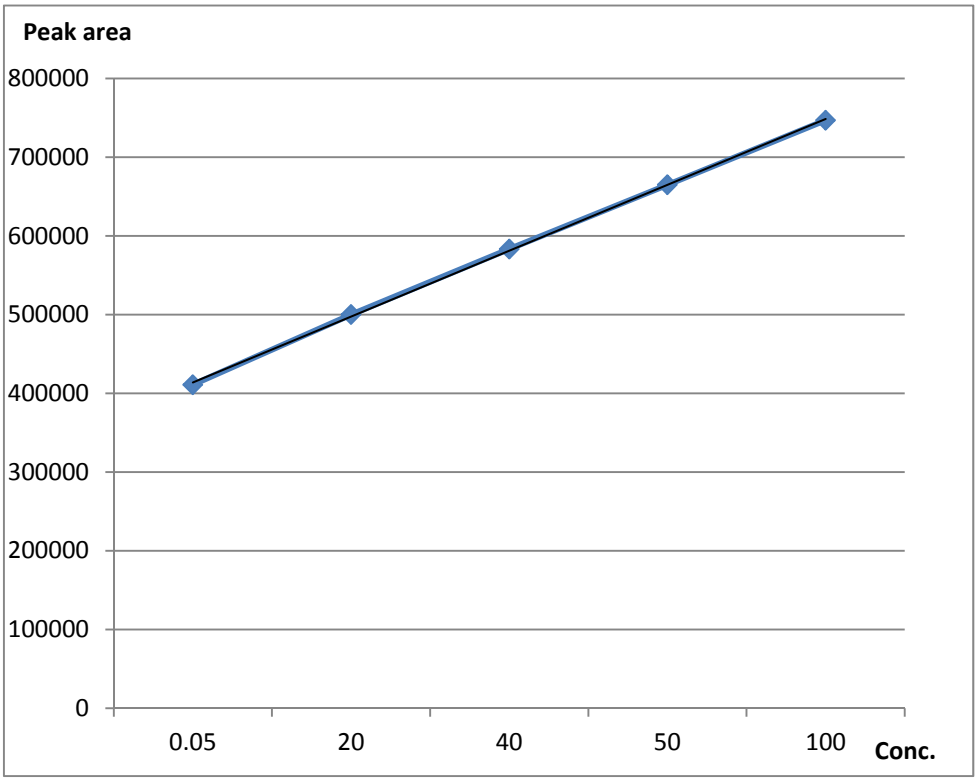

Figure16 Triethyl phosphorothioate calibration graph

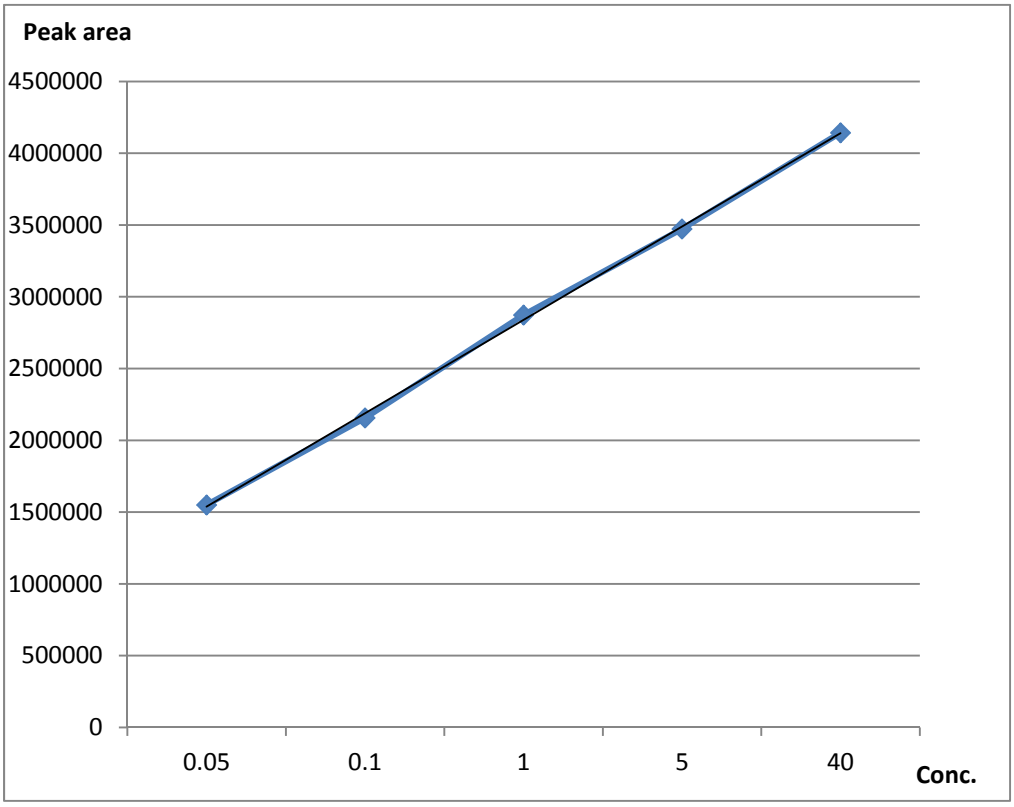

Figure17 Thionazin calibration graph

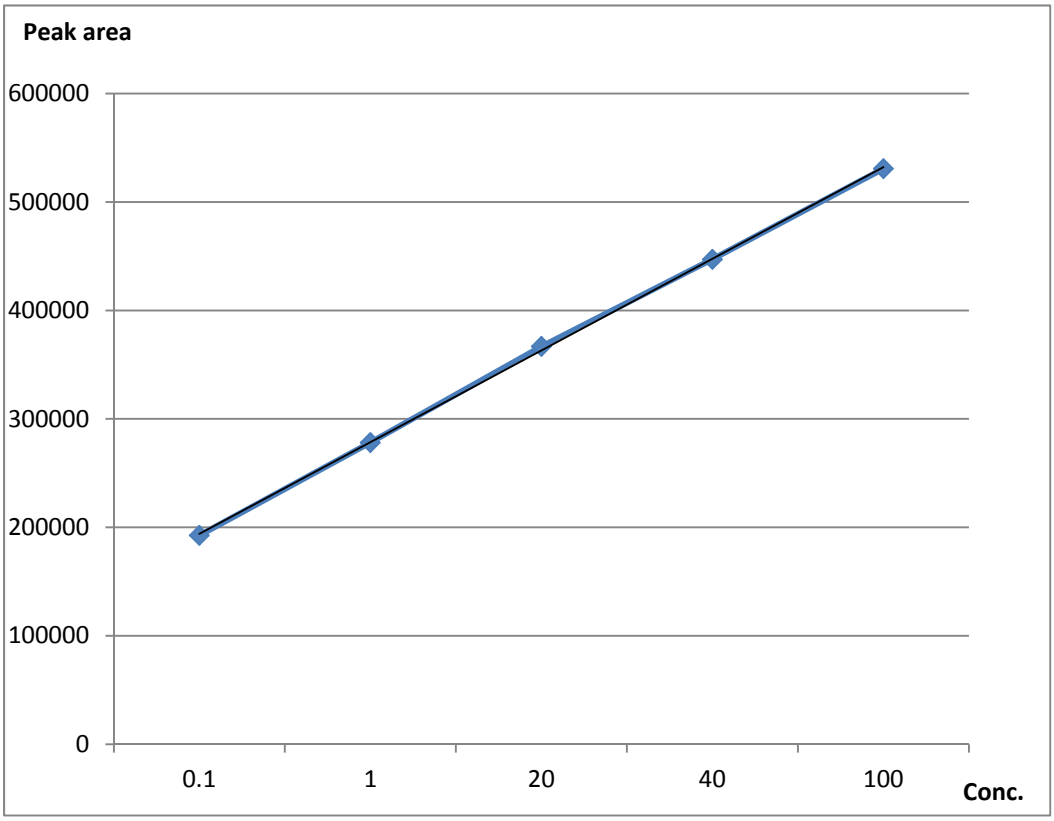

Figure18 Sulfotep calibration graph

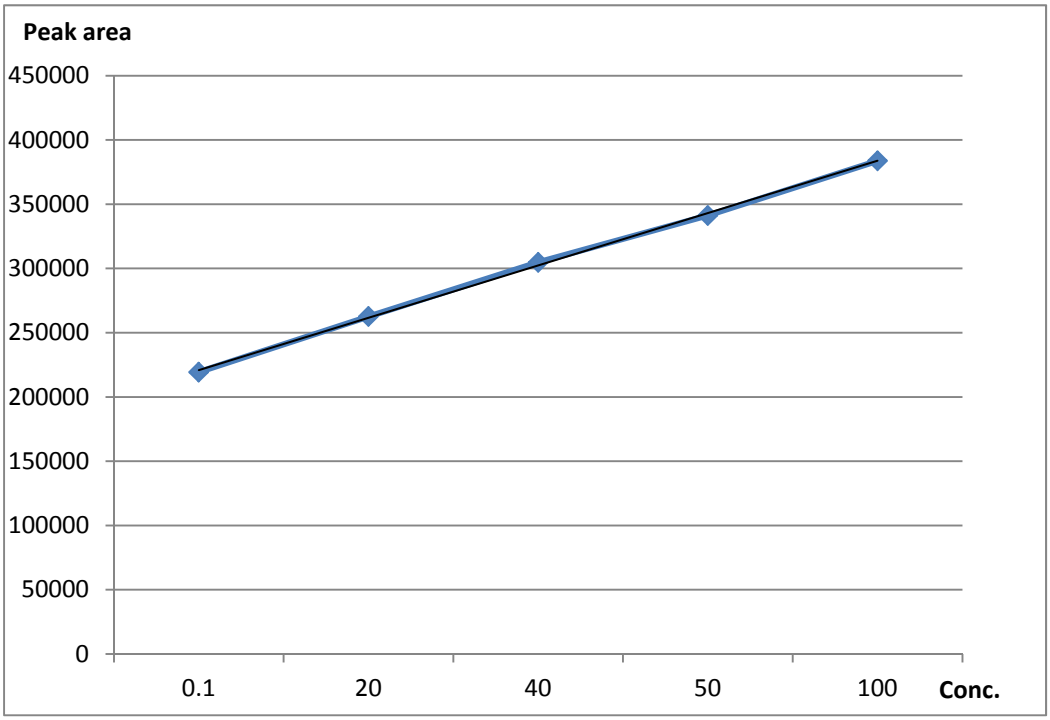

Figure19 Phorate calibration graph

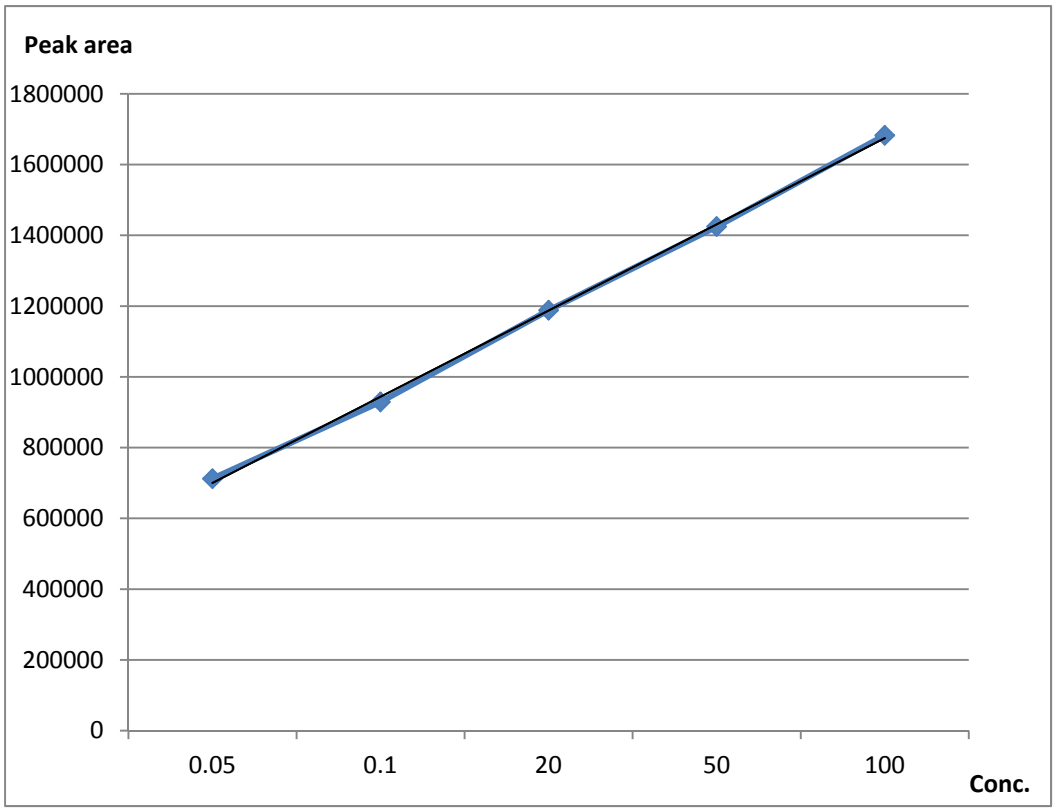

Figure20 Dimethoate calibration graph

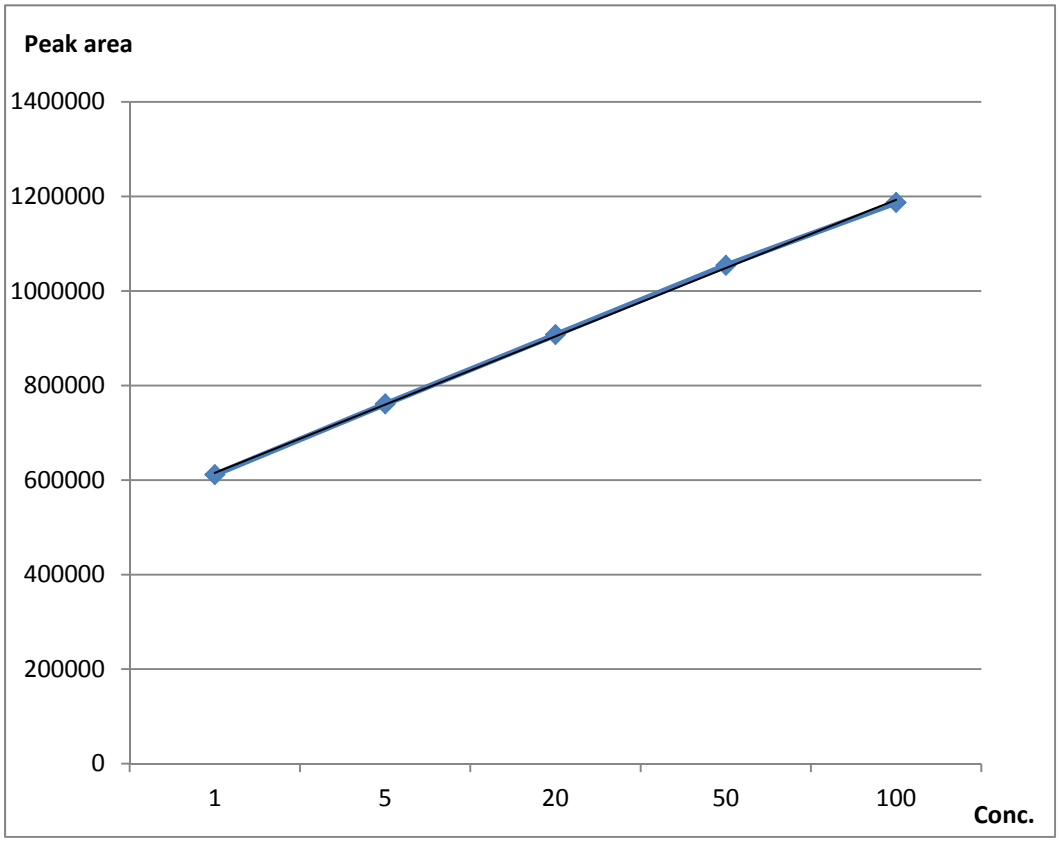

Figure21 Disulfoton calibration graph

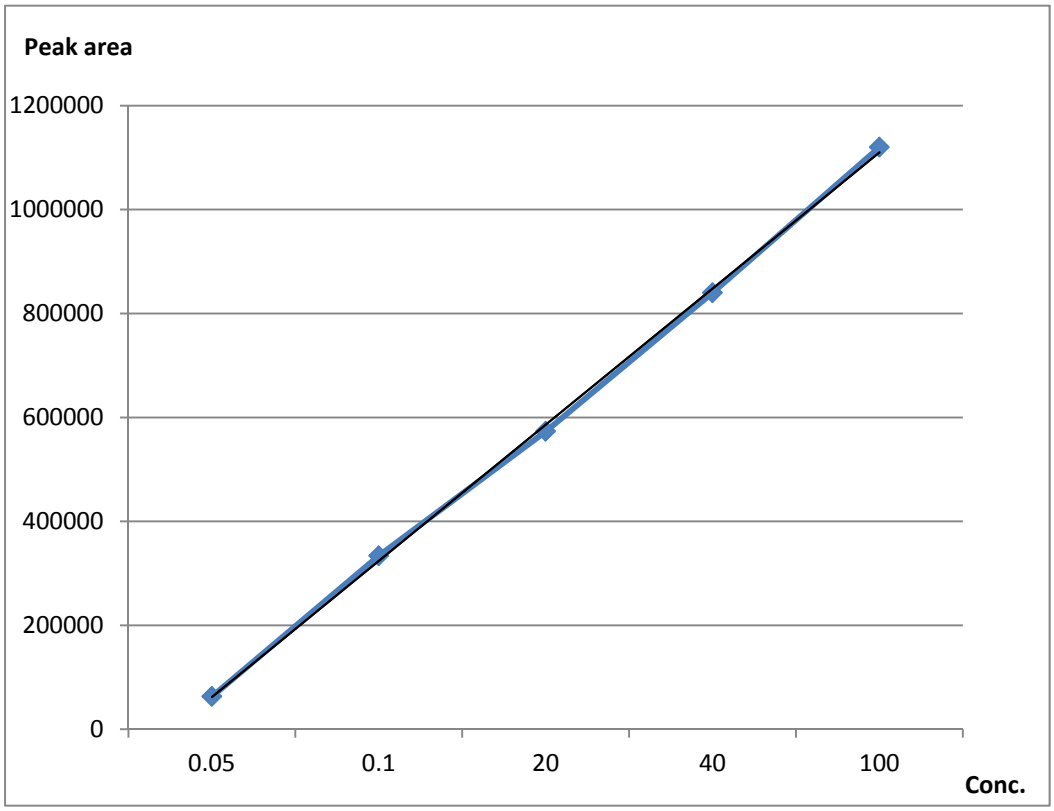

Figure22 Methyl parathion calibration graph

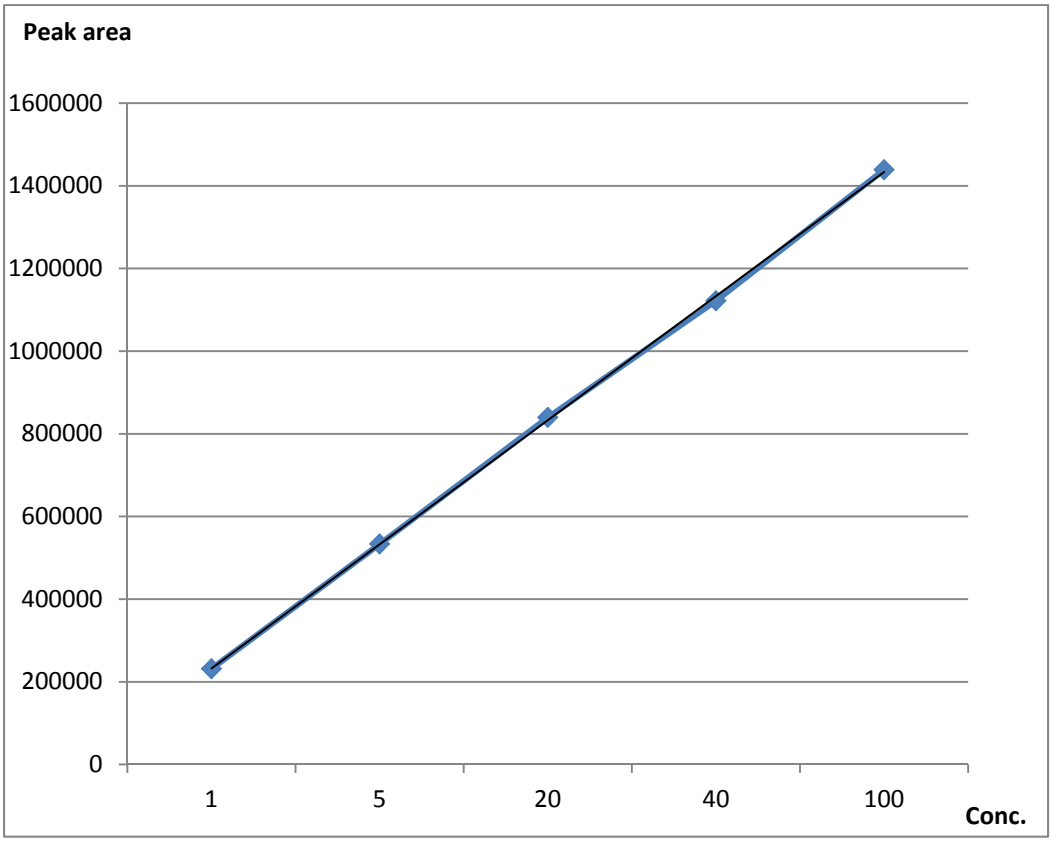

Figure23 Ethyl parathion calibration graph

Table 4 Quantitative parameters of the calibration of OPPs

Lowest standard concentration as follows * (0.05 µg/L), ** (0.1 µg/L), * (1 µg/L)**

OPPs	Linearity range (µg/L)	Correlation of Determination R^2	RDS% (n=3)	LOD (S/N=3) (ng/l)	LOQ (ng/l)
Triethyl phosphorothioate	0.05 – 100	0.9997	0.7 – 4.7	7 *	21
Thionazin	0.05 – 40	0.9994	4.8 – 9.1	0.7 *	2.1
Sulfotep	0.1 – 100	0.9997	1.3 – 8.5	20 **	60
Phorate	0.1 – 100	0.9992	3.6 – 10.7	17 **	56.6
Dimethoate	0.05 – 100	0.9992	3.4 – 8.8	3 *	10
Disulfoton	1 – 100	0.9995	1.9 – 6.5	17 ***	51
Methyl Parathion	0.05 – 100	0.9994	5.5 – 12.9	8 *	24
Ethyl parathion	1 – 100	0.9998	3.3 – 9.7	44 ***	132

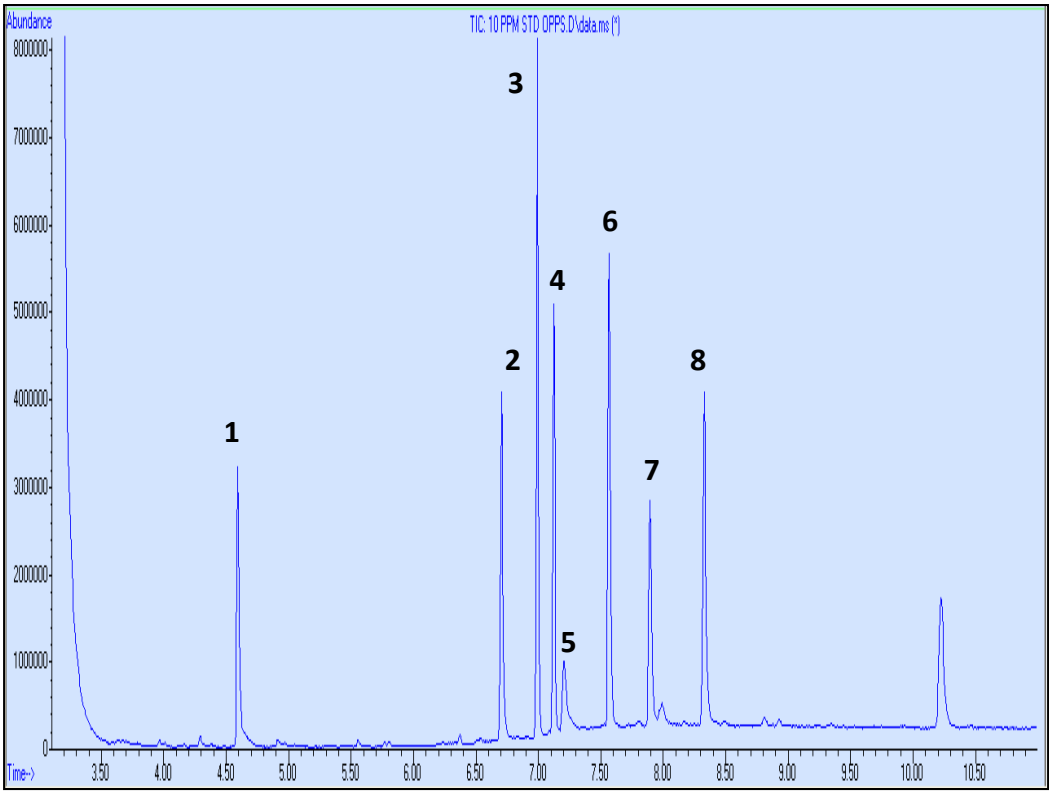

Figure24 OPPs mixture analysed in GC/MS with (SIM) mode
where 1:Triethyl phosphorothioate , 2:Thionazin , 3:Sulfotep , 4:Phorate , 5:Dimethoate , 6:Disulfoton , 7:Methyl Parathion , 8:Ethyl parathion

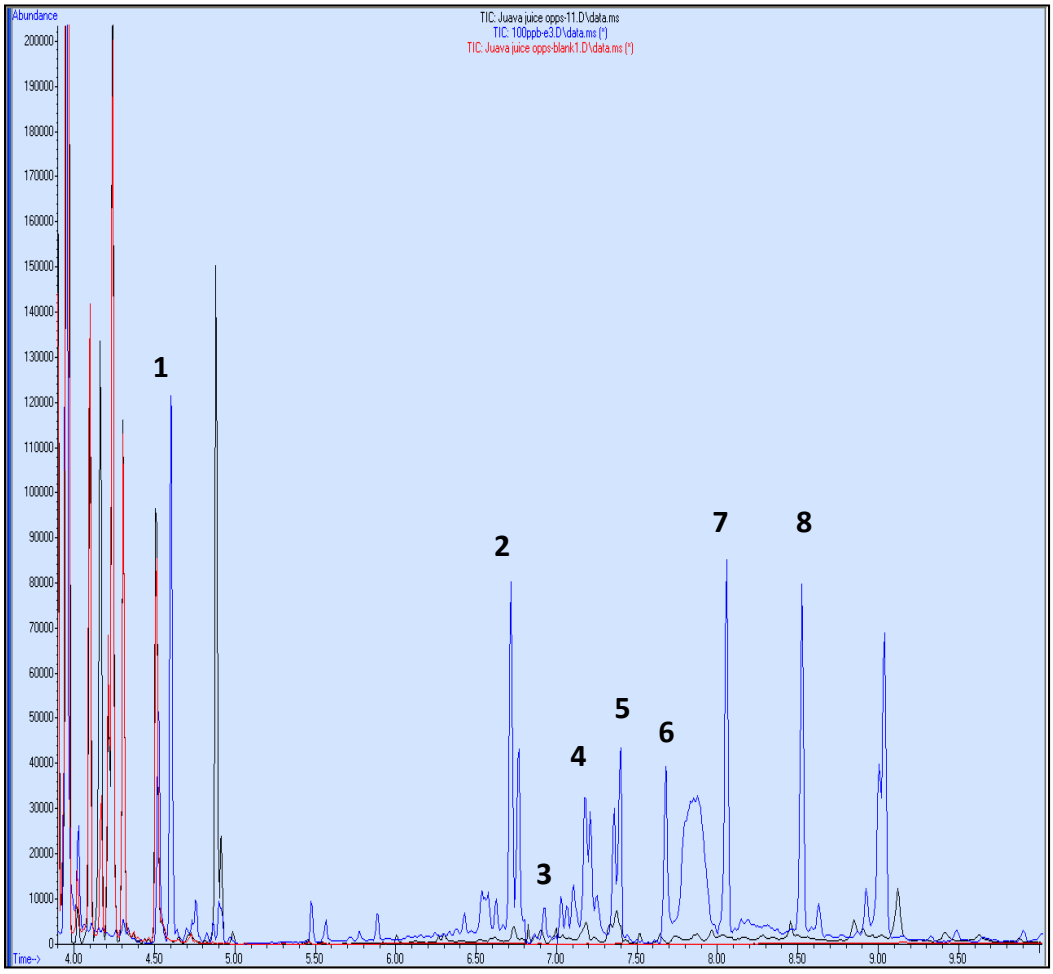

Figure25 In red Guava blank, in black Guava sample and in blue is OPPs 100ppb standard where 1:Triethyl phosphorothioate , 2:Thionazin , 3:Sulfotep , 4:Phorate , 5:Dimethoate , 6:Disulfoton , 7:Methyl Parathion , 8:Ethyl parathion

4.3. Method evaluation

The method performance was compared with the reported SPME/GC with Flame ionization detection (SPME/GC/FID) methods and US EPA 1657, 507, 622 and 622.1. Our adopted method in sample treatment and determination of OPPs clearly had better repeatability than reported methods in literature as our %RSD values were all lower than other reported methods.

As it can be noticed from table 5, the LOD of our method was superior than reported methods in literature in case of Thionazin, Dimethoate and Methyl Parathion. In case of Triethyl Phosphorothioate, BN/LPME/GC/MS [50] method only, was better than our method. In case of Sulfotep and Phorate, our method was only better than SPME/GC/FID [43] method. In case of Disulfoton, our method was better than SPME/GC/FID [43] and EPA methods 1657 [47] and 507 [48] only. In case of Ethyl Parathion, our method was better than SPME/GC/FID [43] and EPA method 622 or 622.1[49] only. All methods were using the 3 (S/N) methods for determination LOD. The chance of our method to achieve lower LOD values might increase significantly if we attempt to modify the values of secondary ions in the selective ion monitoring step in mass spectrometer system.

The linearity was very good between 0.05 µg/L and 100 µg/L with correlation of determination (r^2) between 0.9992 and 0.9998 all were better than the values reported in both BN/LPME/GC/MS which has linearity range 0.5-50 µg/L with correlation of determination (r^2) between 0.994 and 0.999 and SPME/GC/MS which linearity range 0.25-50 µg/L with correlation of determination (r^2) between 0.966 and 0.998 [50].

Our method accomplished better recovery compared to BN/LPME/GC/MS [50] except in the cases of Triethyl Phosphorothioate, Sulfotep, Phorate, Disulfoton where the other method has comparable values. As in comparison with SPME/GC/MS and BN/LPME/GC/MS [50] which was slightly better than our method in only Triethyl Phosphorothioate and Thionazin.

Table 5 Estimated LOD of methods from literature compared to LOD and precision of GC/MS coupled with SBME

OPPs (LODs)	SBME GC/MS (ng/L)	SPME GC/FID (ng/L)	SPME GC/NPD (ng/L)	SPME GC/MS (ng/L)	EPA (ng/L)	BN/LPME GC/MS (ng/L)	SPME GC/MS (ng/L)
Triethyl phosphorothioate	7	4900	260	9	n.a.	1.1	16.6
Thionazin	0.7	430	180	2	1000[49]	4.2	47.7
Sulfotep	20	470	16	2	6[47]	0.3	3.1
Phorate	17	690	11	2	10[47]	1.6	4.7
Dimethoate	3	5200	50	73	27[47]	n.a.	n.a.
Disulfoton	17	900	12	3	32[47] , 300[48]	1.5	5.9
Methyl Parathion	8	1030	114	11	10[47]	11.4	120.5
Ethyl parathion	44	1960	9	5	18[47] , 300[49]	2.7	11.2

4.4. Determination of OPPs

Firstly, attention should be drawn that the concentration unit used in this part of research is microgram per liter (µg\L) or parts per billion (ppb) which also can be expressed as microgram per kilogram (µg\kg) or even one thousandth of milligram per kilogram (0.001mg\kg).

In table 7, samples results are shown including the error on 2×SD basis. DL> stands for values below than detection limits and figure 26 shows the result in graphical display. As it can be noticed from the results; the pesticides: Thionazin, Dimethoate, Disulfoton were not detected in all samples.

O,O,O- Triethyl phosphorothioate was detected in four samples and all detected amounts in samples are not any near to the LC50 (lethal concentration that kills 50% population) for rats by inhaling which is41 mg/L/4hours [33]. Sulfotep has been detected in only 2 samples: strawberry and tomato juices with a concentration of 1.2 µg\L and 1.24 µg\L respectively. These values are almost twelve times more than reported values for environmental and drinking water with maximum allowed concentration of single compound established by European Union (EU) of 0.1 µg/L. Concentration of 0.5 µg/L is the maximum allowed for the total concentration of all OPPs [6, 7, 8], yet still lower than the oral LD50 values of about 5 - 15 mg/kg body weight (rat) [51]. Phorate was found in all samples except Guava and Orange in the concentration range of 0.5 µg/L to 9.06 µg/L. According to EPA, Phorate NOAEL for acute toxicity is 0.25 mg/kg/day and for chronic toxicity is 0.05 mg/kg/day (50 µg/kg/day).

The concentration of this pesticide in the samples is still lower than NOAEL for chronic toxicity.

Methyl Parathion was found in all samples except the lemon juice and it was in the range between 0.96 µg/L and 1.74 µg/L, those values are still bellow the chronic NOAEL, refer to table 6 for more data.

Parathion was found in all samples in concentration range from 0.4 µg/L and 1.25 µg/L, those values are still bellow the chronic NOAEL. Refer to table 6 for more data.

Table 6 Shows the acute and chronic data in rats for (LOAEL) and (NOAEL) of OPPs

OPPs Conc.	Methyl parathion	Parathion
LOAEL Acute	0.53 mg\kg	0.25 g\kg\day
NOAEL Acute	0.11 mg\kg	0.1mg\kg
LOAEL Chronic	0.21 mg\kg	--
NOAEL Chronic	0.02 mg\kg	0.04 mg\kg\day

Table 7 Sample results including the error on 2×SD basis and n.d. stands for values which not detected, (Avg. for n=3)

OPPs Concentration (µg/l)	Orange	Apple	Berry	Grapes	Guava	Lemon	Mango	Mix	Strawberry	Tomato	%recovery
Triethyl phosphorothioate	2.75±0.38	n.d.	18.57±0.29	n.d.	n.d.	2.45±0.52	n.d.	1.9±0.3	n.d.	n.d.	89.76
Thionazin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	83.14
Sulfotepp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2±0.4	1.24±0.24	90.67
Phorate	n.d.	1.76±0.28	0.5±0.1	0.64±0.10	n.d.	1.5±0.3	9.06±0.62	2.4±0.6	1.18±0.19	4.06±0.98	97.05
Dimethoate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	90.53
Disulfoton	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	80.09
Methyl parathion	1.03±0.03	1.17±0.06	1.23±0.09	1.16±0.03	0.96±0.01	n.d.	1.23±0.09	1.74±0.19	1.28±0.06	1.36±0.11	98.95
Parathion	0.50±0.06	0.53±0.05	0.99±0.07	0.54±0.01	0.40±0.03	0.91±0.09	1.09±0.03	1.25±0.19	1.04±0.14	1.15±0.17	96.58

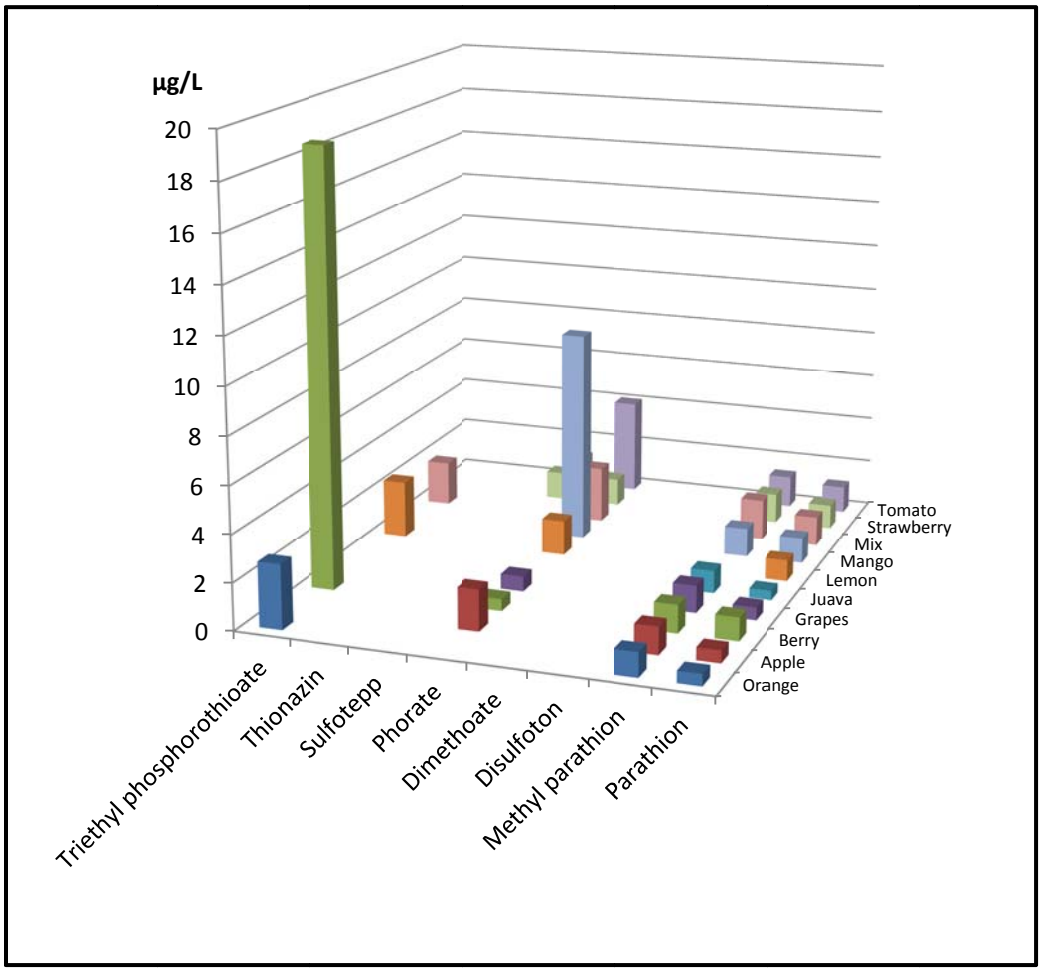

Figure26 Representative graph of sample results in µg/L

4.5. Conclusion and recommendations

The proposed SBME is suitable for determination of ultra-trace quantities of OPPs from fruit juice samples. The procedure provides good repeatability, as well as high recovery and preconcentration factor. Evaporating the solvent is not necessary to reduce the volume of the extracting media.

GC-MS was applied for the analysis and very low LODs was achieved and results were comparable with values reported from using nitrogen-phosphorous detection methods; however using tandem MS may enhance LODs values. Performance of the proposed method in extraction of OPPs from fruit juice was excellent so it can be used as an fast, effective, repeatable, simple and environmental friendly procedure to analyze pesticide residues in fruit juice samples. It can be concluded that the present level of pesticide residues analysis in food beverages represent results of demanding research and development. Since most of the food beverages are imported in the Kingdom regular monitoring of these pesticides in all kind of food juices are required to understand the fate of the OPPs and its bio-accumulative profiles.

CHAPTER 5

EXPERIMENTAL DESIGN FOR BAs

5.1. Chemicals and preparation of solutions

a) Biogenic amines standard solutions:

A stock solution of 1000 μg/L of each; Cadaverine (CAD), Putrescine (PUT), Tyramine hydrochloride (TYR), 2-Phenylethylamine hydrochloride (PEA), Tryptamine hydrochloride (TRY) and Spermidine trihydrochloride (SPD) purchased from Sigma-Aldrich (Steinheim am Albuch, Germany). Individual working standards were prepared by dissolving 100 mg of each BA in 100 mL volumetric flasks and dilute up to the volume with distilled water.

Then the individual working standard or mixed standard were prepared freshly by further dilution of the stock solutions as needed. Figure 27shows the chemical formulas of the mentioned biogenic amines.

b) Dansyl chloride or 5-(dimethylamino) naphthalene-1-sulfonyl chloride was purchased from Sigma Aldrich. The stock solution was prepared by dissolving 1 g of freshly weighed DS-Cl in 100 mL volumetric flask and dilute up to the volume with Acetone and kept sealed in the fridge at 4°C for maximum period

of a month. Figure 28 shows the chemical formula of Dansyl chloride.

c) 2 M Sodium hydroxide solution: prepared by dissolving 8 g of Sodium hydroxide in 100 mL volumetric flask and dilute up to the volume with freshly boiled distilled water.

   To avoid blockage of the flask use plastic stopper rather than the glass stopper.

d) 0.1 M Hydrochloric acid: 86 µL of HCl purchased from J.T. Baker,(New Jersey, USA) diluted with distilled water to 100 mL used for extraction of BAs from solid samples.

e) Saturated Sodium hydrogen carbonate solution: prepared by placing 15 g of Sodium hydrogen carbonate in 100 mL volumetric flask and add distilled water BELOW the volume. Stir vigorously for at least 5 minutes, some undissolved salt will remain, which is normal in case of saturated solutions. Filter the solution into 100 mL volumetric flask and dilute up to the volume with distilled water. To avoid blockage of the flask use plastic stopper rather than the glass stopper.

f) 25% Ammonium hydroxide solution: using the ready concentrated commercial Ammonium hydroxide solution (25 %).

g) 70% Acetonitrile as mobile phase for the HPLC analysis: prepared in 1 L volumetric flask by adding 700 mL of HPLC grade Acetonitrile and 300 mL distilled water. Further preparation can be done if more amounts of the mobile phase are needed.

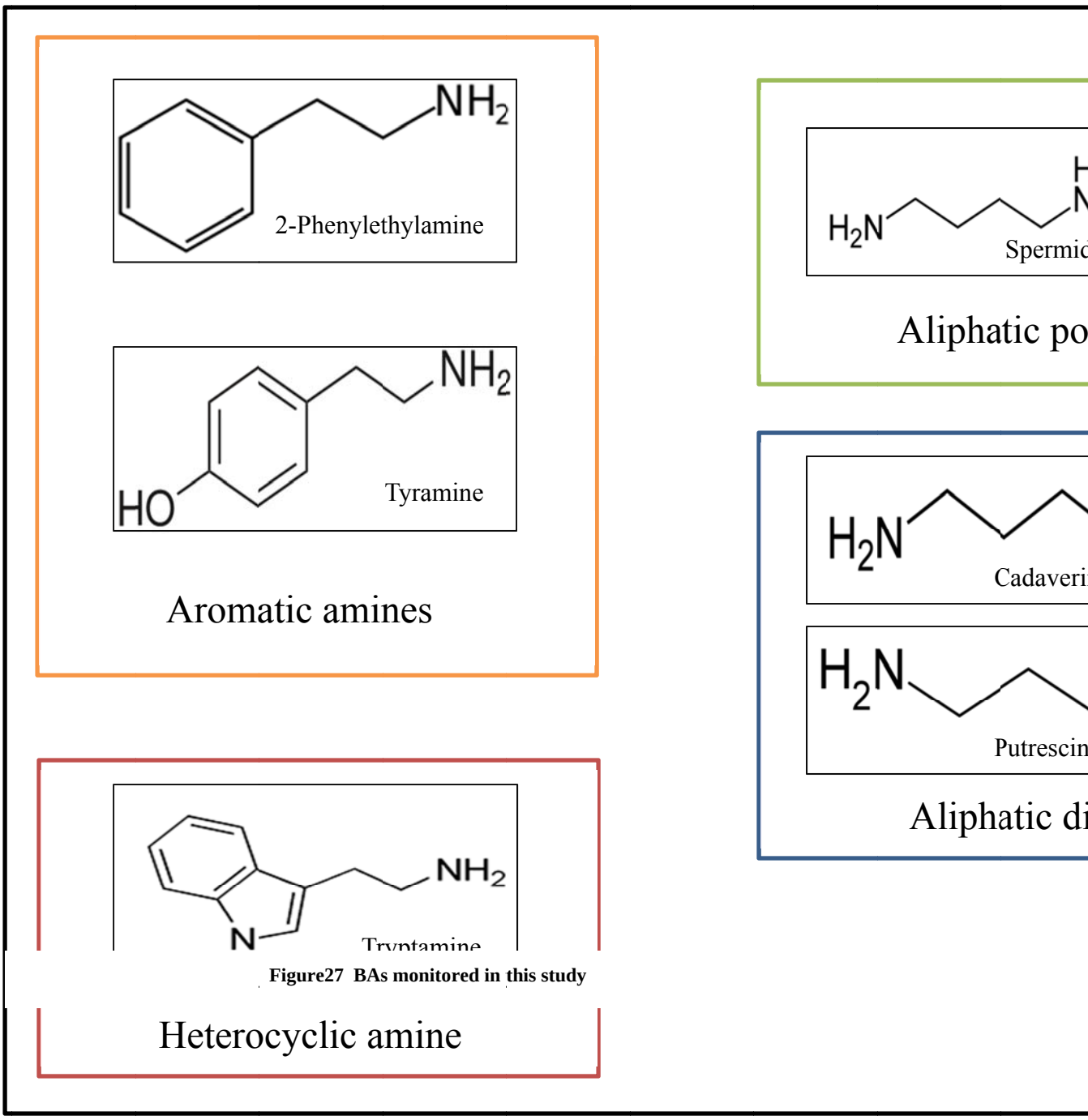

Figure27 BAs monitored in this study

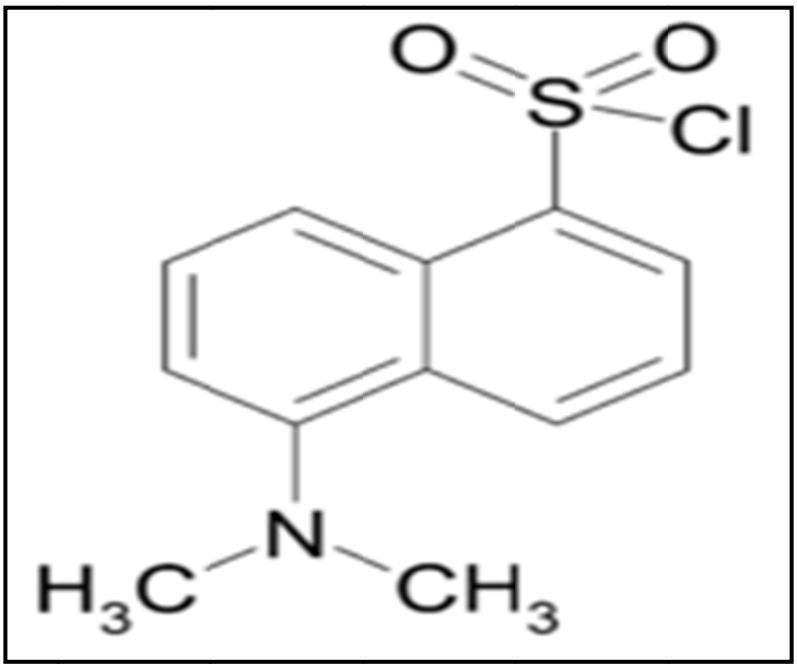

Figure28 Chemical formula of Dansyl chloride or 5-(dimethylamino) naphthalene-1-sulfonyl chloride

5.2. Methodology

Our initial attempt was to use simultaneous extraction and derivatization of BAs with DS-Cl in alkaline buffered media. For the first time, the traditional food consumed in the gulf area will be studied. The BAs are solid polar compounds thus, water soluble, and the samples we are working on are solid as well but not soluble in water except for Debs (dates syrup); therefore, we developed an ultrasonic assisted acidic liquid extraction to selectively extract the basic compounds of the biogenic amines from the complex media. Table 8 shows solubility of targeted BAs in water.

For the pre-concentration step we experimented LLE as a phase separation which was depending on the BAs partitioning between HCl as the aqueous solvent and the variable organic phase. That attempt was not successful.

Previously reported United Nations Environmental Monitoring method (conventional solid-liquid extractions) was modified and used.1 g of the sample was placed in a conical 150 mL flask and 10 mL of 0.1 M Hydrochloric acid were added to the sample flask to carry out the extraction. The flask was stoppered and ultrasonicated for 30 minutes. The Hydrochloric acid in the sample flask was then neutralized with nearly 500 μL of 2 M Sodium hydroxide. Centrifugation followed by filtration might be needed afterwards for clean and clear supernatant.

Calibration graph was constructed using diluted solutions prepared from main stock solution which was prepared from a high purity BAs dissolved in distilled water to give a concentration of 1,000ppm and the method was be optimized by spiking

known amount of solid BAs compounds in food samples. Local traditional wet and dried foods were purchased from local market, check figures 29-35. Solid samples were grinded to tiny particles or powder if possible. Each sample was brought in batches and then will be mixed and randomized as an individual sample. Batches then were divided and BAs were extracted with ultrasonic assisted acidic liquid extraction technique followed by dansylation in basic buffered conditions then analyzed by HPLC-PDA analysis.

Table 8 BAs solubility in water

Biogenic Amine	Formula	Solubility in water (mg/L)
Tryptamine (TRY)	$C_{10}H_{12}N_2$	negligible
2-Phenylethylamine (PEA)	$C_8H_{11}N$	Soluble
Putrescine (PUT)	$C_5H_{14}N_2$	Freely soluble
Cadaverine (CAD)	$C_5H_{14}N_2$	Soluble
Tyramine (TYR)	$C_8H_{11}NO$	1 g in 95 mL at 15°C
Spermidine (SPD)	$C_7H_{19}N_3$	145 g/L at 20 °C

Figure29 Humus (chickpea)

Figure30 Halwa

Figure31 Yagt (goat dried milk)

Figure32 Drabil (sweet rolls made basically of flour, cinnamon and milk)

Figure33 Matai (fried wheat flour with nuts)

Figure34 Debs (date syrup)

Figure35 Raisins

5.3. Derivatization

Since this work is based on Ultraviolet radiation detection and the BAs are not sensitive to UV, a derivatization step was needed to add a chemical group that is sensitive to UV. In this step; the 1 mL from the sample extractant was taken and placed in a reaction tube where 2 mL from 10 g/L Dansyl chloride as the reagent was added. The solution in the reaction tube was then basified by adding 50 µL of 2 M Sodium Hydroxide and 200 µL of saturated Sodium Bicarbonate. Then the reaction tube will be placed in a 70° C water bath for 10 minutes, and complete derivatization reaction was obtained. After cooling the reaction tube the total volume was brought to 2 mL by adding HPLC grade Acetonitirle. A sufficient amount was flirted into HPLC sample vial using waters0.2 µm desk filter in order to deliver it to the HPLC for analysis.

5.4. Instrumentation

HPLC-PDAwas the instrument used for the separation and analysis of BAs in this work. The manifold of the separation instrument of this experiment was Liquid Chromatograph coupled to Photo Diode Array detection system. The liquid chromatograph was Alliance Waters 2695 separation module from Waters and it was equipped with built-in auto sampling system. Waters Spherisorb 10um ODS2, 4.6 x 150 mm analytical column was installed. The Ultraviolet / Visible light detector was Waters 2996 Photodiode Array detector and Empower software was used.

The method parameters were optimized and the degassing system used Helium gas. The elution mode was isocratic with mobile phase composition ratio of 70:30 of Acetonitrile and water and the flow rate was 1 mL/min. Injection volume was 20 µL and the temperature was 35 °C. Detector wave length was 254 nm.

CHAPTER 6

RESULTS AND DISCUSSION OF THE BAs

6.1. Optimization

BAs are high polar, have basic properties and completely soluble in water. Extraction of BAs using non-polar organic solvents would give very low extraction recoveries. Polar, water soluble organic solvents are more obvious choice for the extraction if it was done with organic solvents. Since we plan to use solid samples, extraction with HCl using ultrasonication was performed. Each solid sample was extracted with 10mL of 0.1 M HCl for 30 minutes with the assist of ultrasonication.

6.2. Quantitative information of BAs

Linearity was obtained for BAs compounds by running standard solutions for BAs mixture. The range of the concentration was from 0.05 mg/L to 25 mg/L with correlation of determination between 0.9997 and 0.9999 and RSD not exceeding 7.4 with LOD ranged between 0.7 ng/L and 44 ng/L.

Figure 36 is showing the calibration graphs for BAs and table 9 is showing the quantitative parameters of the calibration of BAs. Figure 37 shows peaks of mixture of 50 mg/L BAs analyzed in HPLC-DAD at 254 nm wavelength and figure 38 shows Yagt (red) and Debs (blue) samples analyzed against 5 mg/L BAs standard at same conditions.

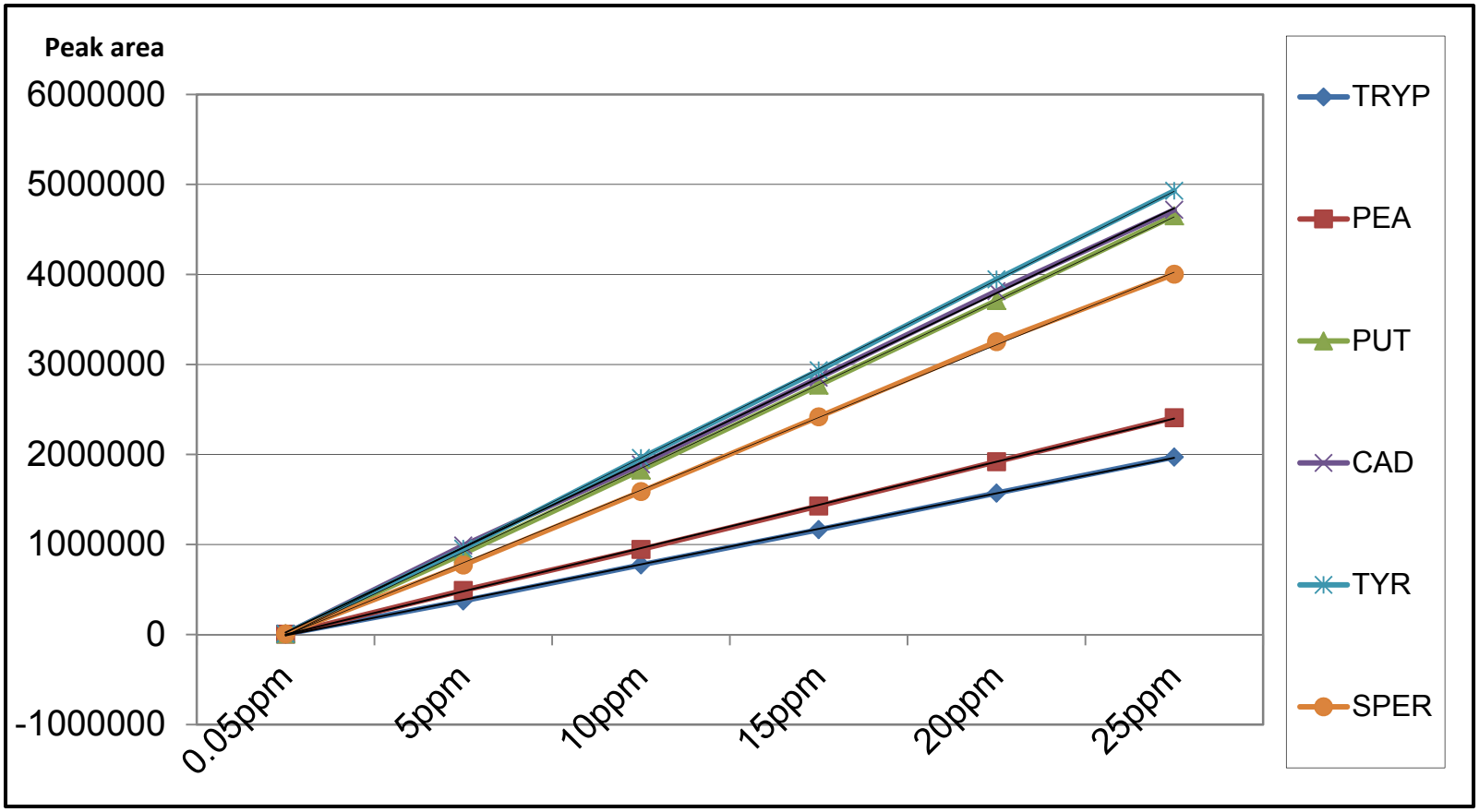

Figure36 Calibration graph for the BAs

Table 9 Quantitative parameters of the calibration of BAs

Biogenic Amine	Linearity range (mg/L)	Coorelation of Determination R^2	RDS% (n=3)	LOD (S/N) (mg/L)	LOQ (mg/L)
Tryptamine (TRY)	0.05 – 25	0.9998	0.3 – 4.2	0.011	0.036
2-Phenylethylamine (PEA)	0.05 – 25	0.9999	0.4 – 1.9	0.014	0.046
Putrescine (PUT)	0.05 – 25	0.9999	1.1 – 5.5	0.008	0.026
Cadaverine (CAD)	0.05 – 25	0.9999	1.2 – 6.1	0.005	0.017
Tyramine (TYR)	0.05 – 25	0.9999	0.3 – 3.4	0.006	0.020
Spermidine (SPD)	0.05 – 25	0.9997	1.7 – 7.4	0.006	0.020

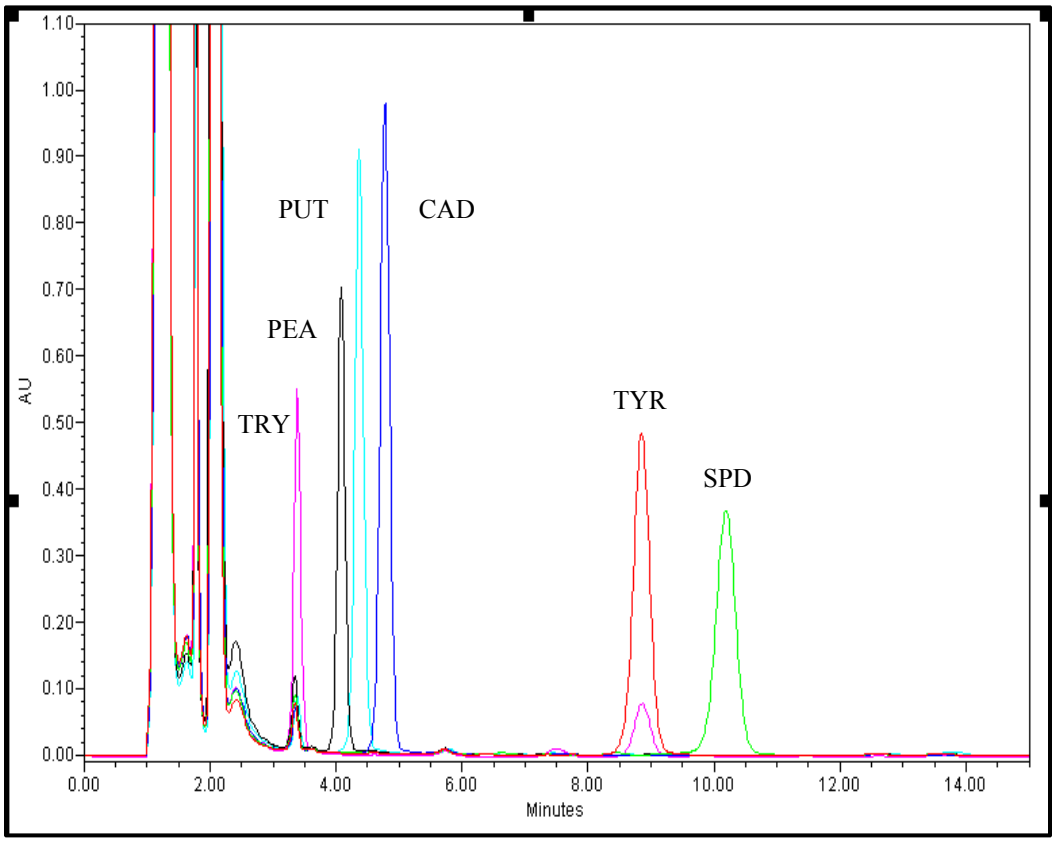

Figure37 Mixture of 50 mg/L BAs analyzed in HPLC-DAD at 254 nm wavelength

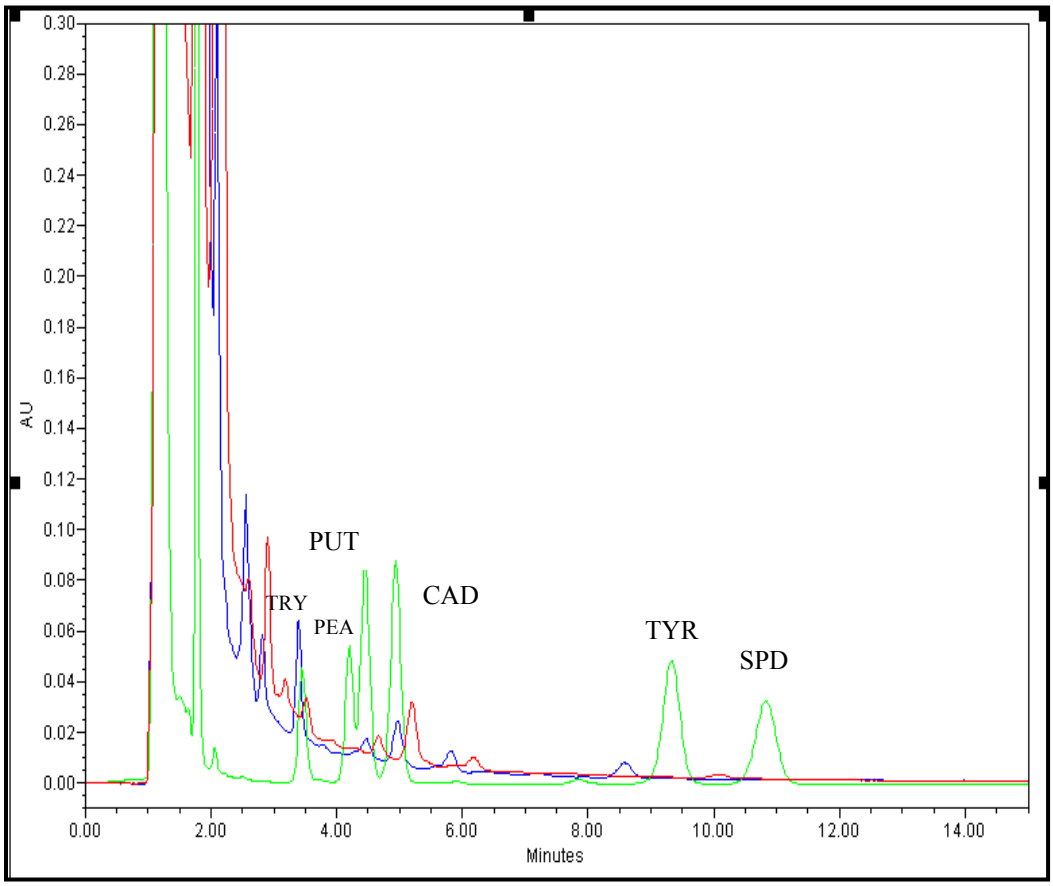

Figure38 Yagt (red) and Debs (blue) samples analyzed against 5 mg/L BAs standard in HPLC-DAD at 254nm wavelength

6.3. Method evaluation

The method performance was compared with the reported Liquid Phase Extraction (LPE)/HPLC/UV-VIS [58], 4-hydroxy-N′-[(E)-(2 -hydroxyphenyl) methylidene] benzohydrazide – based sorbent material (hydroxy)/HPLC/DAD [59], LPE/UPLC/MS/MS [60] and crown ether ligands - based sorbent material (ethers)/HPLC/UV-VIS [61]. Our adopted method in sample treatment and determination of BAs has superior %RSD values than all mentioned methods in case of 2-Phenylethylamine (PEA), Putrescine (PUT) and Tyramine (TYR) and has no data for Cadaverine (CAD) and Spermidine (SPD) yet has comparable values with other methods except sorbent/HPLC/UV-VIS which has lowest values in case of Tryptamine (TRY) as it can be noticed from table 10.

The LOD values of our method was superior than values of LPE/UPLC/MS/MS method and comparable with values of ethers/HPLC/UV-VIS method yet hydroxy/HPLC/DAD method has the lowest LOD values, however as its stated in the source that the large sample volume (50µL) is clearly the reason for low LOD values. All methods were using the 3(S/N) method for determination LOD except LPE/UPLC/MS/MS which calculated LODs and LOQs as 3.3 and 10 α/S, respectively where S is the slope and α is the standard deviation of the y-intercept of the calibration curve, refer to table 11for more details. The chance of our method to achieve lower LOD values might increase significantly if we attempt to modify the derivatization and detection techniques as well as injection volume.

The linearity of hydroxy/HPLC/DAD and ethers/HPLC/UV-VIS methods covered range of 0.001 – 50 mg/L and 0.1 – 250 mg/L respectively which was wider than our method's 0.05 – 25 mg/L. However, our method was having better linearity than LPE/UPLC/MS/MS method which covered only the range of 0.025 – 5 mg/L.

Our method had very competitive correlation of determination (r^2) values between 0.9997 and 0.9999 which was comparable or better than other methods. Table 12 shows more details.

Table 10 RSD values for the tested samples

BAs RSD	Matai	Humus	Raisin	Debs	Halwa	Darabil	Yagt
Tryptamine (TRY)	6.2	3.2	3	6.9	3.7	1.9	4.3
2-Phenylethylamine (PEA)	0.9	2.4	0.9	≈ 0.0	1.5	≈ 0.0	N.A.
Putrescine (PUT)	≈ 0.0	N.A.	N.A.	0.6	N.A.	N.A.	N.A.
Cadaverine (CAD)	N.A.	N.A.	N.A.	0.5	2.5	0.6	N.A.
Tyramine (TYR)	0.6	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Spermidine (SPD)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

Table 11 Estimated LOD of methods from literature compared to LOD and precision of HPLC/DAD coupled with LPE
*** final volume wasn't mentioned, + injection of large sample volume (50 µL)**

BAs (LODs)	Ultrasound-assisted LPE/HPLC/DAD (µg\L)	LPE/HPLC/UV-VIS (ng)*	hydrazon/HPLC/DAD (µg\L)+	LPE/UPLC/MS/MS (µg\kg)	crown ether /HPLC/UV-VIS (µg\L)
Tryptamine (TRY)	11	0.0028	0.13	17.3	5.4
2-Phenylethylamine (PEA)	14	0.0025	0.19	32	N.A.
Putrescine (PUT)	8	0.0009	0.09	20.3	4.4
Cadaverine (CAD)	5	0.0011	0.09	39.6	N.A.
Tyramine (TYR)	6	0.0015	0.09	14.9	7.3
Spermidine (SPD)	6	0.0011	0.07	20.3	7

Table 12: Calculated linearity of methods from literature compared to HPLC/DAD coupled with LPE method
all numbers between brackets are for (R^2) unless otherwise stated, * no final volume mentioned, ** stated in the source as (µg/kg)

BAs (Linearity)	Ultrasound-assisted LPE/HPLC/DAD (mg/L)	LPE/HPLC/UV-VIS (ng)*	hydazon/HPLC/DAD (mg/L)	LPE/UPLC/MS/MS (mg/kg)**	crownether/HPLC / UV-VIS (mg/L)
Tryptamine (TRY)	0.05 – 25 (0.9998)	2.0–509.1 (0.9998)	0.001 – 50 (1.0000)	0.025 – 5 (0.9908)	0.1–250 (0.9989)
2-Phenylethylamine (PEA)	0.05 – 25 (0.9999)	1.9–480.4 (0.9999)	0.001 – 50 (1.0000)	0.05 – 2 (0.9941)	N.A.
Putrescine (PUT)	0.05 – 25 (0.9999)	1.4–342.1 (0.9990)	0.001 – 50 (0.9999)	0.05 – 2.5 (0.9948)	0.1–100 (0.9999)
Cadaverine (CAD)	0.05 – 25 (0.9999)	1.5–364.7 (0.9998)	0.001 – 50 (1.0000)	0.05 – 2.5 (0.9981)	N.A.
Tyramine (TYR)	0.05 – 25 (0.9999)	2.0–493.7 (0.9998)	0.001 – 50 (1.0000)	0.025 – 2.5 (0.9986)	0.1–200 (0.9995)
Spermidine (SPD)	0.05 – 25 (0.9997)	1.4–356.5 (0.9998)	0.001 – 50 (1.0000)	0.05 – 2.5 (0.9867)	0.1–130 (0.9979)

6.4. Determination of BAs

Firstly, attention should be drawn to the point that the concentration unit used in this part of research was milligram per liter (mg\L) or parts per million (ppm) which also can be expressed as milligram per kilogram (mg\kg). If the concentration unit used microgram per liter (µg\L) or parts per billion (ppb) which also can be expressed as microgram per kilogram (µg\kg) or even one thousandth of milligram per kilogram (0.001mg\kg). In table 13, samples results are shown including the error on 2×SD basis. DL> stands for values below than detection limits and figure 39 shows the result in graphical display.

All samples were purchased from Local Thursday Traditional Market in Qatif city. Samples pictures were given in the previous chapter with brief details about their nature. Matai, Humus, Drabil and Yagt all were solid samples that needed to be grinded and homogenized in a machine before extraction. Halwa and Raisin both were also solid but soft samples which only needed a spatula to cut and smash to small pasty pieces. Debs in the other hand was the only viscous liquid sample that totally was miscible with water and did not need pretreatment.

Tryptamine (TRY) was present in al samples except Debs in concentration range of 0.13 - 4.8mg\L which was relatively low; however there is no regulation on the maximum amount of Tryptamine consumption in some food such as sausage in some countries.

2-Phenylethylamine (PEA) was found in all samples except Yagt (dried goat milk) in concentration range of 0.99 - 1.27mg\L which was still lower than the 30 mg/kg reported as toxic dose in foods. Putrescine (PUT) was found only in three samples; Matai, Debs and Darabil; in concentration range of 0.99 - 1.04mg\L which was much lower than (NOAEL) of 2000 mg\L. In the case of Cadaverine (CAD), it was found only in three samples; Debs, Halwa and Darabil; in concentration range of 1.04 – 1.88mg\L which was again much lower than (NOAEL) of 2000 mg\L. Tyramine (TYR) was found only in Matai with concentration of 1.04 mg\L which is again much lower than (NOAEL) of 2000 mg\L, still Tyramine alone at high levels can be toxic and cause a reaction known as the cheese reaction. Spermidine was not found in any sample.

Table 13 Sample results including the error on 2×SD basis and n.d. stands for values which not detected (Average for n=3)

BAs Concentration (mg/L)	Matai	Humus	Raisin	Debs	Halwa	Darabil	Yagt	%Recovery
Tryptamine (TRY)	0.34±0.04	1.31±0.08	0.77±0.04	n.d.	4.8±0.4	1.05±0.04	0.13±0.01	94.8
2-Phenylethylamine (PEA)	1.07±0.02	1.27±0.06	1.15±0.02	0.99±0.00	1.16±0.03	1.01±0.00	n.d.	93.6
Putrescine (PUT)	0.99±0.00	n.d.	n.d.	1.04±0.01	n.d.	1.04±0.00	n.d.	100.5
Cadaverine (CAD)	n.d.	n.d.	n.d.	1.18±0.01	1.88±0.09	1.04±0.01	n.d.	98.8
Tyramine (TYR)	1.04±0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	90.3
Spermidine (SPD)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	97.8

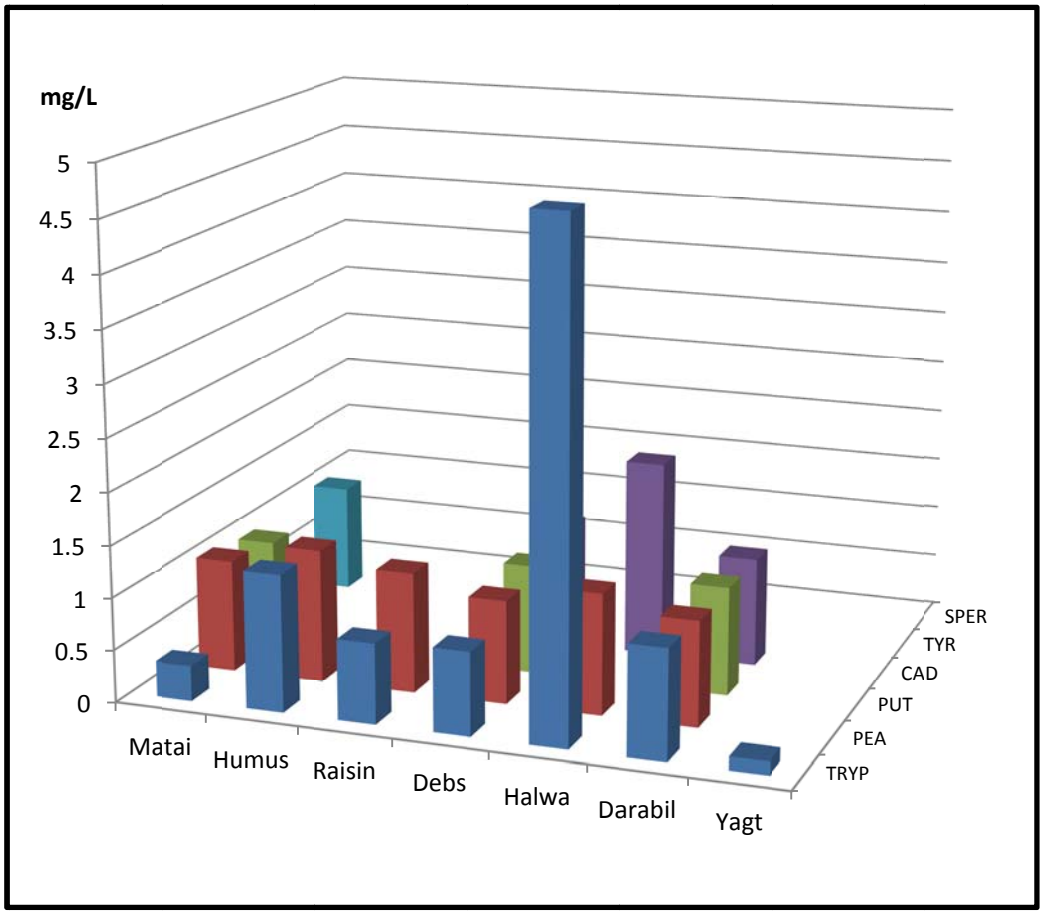

Figure39 Quatification of BAs in each sample in mg/L unit

6.5. Conclusion and recommendations

The ultra sound assisted liquid extraction procedure has been evaluated for the extraction of six BAs (TRP, PUT, CAD, TYR, PEA and SPD) from solid samples. Results obtained showed that extraction procedure exhibit high throughput and excellent linearity. The proposed method for extraction demonstrated high repeatability and reasonable recoveries when different food matrices were used. HPLC-UV was applied for the analysis and low LODs were achieved even better with values reported from using tandem MS. However, derivatization by DS-Cl (dansylation) was needed to enhance the UV detection step since BAs are not sensitive toward UV detection.

It is well known that excess intake of BAs causes several kinds of diseases like migraine, heart failure, hypertension and flushing. In this study, we determined the overall amounts of biogenic amines in several traditional foods consumed locally. Results indicate that these foods contain very low amounts of BAs that may not pose a danger for general health. Therefore, strict quality control with regular monitoring of BAs in the food import is needed to improve the quality of food. To improve the analytical sensitivity, we recommend a multiple column coupling in reverse phase HPLC for further separation and determination of BAs.

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