REPUBLIC OF TURKEY

YILDIZ TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF SCIENCE AND ENGINEERING

DEVELOPMENT OF ANALYTICAL STRATEGIES FOR ACCURATE AND PRECISE DETERMINATION OF ENDOCRINE DISRUPTIVE CHEMICALS IN BIOLOGICAL AND ENVIRONMENTAL MATRICES

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MASTER OF SCIENCE THESIS

Department of Chemistry

Analytical Chemistry Program

Advisor

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Sezin ERARPAT

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LIST OF SYMBOLS

%	Percentage
$\sum R_x$	Total isotope ratios of x (x=N, N* or D)
µl	Microliter
µm	Micrometer
A	Volume of DCM
B	Volume of Ethanol
C	Volume of $NaBEt_4$
D	Isotopic Labelled Material
d	Deuterium
g	Gram
g	g force
h	Hour
K	Kelvin
k	Factor number
K	Mass bias calibration factor
kg	Kilogram
L	Liter
L	Levels
M	Molar
m/z	Mass to Charge Ratio
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
Mx	Molar mass of x (x=N, N* or D)
$m_{x(xy),aq}$	Mass of the solution of x in xy blend (x,y=N,N* or D)
n	Number of center points
N	Sample
N*	Primary standard of natural isotopic composition

N*D-x	Calibration Blend (x=1, 2 or 3)
ND	Sample Blend
ng	Nano gram
N_W	Total number of white marbles
n_x	Amount of x (x=N, N* or D)
^oC	Celsius Degree
p	Size of fraction
psi	Per square inch
R^2	Correlation coefficient
R_nSnX_{4-n}	R and X represent alkyl or aryl groups and anionic species or groups
rpm	Revolutions per minute
R_x	Isotope amount ratio of x (x=N, N* or D)
s	Second
T	Vortex period
v/v	Volume/Volume
w/v	Weight/Volume
w/w	Weight/Weight
w_x	Mass fraction of x (x=N, N* or D)
X	Volume of Protonated DMBA
Y	Concentration of NaOH
Z	Volume of NaOH
α	Volume of Dichloromethane + 1,2-Dichloroethane
β	Volume of Ethanol
γ	Vortex Period

LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrometry
ACN	Acetonitrile
ACT	Acetone
AED	Atomic Emission Detector
ANOVA	Analysis of Variance
APEOs	Alkylphenol Ethoxylates
APs	Alkylphenols
BBD	Box Behnken Design
BS-LPME	Binary Solvent Liquid Phase Microextraction
BSTFA	N,O Bis(trimethylsilyl)trifluoroacetamide
CE	Capillary Electrophoresis
CHL	Chloroform
CI	Chemical Ionization
D	Isotopically Labelled Material
DBT	Dibutyltin
DCE	1,2-Dichloroethane
DCM	Dichloromethane
DI-SDME	Direct Immersion Single Drop Microextraction
DLLME	Dispersive Liquid-Liquid Microextraction
DMBA	N,N-Dimethylbenzylamine
ECD	Electron Capture Detector
EDCs	Endocrine Disruptive Chemicals/Compounds
EI	Electron Impact Ionization
ESI	Electrospray Ionization
ESI-MS	Electrospray Ionization Mass Spectrometry
EtOH	Ethanol
FAB	Fast Atom Bombardment
FID	Flame Ionization Detector
GC-AFS	Gas Chromatography Atomic Fluorescence Spectrometry

GC-FID	Gas Chromatography Flame Ionization Detector
GC-MS	Gas Chromatography Mass Spectrometry
GC-PFPD	Gas Chromatography Pulsed Flame Photometric Detection
GLC	Gas Liquid Chromatography
GSC	Gas Solid Chromatography
HPLC	High Performance Liquid Chromatography
HPLC-MS	High Performance Liquid Chromatography Mass Spectrometry
HS-SDME	Headspace Single Drop Microextraction
ICP-MS	Inductively coupled plasma mass spectrometry
ICR	Ion Cyclotron Resonance
ID^2MS	Double or Reverse Isotope Dilution
ID^3MS	Triple Isotope Dilution Mass Spectrometry
ID^4MS	Quadrupole Isotope Dilution Mass Spectrometry
IDMS	Isotope Dilution Mass Spectrometry
IPA	Isopropyl alcohol
IR	Infrared Spectroscopic Detector
LC	Liquid Chromatography
LC50	Lethal concentration required to kill 50% of the population
LC-ESI-MS-MS	Liquid Chromatography-Electrospray-MS-MS System
LC-UV	Liquid Chromatography Ultraviolet Detection
LLE	Liquid-Liquid Extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LPME	Liquid Phase Microextraction
MALDI	Matrix-Assisted Laser Desorption Ionization
MBT	Monobutyltin
MCF-7	Estrogen-dependent breast cancer cells
MeOH	Methanol
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MTBSTFA	N-tert-butyldimethylsilyl-N-methyltrifluroacetemide
$NaBEt_4$	Sodium Tetraethylborate
NMIs	National Metrology Institutes
NMR	Nuclear Magnetic Resonance

Q	Quadrupole
RSD	Relative standard deviation
SA-ID^{2}MS	Standard Addition Double Isotope Dilution Mass Spectrometry
SA-ID^{3}MS	Standard Addition Triple Isotope Dilution Mass Spectrometry
SA-IDMS	Standard Addition Isotope Dilution Mass Spectrometry
SD	Standard deviation
SDME	Single Drop Microextraction
SFODME	Solidified Floating Organic Drop Microextraction
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SS-LPME	Switchable Solvent Liquid Phase Microextraction
TBT	Tributyltin
TCD	Thermal Conductivity Detector
THF	Tetrahydrofuran
TOF	Time of Flight
TPhT	Triphenyltin
UA-DLLME	Ultrasound Assisted Dispersive Liquid-Liquid Microextraction
UV	Ultraviolet
UV-VIS	Ultraviolet-Visible
VA-DLLME	Vortex Assisted Dispersive Liquid-Liquid Microextraction

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ABSTRACT

Development of Analytical Strategies for Accurate and Precise Determination of Endocrine Disruptive Chemicals in Biological and Environmental Matrices

Sezin ERARPAT

Department of Chemistry

Master of Science Thesis

Advisor: Prof. Dr. Sezgin BAKIRDERE

Endocrine disruptive chemicals (EDCs) have a lot of importance due to their unfavorable effects on human health and environment even at trace levels. To date, it has become crucial in analytical chemistry to detect EDCs at trace levels with high accuracy and precision. In the scope of this thesis, 4-*n*-nonylphenol, monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT) were determined by gas chromatography mass spectrometry (GC-MS) after some preconcentration, derivatization, isotope dilution strategies.

Firstly, switchable solvent liquid phase microextraction (SS-LPME) was utilized for preconcentration of trace 4-*n*-nonylphenol from wastewater samples. With the SS-LPME conditions optimized, detection limit of direct GC-MS for the analyte was enhanced by 115 folds by the SS-LPME-GC-MS method. To achieve high accuracy and precision, quadrupole isotope dilution mass spectrometry (ID^4MS) was integrated to the optimized SS-LPME-GC-MS method. When the SS-LPME-ID^4MS was

applied to wastewater sample, percent recovery result was computed as 101.5 ±0.38% at 40 ng/g.

4-*n*-nonylphenol was also detected by combining binary solvent liquid phase microextraction (BS-LPME) technique and standard addition triple isotope dilution mass spectrometry (SA-ID^{3}MS). After performing optimization studies, 495.4 times enhancement in detection power was attained by the BS-LPME-GC-MS method. Recovery studies for infant formula and wastewater samples proved positive interferences in chromatographic signals. To suppress matrix effects, SA-ID^{3}MS method was applied to both samples at 50 ng/g levels. Percent recovery results for wastewater and infant formula samples were calculated as 100.3±1.6 and 99.4±1.7%, respectively.

The last study in this thesis was conducted for trace determination of MBT, DBT and TBT. Tetraethyl borate derivatization reaction and dispersive liquid-liquid microextraction (DLLME) technique were employed. After optimizing significant parameters, enhancements in detection power were recorded as 191.5 folds for MBT, 83.6 folds for DBT and 70.9 folds for TBT. Developed method was applied to anchovy and mytilus galloprovincialis samples with high percent recovery results.

Keywords: 4-*n*-nonylphenol, butyltin compounds, GC-MS, isotope dilution

ÖZET

Biyolojik ve Çevre Örneklerinde Bulunan Endokrin Bozucu Kimyasalların Yüksek Doğruluk ve Duyarlılıkta Tayinlerine Yönelik Analitik Stratejilerin Geliştirilmesi

Sezin ERARPAT

Kimya Anabilim Dalı

Yüksek Lisans Tezi

Danışman: Prof. Dr. Sezgin BAKIRDERE

Endokrin bozucu kimyasallar (EBK) eser seviyelerinin bile insan sağlığı ve çevreye olan olumsuz etkilerinden dolayı oldukça önem arz etmektedir. Bugüne kadar EBK'nın eser seviyelerde yüksek doğruluk ve kesinlikte ile tayini analitik kimya için çok önemli olmuştur. Bu tez kapsamında, 4-*n*-nonilfenol, monobütil kalay (MBK), dibütil kalay (DBK) ve tribütil kalay (TBK) bazı ön deriştirme, türevlendirme ve izotop seyreltme stratejileri sonrasında gaz kromatografisi kütle spektrometresi (GC-MS) ile tayin edilmiştir.

İlk olarak akıllı çözücü sıvı faz mikroekstraksiyon (AÇ-SFME) yöntemi eser seviyede 4-*n*-nonilfenol'ün atık su örneklerinden ön deriştirilmesi için kullanılmıştır. Optimize edilen AÇ-SFME koşulları ile ilgilenilen analit için GC-MS'in tespit gücü AÇ-SFME-GC-MS metodu ile 115 kat iyileştirilmiştir. Yüksek doğruluk ve kesinlik elde etmek amacıyla dörtlü izotop seyreltme kütle spektrometrisi (İS^{4}KS) optimize edilen AÇ-SFME-GC-MS metodu ile birleştirilmiştir. AÇ-SFME-İS^{4}KS atık su örneğine

uygulandığında yüzde geri kazanım sonucu 40 ng/g için %101,5 ±0,38 hesaplanmıştır.

4-*n*-nonilfenol ayrıca ikili çözücü sıvı faz mikroekstraksiyon (İÇ-SFME) tekniği ve standart katma üçlü izotop seyreltme kütle spektrometrisi (SK-İS^{3}KS) birleştirilerek tayin edilmiştir. Optimizasyon çalışmalarının gerçekleştirilmesinin ardından İÇ-SFME-GC-MS metodu ile tayin gücünde 495,4 kat iyileştirme elde edilmiştir. Bebek maması ve atık su örnekleri için geri kazanım çalışmaları kromatografik sinyallerde pozitif girişim olduğunu kanıtlamıştır. Matriks etkisini azaltmak amacıyla SK-İS^{3}KS metodu her iki örneğe 50 ng/g seviyesinde uygulanmıştır. Atık su ve bebek maması örnekleri için yüzde geri kazanım sonuçları sırasıyla %100,3±1,6 ve %99,4±1,7 olarak hesaplanmıştır.

Tez kapsamındaki son çalışma, eser seviyede MBK, DBK ve TBK'nın tayinleri için yapılmıştır. Tetraetil borat türevlendirme reaksiyonu ve dağıtıcı sıvı-sıvı mikroekstraksiyon (DSSME) tekniği kullanılmıştır. Önemli koşullar optimize edildikten sonra tayin gücündeki iyileştirme MBK için 191,5 kat, DBK için 83,6 kat ve TBK için 70,9 kat olarak kaydedilmiştir. Geliştirilen metot ançüez ve Akdeniz midyesi örneklerine yüksek yüzde geri kazanım ile uygulanmıştır.

Anahtar Kelimeler: 4-*n*-nonilfenol, bütil kalay bileşikleri, GC-MS, izotop seyreltme

1

Introduction

1.1 Literature Review

Humans, animals and all other living organisms have sophisticated and extensive chemical signaling systems that enable their biological developments and perform regular activities. To date, many studies have focused on environmental pollutants that have abilities to damage these sensitive and vital systems [1]. Two substantial systems that are responsible for communication between cells and regulation of muscle and organ activities are the nervous and endocrine systems. Specific hormones are secreted from endocrine glands like hypothalamus and thyroid and then give response to some stimuluses [2]. However, some pollutants can perturb the function of the endocrine system in living organisms. These pollutants and chemicals are known as endocrine disruptive chemicals/compounds (EDCs). These chemicals can alter, inhibit or affect the mechanism of hormone synthesis, secretion, transportation and metabolism [3].

There are many types of EDCs that are used in industrial, pharmaceutical or agricultural areas. Some examples of EDCs are phthalates, pesticides, organotin compounds, alkylphenols, dioxins, furans, parabens and polycyclic aromatic hydrocarbons. These kind of chemicals can enter the body from different sources such as water bodies, soils, sediments, personal care products and pharmaceuticals [2].

It has been reported that EDCs are found at trace levels in environmental resources [4]. Accurate and precise instrumental detection of EDCs is needed due to their occurrence in the environment and potential harm to humans, mammals and other organisms. Their detections at trace levels are commonly based on mass spectroscopic techniques and some hyphenated techniques such as gas chromatography mass spectrometry (GC-MS), high performance liquid

chromatography mass spectrometry (HPLC-MS) and liquid chromatography ultraviolet detection (LC-UV) [5]. Their occurrence at trace levels and the complexity of sample matrices resulted in the pursuit to develop effective analytical techniques for their quantification [6]. Determination of EDCs in complex matrices requires a sample preparation technique before their instrumental detections. Extraction is a sample preparation technique and it is broadly categorized into liquid-liquid extraction (LLE) and solid phase extraction (SPE). In the past three decades, very low amounts of extraction solvents and sorbents resulting in liquid phase microextraction (LPME) [7] and solid phase microextraction (SPME) [8] have been used for the extraction of analytes and high preconcentration. In some cases, derivatization techniques are employed in order to convert the analyte into a chemical form which is suitable for detection by the selected instrumental technique. For instance, volatility problems for GC systems and detection problems for LC systems can be solved by various derivatization techniques [9]. In addition to these problems, instrumental detections and sample preparation techniques affect the accuracy and precision of measurements [10]. However, isotope dilution strategies which are primary analytical techniques can be used to improve the accuracy of results and mitigate the effects of analyte loss in sample preparation and instrumental measurement [11].

1.2 Objective of the Thesis

The principal aim of this thesis is to quantify trace levels of selected EDCs at trace levels by GC-MS system. Since this instrumental system suffers from sensitivity related problems for trace determinations, different microextraction techniques were used to boost the sensitivity of this instrument for the selected analytes. GC-MS also requires volatile and thermally stable species which is a restrictive point for nonvolatile compounds. Derivatization methods were also applied for the determination of nonvolatile analytes for convenient determination by the GC-MS system. Accuracy, precision and mitigation of matrix effects were also improved by employing different isotope dilution strategies. The developed methods were validated and their applicability to different environmental and food samples was determined.

1.3 Hypothesis

4-*n*-nonylphenol, monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT) are examples of EDCs that are detrimental to humans, aquatic organisms and plants even at trace levels. For this reason, their determination at trace levels are extremely important in order to prevent environmental pollution and health disorders. GC-MS is a commonly used instrumental technique for the determination of these EDCs but is not proper for ppb or ppt level detections of analyte(s). Hence, some microextraction techniques should be coupled to GC-MS system in order to lower detection limits of the analytes. These microextraction techniques include switchable solvent liquid phase microextraction (SS-LPME), binary solvent liquid phase microextraction (BS-LPME) and dispersive liquid-liquid microextraction (DLLME), all known as green sample preparation techniques used in analytical chemistry. Optimization studies were performed for each system via a multivariate or univariate approach to achieve high extraction yields. Analytical figures of merit were evaluated and determined for all microextraction and isotope dilution systems. Wastewater samples were analyzed by SS-LPME and BS-LPME methods. Infant formula sample was analyzed by BS-LPME method while anchovy and mytilus galloprovincialis samples was analyzed by DLLME method. Spiking experiments were also conducted to verify accuracy and applicability of the proposed methods. Quadrupole isotope dilution mass spectrometry (ID^4MS) was applied to enhance the accuracy, precision and recovery results for 4-*n*-nonylphenol determination. Additionally, standard addition triple isotope dilution mass spectrometry (SA-ID^3MS) was used to overcome complicated matrix effects of wastewater and infant formula.

2

Literature Review

2.1 4-*n*-nonylphenol

4-*n*-nonylphenol is an alkylated phenolic compound and its physical state at room temperature is a yellow viscous liquid. It has low soluble in water medium (11.0 mg/L at 20 ^{0}C) [12]. 4-*n*-nonylphenol is a human-made compound and it is one of the alkylphenols (APs) produced by the degradation of alkylphenol ethoxylates (APEOs) [13], [14]. Its polyethoxylated compound is mainly used as surfactant due to its hydrophobic alkylphenol chain and hydrophilic ethylene oxide units [15]. There are several application areas of APEOs such as the detergent industry, paint ingredients, pesticide production and cosmetics [13]. However, 4-*n*-Nonylphenol has some endocrine disrupting properties even at low concentration levels which influences the growth and fertility of organisms [16].

The European Union (Commission Directive 2000/60/EC) listed 4-*n*-nonylphenol as a member of priority hazardous substances [17]. In spite of its adverse effects on human health and the environment, some countries still make use of this compound, leading to its wide occurrence in drinking water, wastewater and living organisms [18]–[20].

In the early 1930s, para-alkylated phenolic compounds like 4-*n*-nonylphenol were demonstrated as an estrogenic active compound [21]. It was established that para-alkylated compounds are capable of removing estrogen from the estrogen receptors [22]. As shown in Figure 2.1, nonylphenol and 17-β-estradiol resemble each other in terms of their molecular structures [23], [24]. Nonylphenol can react with estrogen receptor instead of the estrogen hormones because of this molecular similarity. Hence, nonylphenol and some nonylphenol ethoxylates are categorized in the list of EDCs and priority pollutants [17], [25], [26].

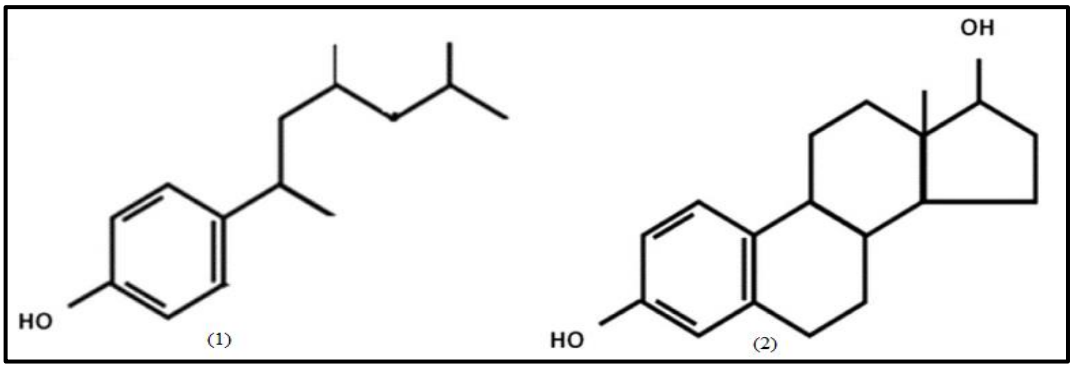

Figure 2.1 Molecular structures of nonylphenol (1) and 17-β-estradiol (2) [27]

It was also report that para-nonylphenols in polystyrene can stimulate the receptors which are responsible for estrogen-dependent breast cancer cells (MCF-7)[28] Another study proved that para-nonylphenols could trigger the feminization of male fishes with the production of vitellogenin in their bodies [29]. Nonylphenols or nonylphenol ethoxylates can enter the human bodies through cutaneous absorption, ingestion or inhalation [30]. Among these exposure ways, drinking water is mainly responsible for exposure to 4-*n*-nonylphenol [31].

4-*n*-nonylphenol has a half-live shorter than 24 hours in the human body. After its entry into the body, it is excreted as its glucuronides and sulfate conjugates [32]. Tolerable daily intake of 4-*n*-nonylphenol has been specified as 5.0 μg kg^{-1} body weight [33]. Its presence in the human body may cause a decline in sperm counts, and the formation of breast cancer [34].

A study on carcinogenicity for 28 weeks of exposure to this chemical showed the occurrence of lung adenomas and carcinomas in rats. Additionally, it was proved that 4-*n*-nonylphenol is highly dangerous for aquatic species. For example, LC_{50} values for *cyprinodon variegatus* (marine fish) and *salmo gairdneri* (freshwater fish) are stated as 0.31 mg/L and 0.56–0.92 mg/L, respectively [12]. For these reasons, 4-*n*-nonylphenol has to be detected at trace levels to alleviate or eliminate its estrogenic impacts.

2.2 Butyltin Compounds

Compounds composed of one or several covalent bonds between carbon and tin atoms are known as organotin compounds. Their presentative formula can be written as R_nSnX_{4-n} where R and X represent alkyl or aryl groups and anionic species

or groups, respectively. Tin is mostly found in its Sn(IV) form rather than Sn(II) form [35]–[37]. Alkylation or arylation degrees and number of carbon atoms in alkyl chain determines the hydrophobicity of these compounds [38]. Organotin compounds are produced annually at about 60,000 tons, making them one of the most used organometallic compounds. Their applications range from agriculture to medicine, industry to cancer chemotherapy [39]–[42].

Diethyltin diiodide was the first laboratory-made organotin compound, synthesized by Frankland in 1849. However, the number of studies on organotin compounds increased in the 1940s after the expansion of the plastic industry. Their applications in the plastic industry was to add thermal and light stable properties and to catalyze silicone and polyurethane foams [43]. In the 1960s, organotin compounds were commonly implemented in the production of antifouling agents for vessels [43]–[45]. Furthermore, it has been of great use in the production of disinfectant agents, algicides and pesticides [46]. Triphenyltin acetate (Brestan), triphenyltin hydroxide (Duter) and tricyclohexyltin hydroxide (Plictran) are sold as fungicides and insect killers. It is known that their biodegradation is quite slow and their accumulation in soil and water causes pollution [40], [47], [48].

High persistency in environmental bodies, toxicity and migration through the trophic levels are some major concerns on the widespread usage of organotin compounds [43], [49]. Various studies on the toxicity of these compounds revealed that 1.0 ng/L of TBT and triphenyltin (TPhT) compounds result in damages to the endocrine disrupting system of marine organisms [50]. For example, it is proved that there is a relationship between TBT concentration and reproductive organs failure in female gastropods on a coastal region of Portugal [51]. Other symptoms of marine organisms subjected to organotin compounds are low growth rate, low photosynthesis and cell death. Smith and his coworkers investigated the behavior of adult corals when exposed to sediments with 160 mg/kg of TBT and the study was concluded with 38% death of the corals [52].

Organotin compounds have different pathways to the human body. Marine species (oyster, salmon, mussel, etc.) and plants contaminated with these toxic compounds can find a place in the cuisines of coastal countries. TBT, DBT, MBT and TPhT were detected in some seafoods on the Portuguese market. Mussel samples contained the

highest concentration of organotin compounds with an average of 170 ng/g in a study performed by Santos *et al*. Thereby, these compounds are picked up by marine organisms, enter the food chain and eventually reach humans [53]. Several studies have focus on the TBT compound and its degradation products in marine organisms and the environment. The occurrence of butyltin compounds in mussels, fish, snails and dolphins prove their persistence in the environment [54], [55].

Organotin compounds can accumulate in organs such as the liver, brain and kidneys [56]of higher species and pass through the skin, placenta and blood brain barrier [57], [58]. These compounds can affect the central nervous system, cardiovascular system, skin, respiratory system and irritate the eyes [59]. The toxicity level of organotin compounds is associated with the length of alkyl chain they are attached to. Among them, trialkylated forms are the most toxic, followed by the dialkylated and monoalkylated forms. Organotin with ethyl groups are known as the most toxic derivatives [42]. Organotin compounds with short alkyl chains such as trimethyltin and triethyltin chlorides lead to neuronic degradation, and cerebral edema, whereas compounds with moderate number of alkyl chains such as tributyltin and tripropyltin chlorides have immunotoxic properties [60]. These lipophilic compounds are more toxic due to the reduction of thymidine integration at micromolar concentrations and cytocidal toxicity to thymocytes [61].

The first actions to minimize TBT usage was undertaken in France in 1982 and then in the UK in 1985. A sharp decrease in the number of oysters was associated with TBT impacts on oysters in the coastal regions of France in the 1970s. The reduction of oyster numbers was related to high yachting activity in the UK. According to British legislations, TBT-containing paints are prohibited and the maximum level of organotin concentration has been set to 20 ng L^{-1} in water [62]–[64]. Some European countries have also regulated the usage of TBT-containing antifouling paints [17] and the International Maritime Organization prohibited the shipment of organotin based paints in 2008 [65], [66]. Even though many attempts to eliminate organotin compounds from industrial, agricultural and environmental areas, their persistency in water, soil and other parts of the environment requires sensitive, accurate and green determination methods.

2.3 Sample Preparation

An analytical procedure comprises of different steps; sampling, sample preparation, purification of analyte(s), classical or instrumental analysis and data processing. There are various types of sample preparation techniques and examples are given in Table 2.1 according to their working principles [67].

Table 2.1 Some examples of sample preparation methods with their main principles [67]

Methods	**Main Principle**
Grinding	Mechanics
Blending	
Sieving	
Centrifugation	Gravity
Sedimentation	
Methylation	Derivatization
Silylation	
In situ labelling	Labelling
On column labelling	
Edman degradation	Degradation
Sanger reaction	
Liquid-liquid extraction	Phase partition
Cloud point extraction	
Soxhlet extraction	
Solid phase extraction	Sorption
Stir bar sorptive extraction	

Sample preparation methods are broadly classified as chemical and non-chemical processes. Labelling, derivatization and decomposition are chemical processes used to make the analyte(s) detectable by an instrument. Grinding, blending and sieving are mechanical and non-chemical processes preferred for solid samples. Centrifugation and sedimentation are also non-chemical processes used to separate heterogeneous mixtures. Phase partitioning is a method used to separate analyte(s)

from one phase to the other before their determination by classical or instrumental techniques [67].

Sample matrices with various complexities are one of the most common problems in qualitative/quantitative determination of analytes. It is a challenging task to quantify trace levels of analytes especially in environmental samples which are usually known for their complexity. Sample preparation prior to instrumental analyses is a significant step because of complicated sample matrices and low concentration of analyte(s) [68], [69]. This step can mainly reduce/eliminate interferences from matrix constituents, isolate and preconcentrate the analyte(s), and convert the analyte(s) into a chemical form consonant with a detector [67], [70].

2.3.1 Microextraction Techniques

When developing an analytical method, the choice of sample preparation method has great importance in terms of results reliability and accuracy [71]. Selective extraction methods are required to obtain measurable levels of analyte(s) from different types of samples [72]. In analytical chemistry, liquid-liquid extraction (LLE) and solid phase extraction (SPE) are well-known extraction methods used to purify analytes from sample matrices [71], [73].

LLE is dependent on partition of analyte(s) from aqueous sample into an organic solvent. It is important to select an appropriate organic solvent as extraction solvent for the collection of analyte(s) from the aqueous solution because the analyte should be more soluble in the extraction solvent. In addition, the organic solvent used in LLE processes should have a different density from the aqueous phase to attain a complete separation of phases [74]. SPE needs an adsorbent filled in a column, a cartridge or a disk in which sample solution is loaded into these apparatuses and the analyte(s) of interest is held onto the adsorbent. In this extraction method, intermolecular forces play important roles to get interactions between the adsorbent and analyte(s) [75].

In recent years, the concept of green chemistry has been considered and incorporated into the conventional extraction methods. Hence, researchers seek new and green alternatives to toxic organic solvents, or employ techniques that consume very low solvent and sample volumes [76]. Microextraction techniques

firstly proposed by Pawliszyn *et al*. avoid large volume of toxic chemicals and high volumes of sample solutions. Less extraction steps facilitate faster sample preparation and less errors resulting from other chemicals used in the extraction protocol [77]. Solid phase microextraction (SPME) and liquid phase microextraction (LPME) are the main categories of microextraction techniques [78].

2.3.1.1 Solid Phase Microextraction (SPME)

Solid phase microextraction is one of the significant methods for the isolation of analyte(s) from solid, liquid and gas samples. Its first introduction to literature was by Pawliszyn *et al.* [79]. This method includes two stages which are partition of the analyte(s) between extractant and sample, and desorption of the analyte(s) [80]. Analyte is adsorbed onto a sorbent and then is desorbed for its instrumental detection. Its superiority to the conventional SPE technique is that SPME devices are proper for on-site analysis and their adsorption-desorption processes are fairly easy [81].

SPME provides simultaneous extraction, sample preparation and sample introduction in only one step, thus being an important part of an analytical protocol in routine analysis [82], [83]. In 1993, Supelco started to commercialize SPME devices with different coatings such as polydimethylsiloxane, polyacrylate and carbowax divinylbenzene. The SPME method has had several breakthroughs in terms of its geometry, coating materials and configurations [84]. Another important feature about this technique is the compatibility with different sophisticated instruments like GC, LC and capillary electrophoresis (CE). They can also be employed as an extraction, preconcentration and sampling, thereby increasing the demand for SPME devices [85]. On the other hand, SPME devices have short life cycle and easily degrade, making the method quite expensive [86].

2.3.1.2 Single Drop Microextraction (SDME)

In the mid of 1990s, Dasgupta and his research group used a liquid drop in order to extract ammonia and sulfur dioxide from air samples [87]. This group also generated a silica capillary tube as a supporter of the drop to gather gas form analytes [88]. However, the first publication regarding single drop microextraction coupled to chromatographic analysis was suggested by Cantwell's group. In that

study, a Teflon rod was used to hold 8.0 µL droplet of octane in a stirring sample solution and the solvent on the rod was injected into GC system [89], [90].

In this extraction/preconcentration method, analyte(s) can be collected into microliter volumes of solvent but diffusion rate of analyte(s) molecules, temperature of sample solution and viscosity of the solvent are the most influential parameters. Nowadays, magnetic stirring, mechanical and syringe plunger motions have been used to improve analyte diffusion from liquid or gas samples. SDME is applied under non-equilibrium (kinetically controlled) conditions because the equilibrium can only be established according to contact area, equilibrium constant, extracted volume, etc. [91].

There are several advancements that have been made for SDME method to improve its applicability and operation. SDME is divided into two groups namely direct-immersion single drop microextraction (DI-SDME) (illustrated in Figure 2.2) and headspace single drop microextraction (HS-SDME). The former is designed for non-volatile species while the latter is developed for semi-volatile and volatile species [92].

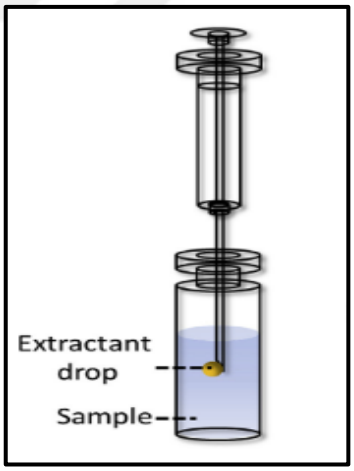

Figure 2.2 A representation of DI-SDME technique [93]

SDME techniques are compatible with green chemistry which promotes minimal volume of solvents and low amount waste products. However, it poses great challenge to successfully immerse the µL drop into a stirred solution [92].

2.3.1.3 Solidified Floating Organic Drop Microextraction (SFODME)

This microextraction method established by Khalili Zanjani *et al.* offers simplicity and high preconcentration factors with a good separation of analytes. It requires water immiscible solvents with low density and melting points at room temperature. Some solvents that meet these criteria include 1-undecanol, 1-dodecanol and n-hexadecane. After injection of these solvents into an aqueous solution, the solution is kept under temperatures lower than their melting points. A solid droplet is observed at the top of the solution due to the solvent's low density. This solid can be easily taken into a vial and allowed to melt before instrumental determination [94]. A major setback to this method is the usage of high viscous solvents which may cause problems to the sample introduction unit of some instruments especially for flame atomic absorption spectrophotometer. Low molecular weight alcohols can be added to make the solvents less viscous and suitable for instrumental measurement [95].

2.3.1.4 Liquid Phase Microextraction (LPME)

Liquid phase microextraction was introduced to the scientific community in order to eliminate or minimize challenges of the SPME technique [96]. Its principle is based on microliter level of a water immiscible solvent which can collect the analyte(s) of interest from the aqueous solution [97]. Low usage of organic solvents, fastness and simplicity are some benefits of the LPME technique relative to the traditional LLE method. It has evolved with different modifications to fulfil analytical, instrumental or operational needs for years [98]. Some types of LPME methods are dispersive liquid-liquid microextraction (DLLME) [99], switchable solvent liquid phase microextraction (SS-LPME) [100] and binary solvent liquid phase microextraction (BS-LPME) [101].

LPME has become an indispensable technique used to extract and concentrate target analyte(s) from environmental [102], [103], food [104], [105] and pharmaceutical samples [106], [107].

- **Dispersive Liquid-Liquid Microextraction (DLLME)**

Dispersive liquid-liquid extraction was firstly reported by Rezaee *et al.* for the determination of sixteen polycyclic aromatic hydrocarbons in water samples by gas

chromatography flame ionization detector (GC-FID). One novelty of this method was the usage of dispersive solvents to increase the distribution of extraction solvents throughout an aqueous solution. The extraction solvents are usually water immiscible solvents like chloroform, carbon tetrachloride and tetra chloroethylene. Dispersive solvents should be soluble in both selected extraction solvent and aqueous phase. Mass transfer of analyte(s) occurs between extraction solvent and aqueous solution with the help of dispersive solvent [99]. DLLME method consists of steps illustrated in Figure 2.3 as injection of extraction and dispersive solvent into the aqueous solution, formation of a cloudy solution, centrifugation for phase separation and collection of the organic phase for instrumental determination of analyte(s).

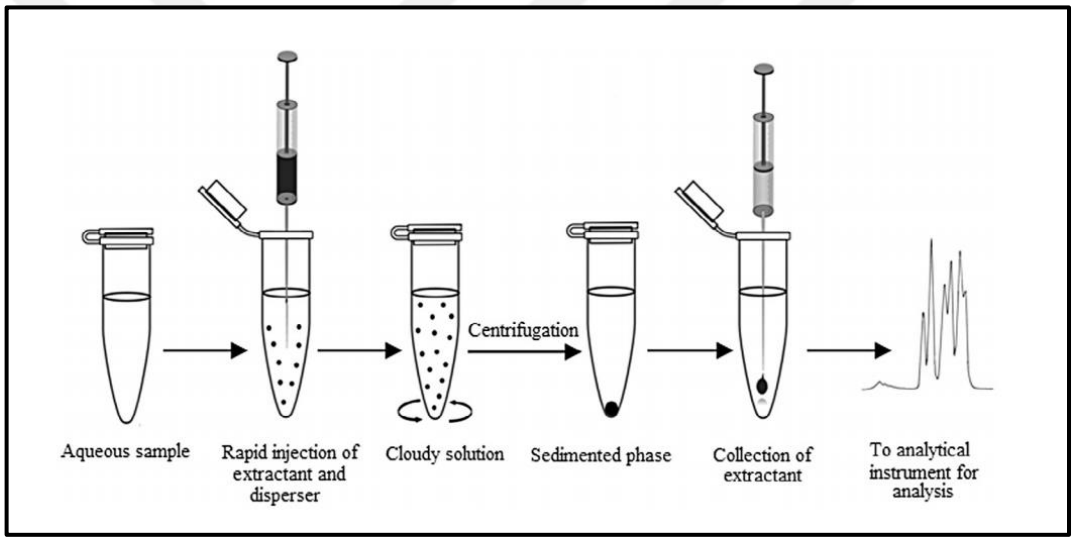

Figure 2.3 Schematic illustration of DLLME technique [108]

Extraction efficiency of this method is dependent on several factors such as type and volume of extraction solvent, type and volume of dispersive solvent, volume of sample solution, extraction period, pH value and ionic strength. The most important part of DLLME technique is the selection of a proper extraction and dispersive solvent. Organic solvents with densities higher or lower than water can be viable candidates as extraction solvent while dispersive solvents should have miscibility in both extractant and aqueous phases [109]. In addition to these solvents, different types of mixing processes have been performed to elevate the interaction between organic and water phases. Vortex assisted dispersive liquid-liquid microextraction (VA-DLLME) [110], ultrasound assisted dispersive liquid-liquid microextraction

(UA-DLLME) [111] and magnetic stirring assisted dispersive liquid-liquid microextraction [112] are some sample agitation process used. The pH value of a solution has a considerable impact on the extraction yields. Specifically, metal determinations with complex formation reactions are highly dependent on pH value in terms of complex formation and stability [78].

In recent years, this microextraction strategy has gained momentum thanks to its low consumption of solvents, easy application and high extraction outputs [109], [113]. Trace levels of organic and inorganic analytes can be determined by the DLLME technique with several instruments. Pesticides [114], flame retardants and plasticizers [115], organotin compounds after derivatization with sodium tetraethylborate [116], herbicides [117], fatty acids after derivatization with ethyl chloroformate [118] are some examples of DLLME combined with gas chromatography. Cadmium [119], selenium [120], gold [121], copper [122] and other heavy metals have been preconcentrated by DLLME for determination by atomic absorption spectrometry with different atomization methods.

Polar and non-volatile compounds can also be determined by DLLME-GC methods after some derivatization reactions. Pre-DLLME, post-DLLME and in situ DLLME derivatization modes are some types of the combination of DLLME and derivatization methods. Pre-DLLME derivatization can be applied when the extraction rate is higher than the derivatization reaction. Post-DLLME derivatization is proper for highly reactive derivatization reagents to matrix components. In situ DLLME derivatization can be employed if there is no influence caused by matrix components on the derivatization reaction [123].

- **Switchable Solvent Liquid Phase Microextraction (SS-LPME)**

Several chemical processes include different reagents, catalysts, products which generally demand different polarity solvents. These kinds of processes can lead to high amounts of generated waste and excess cost [124]. It is a general prospect that waste products should be recycled or reduced in some ways. In this regard, Jessop *et al.* [125] discovered that the polarity of some solvents can be switched reversibly by adding some reagents. A nonpolar solvent was treated with 1.0 bar CO_2 which can react with alcohol, amidine or guanidine groups of the relevant solvent. After this reaction, the polarity of solvent was turned into a polar form proposed as its

alkylcarbonate anion. The polar form can be reversed back to its nonpolar form via elimination of CO_2 and/or introduction of nonacidic gas like $N_{2(g)}$ [124]. For these reasons, switchable solvents have water miscible property with the occurrence of CO_2 and immiscible property if some triggers like $N_{2(g)}$, $Ar_{(g)}$, air, acid and base are mixed with these solvents as presented in Figure 2.4 [126]. In this reaction, CO_2 is generally preferred in terms of its agreement to the concept of greenness [127]. Switchable solvents also provide energy saving extraction processes because there is no need to evaporate the extract after extraction [128].

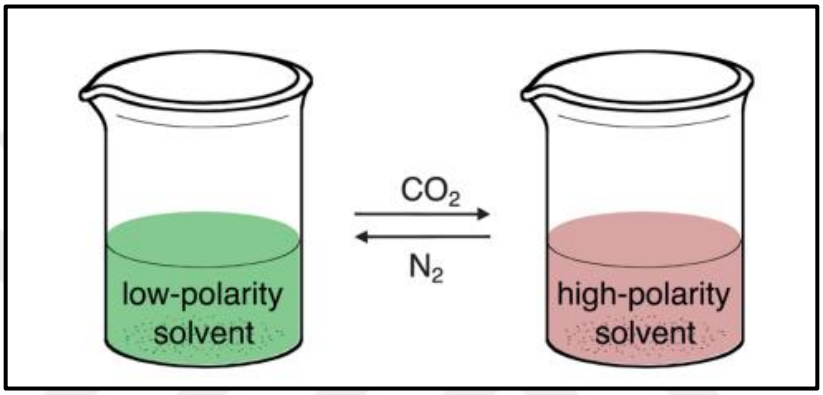

Figure 2.4 A representation of switchable solvent production [124]

Switchable solvents can be employed for different purposes such as catalyst in a reaction medium, separation and extraction. In particular, these solvents can be utilized in multiple processes when a specific solvent needs to be used and removed before the next step of the process [129]. In microextraction methods, switchable solvents diminish extraction steps and usage of toxic solvents [130]. Switchable solvent liquid phase microextraction (SS-LPME) is an efficient method for the extraction of organic and inorganic analytes. In 2015, cadmium in water, vegetable, fruit and cigarette samples was determined by SS-LPME-FAAS system. A limit of detection (LOD) value of 0.16 ng/mL was achieved with 93-100% recovery results. A tertiary amine (triethylamine) was turned into its carbonate form for the production of switchable solvent [127]. In another study, chlorflurenol and procymidone were preconcentrated by a switchable solvent synthesized with the tertiary amine N,N-dimethylbenzylamine, water and dry ice. About 21 and 159 folds improvement in detection power for chlorflurenol and procymidone were recorded for the SS-LPME-GC-MS method [131].

- **Binary Solvent Liquid Phase Microextraction (BS-LPME)**

Binary solvent liquid phase microextraction, reported by Maham *et al.* [132], is totally based on the DLLME principles that provide preconcentration of organic and inorganic analytes using a mixture of extraction and dispersive solvent. However, analytes with different polarity can be efficiently extracted by different organic solvents when a simultaneous determination of these analytes is conducted. Generally, common extraction solvents are selected in order to extract all analytes but this could lead to low preconcentration efficiency for some analytes. In literature, some authors solved this problem by combining two or more extraction solvents [132]–[135]. For example, diuron ($\log K_{ow}$=2.9), teflubenzuron ($\log K_{ow}$=4.3), atrazine ($\log K_{ow}$=2.7), desisopropylatrazine ($\log K_{ow}$=1.1) and desethylatrazine ($\log K_{ow}$=1.5) were preconcentrated by a mixture of chloroform and dichloromethane with acetonitrile as a disperser solvent [136]. In another study, BS-LPME method was applied to some herbicides for their separation and preconcentration. Dichloromethane and 1,2-dichloroethane were employed as extraction solvents to improve the overall extraction efficiency of the herbicides studied [137].

BS-LPME is an ecofriendly analytical method that provides high extraction efficiency for the preconcentration and separation of various analytes. In addition to these advantages, solvents lighter and heavier than water can be combined in the same extraction procedure. As an example, patulin was extracted from aqueous solution with a mixture of ethyl acetate and chloroform, and compared to an extraction with only chloroform. The extraction output of the solvent mixture gave results seven times higher than that of chloroform. Hence, light organic solvents that settle as upper phase in DLLME procedures can be selected as one of the extraction solvents in the BS-LPME method [132].

2.3.2 Derivatization Techniques

Derivatization is one of the most important branches in analytical chemistry. Spectroscopic techniques such as fluorimetry, ultraviolet-visible (UV-VIS), and nuclear magnetic resonance (NMR), mass spectroscopic techniques and chromatographic techniques frequently require a derivatization step before instrumental analyses [138]. The main principle of derivatization is any

transformation to change the physical or chemical property of an analyte(s) by a proper reaction. It can be done for an improvement in chromatographic behavior, peak resolution or instrumental detection. The nature of an analyte(s) and the analytical method are the most crucial factors in choosing a suitable derivatization reaction. Different derivatization techniques can be compatible with a variety of extraction methods such as SPME and SDME [139], several instrumental techniques such as GC [140], matrix laser desorption ionization (MALDI) [141] and liquid chromatography (LC) [142]. The main purpose of employing derivatization in GC analyses is to generate less polar, thermally stable and volatile derivatives of the analyte(s). In liquid chromatography, some groups can be added to the analyte(s) for efficient detection [143].

2.3.2.1 Derivatization for Liquid Chromatographic Techniques

Generally, liquid chromatographic systems require derivatization reaction before or after separation on column in order to overcome detection-related problems. In this regard, different derivatization reagents can be used to enable the detection of analyte(s) by several HPLC detectors [144].

HPLC is a good choice for nonvolatile compounds but it has a problem to detect trace level analytes in biological matrices or analytes having low ultraviolet (UV) absorption. For these reasons, some chromophore groups can be added to the analyte molecule to augment its UV detection. For example, aliphatic amines or amino acids can be chemically derivatized by m-toluoyl chloride and benzoyl chloride to increase UV absorption [143].

HPLC with fluorometric detection is a highly sensitive LC technique and it is mainly used in biomedical analyses because analytes like drugs and metabolites are present in complex matrices at pg mL^{-1} and ng/mL levels. Dansyl chloride, fluorescamine, 1- or 9-anthroylnitrile are frequently used reagents to produce fluorescent derivatives of analytes [138]. For a specific example, 9-fluorenylmethyl chloroformate can be used for the derivatization of free amino acids [145].

Ion pairing is another technique used to increase extraction output of ionic species into organic solvents in LC systems. It is a pre-column derivatization technique that changes the physical or chemical nature of analytes in the mobile phase. It can

eliminate peak tailing, enhance peak resolution and detection outputs. Note that, trifluoroacetic acid was used as an ion pairing reagent for the determination of amphetamine by reversed phase liquid chromatography-electrospray tandem MS system (LC-ESI-MS/MS) and tailing caused by the interaction between C18 column and protonated analyte was eliminated [143]. Chemiluminescence detectors are also coupled to HPLC systems but it requires derivatization with a chemiluminescent reagent such as N-(4-aminobutyl)-N ethylisoluminol, lucigenin or peroxyoxalate [138]. Electrochemical detectors provide high sensitivity in HPLC systems on condition that the analyte has an electroactive property. In order to make an analyte electroactive, some reagents such as 2,4,6-trinitrobenzenesulfonic acid for amines, 2,4-dinitrophenylhydrazine for aldehydes and 4-nitrobenzyl bromide for carboxyl groups can be used to derivatize the analytes [138].

2.3.2.2 Derivatization for Gas Chromatographic Techniques

Derivatization is a requirement for gas chromatographic techniques for three main reasons; The first one is to obtain volatile species of the analyte [146]. The second is to block hydroxyl groups to minimize tailing problems related to the interaction between hydroxyl groups and capillary column used in GC. The third is to obtain thermally stable compounds for GC analyses. The overall effect of derivatization enhances peak shapes, resolution, chromatographic behaviors and sensitivity of analytes [9].

- **Silylation Reactions**

Hydroxyl, amine, thiol, carboxylic acid, and phosphate functional groups that pose separation and detection problems can be reacted with silyl compounds like N-tert-butyldimethylsilyl-N-methyltrifluroacetemide (MTBSTFA) and N,O-bis(trimethylsilyl)-trifluoroacetamide [147]. Acidic hydrogen is replaced by an alkyl silyl group as shown in Figure 2.5. However, silylation reactions generally taken place in aprotic solvent medium [143].

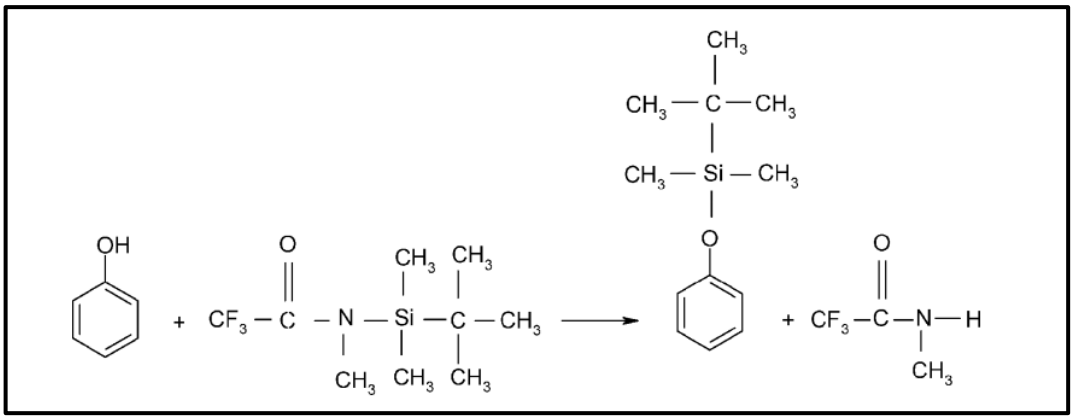

Figure 2.5 An example for derivatization of hydroxyl groups by MTBSTFA [148]

- **Acylation Reactions**

This type of reactions is an alternative to silylation reactions in terms of getting high volatility and low polarity forms of analytes. Hydroxide, sulfhydryl and amine groups can be transformed into esters, thioesters and amides, respectively. Hence, thermal stability and nonpolar nature can be gained by the analyte molecule as illustrated in Figure 2.6 [143].

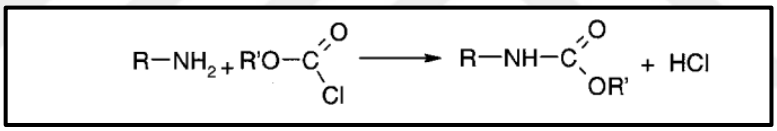

Figure 2.6 An example of acylation reaction of amines [149]

Perfluoracyl groups are common reagents for derivatization of analytes which are to be detected by electron capture detector (ECD). Perfluoro acid anhydrides, fluoracylimidazoles and nonhalogenated groups are well-known acylation reagents that give the analyte molecule nonpolar features [143].

- **Alkylation Reactions**

It is based on nucleophilic displacement of an active hydrogen in carboxylic acid, alcohol, thiol, amine or amides with an alkyl group. For the derivatization of carboxylic acids, there is an advantage over their silylation reaction in that it gives more stable derivatives. Pentafluorobenzyl bromide is a common reagent used to convert acids and phenols into ester and ether derivatives, respectively [143].

- **Esterification Reactions**

Esterification reaction which is known as a reaction between acid and alcohol groups with the help of a catalyst can be employed as a derivatization reaction for ester derivatives of analytes having carboxylic acid and other acid groups, as given in Figure 2.7 [150]. It is a rapid reaction and the length of alkyl groups can be changed for different chromatographic retentions [151].

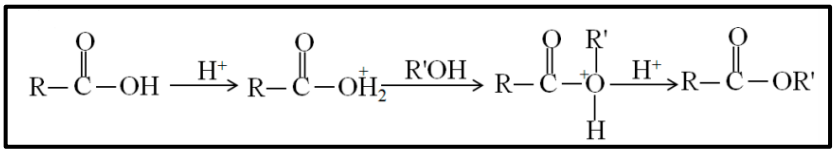

Figure 2.7 An example of esterification reaction [152]

- **Other Reactions**

There are additional derivatization techniques for specific group of molecules. Diazomethanes, chloroformate, borohydride and tetraethylborate reactions will be discussed below.

- **Diazomethane Reaction**

Diazomethane (CH_2N_2) is used for the derivatization of carboxylic acids even though it has toxic and carcinogenic properties. At the end of this reaction, methyl ester derivatives are produced at room temperature [143]. It is useful for the extraction and analysis of chlorinated phenol fungicides, hydroxyether bactericides, and chlorophenoxy acid herbicides from biological and environmental matrices [153].

- **Chloroformate Reaction**

Chloroformates possess similar reactivity to acyl chlorides. Their function is to produce carbamates from the analytes with hydroxyl or amine groups. This reaction can be implemented in both organic and aqueous medium. It is an advantage for simultaneous derivatization and extraction of analytes [154].

- **Borohydride Reactions**

Metallic species are derivatized by different reagents to improve their chromatographic separation, thermal stability and make them identifiable by GC-MS systems [143]. Borohydride reaction can be applied to organometallic species when

the final detection is done by atomic absorption spectrophotometry (AAS) systems. A typical example of this application the derivatization of mercury and organomercury species by sodium borohydride and then extracted with a dry solvent. Hydride species were detected by gas chromatography-atomic fluorescence spectrophotometry GC-AFS [155]. An example of borohydride reaction can be seen in Figure 2.8 for organotin compounds [156].

$$R_nSn^{(4-n)+} + NaBH_4 + H^+ \rightarrow R_nSnH_{(4-n)} + H_2$$

Figure 2.8 An example of borohydride reaction for organometallic species [156]

2.4 Chromatographic Techniques for the Determination of Organic and Organometallic Compounds

Chromatography is basically described as an analytical technique used to separate analyte(s) of interest from different matrices. Analytes are dissolved in liquid, gas or supercritical fluid mobile phases and then travel on a solid or viscous liquid stationary phase. This process as illustrated in Figure 2.9 shows different migration rates of analytes which leads to their separation from each other [157].

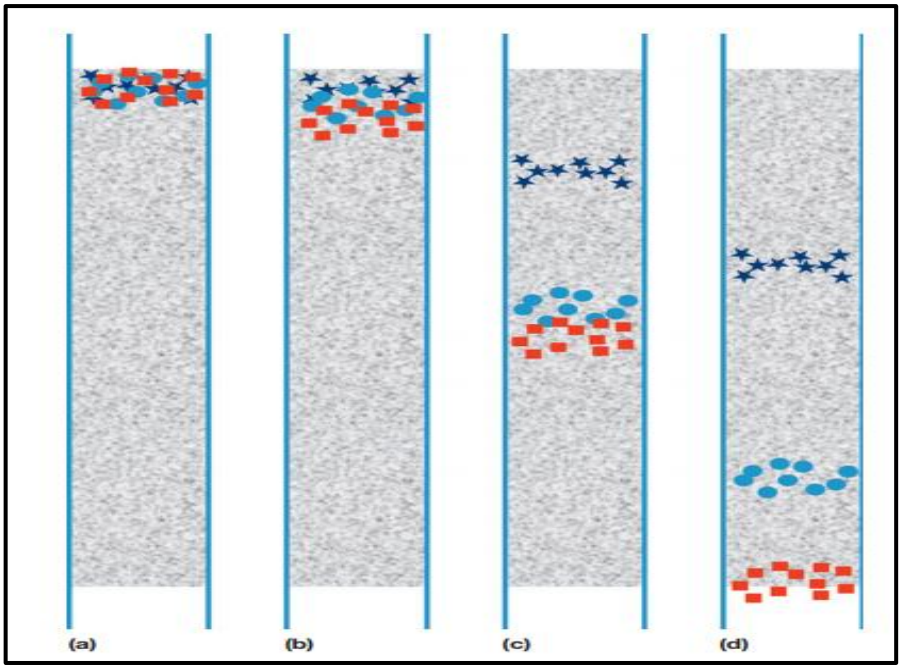

Figure 2.9 The basis of chromatography a) sample is introduced to the column, b-d) mobile phase is poured into the column and analytes travel through the column with different times [157]

Chromatographic techniques can be categorized according to their physical setup of stationary phase, physical state of mobile phase and separation mechanism. The first one has two subcategorizes: planar and column chromatography. The second has three physical state of eluent which are gas, liquid and supercritical fluid. The last one has five mechanisms of separation and these are adsorption, partition, ion exchange, size exclusion and affinity [157], [158].

The principle of *like dissolves like* can be said to be the fundamental principle of partition chromatography, where analytes are adsorbed onto a stationary phase. Nonpolar analytes are highly adhered to the nonpolar stationary phase and vice versa. In ion exchange chromatography, ions are separated according to their charges. Coulombic interactions are effective in the separation of ionic analytes. The other chromatographic separation technique is size exclusion or gel permeation chromatography, which separates analytes in terms of their molecular sizes [157].

2.4.1 Liquid Chromatography (LC)

Liquid chromatography (LC) was firstly applied for the separation of plant pigments by M.S. Tswett, who was a Russian-Italian botanist studying on cell physiology. A calcium carbonate adsorbent was employed as stationary phase while petroleum ether and ethanol were utilized as mobile phases in his study [159]. Today, LC is associated with high performance liquid chromatography (HPLC) which has a wide range of application including food, pharmaceutical and clinical areas. Analytes are separated according to the type of stationary phase employed. When a polar stationary phase like silica is chosen, analytes are separated from each other by dipole-dipole and hydrogen bonding interactions. An appropriate mobile phase is selected from nonpolar solvents like chloroform, hexane and dichloromethane. This chromatographic mode is known as normal phase liquid chromatography. Another mode is called reverse phase liquid chromatography and it consists of nonpolar stationary phases and polar mobile phase. The interaction between the nonpolar stationary phase and analytes is mainly non-specific hydrophobic interactions [160]. A general scheme of an HPLC system is given in Figure 2.10.

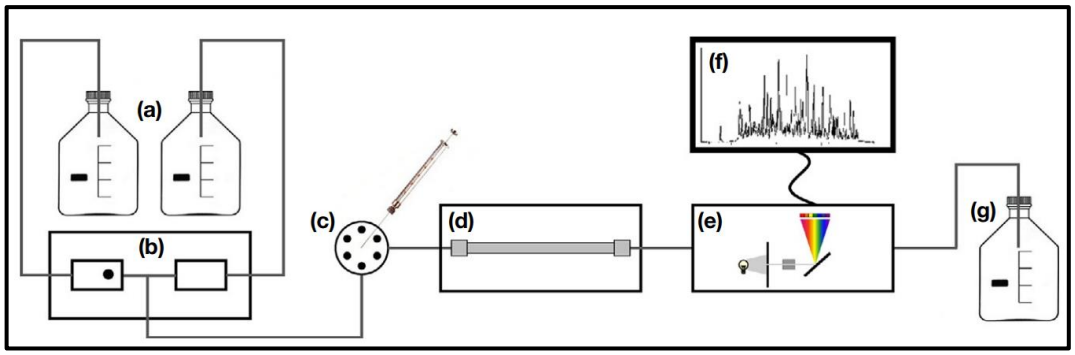

Figure 2.10 A schematic diagram for an HPLC system; a) Solvent reservoir; b) pump; c) sample injection; d) column; e) detector(s); f) data processing; g) waste [160]

In HPLC systems, analytes are eluted from the column based on their interaction with the stationary phase. More interactions lead to a late elution of the analyte while less interactions results in a quick elution. Different eluents such as organic solvents and buffer solutions can be selected as mobile phase [160]. Many types of detectors such as UV, florescence, electrochemical, mass spectrometer and refractive index can be integrated into HPLC systems [161].

2.4.2 Gas Chromatography (GC)

Gas chromatography based on the separation of the vaporized sample components thanks to the distribution between a mobile gaseous phase and a liquid or a solid stationary phase. In this type of chromatography, mobile phase transfers the analyte(s) through the column without interacting with them. Gas liquid chromatography (GLC) and gas solid chromatography (GSC) are the types of gas chromatography but the former has few applications. The analyte is partitioned between a gaseous mobile phase and a liquid phase [162].

GC has an important role and many applications in modern day analytical chemistry. It was invented by Martin and James in 1952, whose study was about the separation of volatile fatty acids with the principle of partition chromatography [163].

GC has is mainly used for the separation, identification and quantification of volatile and semi volatile compounds. Its separation principle is based on the vaporization of the sample and then the separation of the analytes according to their partition between the stationary phase and mobile phase in a column. The sample constituents elute through of gas stream and are converted to a signal by an

appropriate detector. Analyte(s) is sample can be identified in terms of their retention times after matching with their pure standards analyzed under the same conditions as the sample [164], [165].

GC can be performed for the analysis of mixture that consists of thermally stable compounds with boiling points from zero to over 700 K. Derivatization to enhance the volatility property of a compound may change this temperature range. The basic components of GC system comprises of an injection port, a column (in an oven), a carrier gas, a detector and a data processing system [166].

2.4.2.1 Injection port

In the injection port, the sample should be injected in a short time and residues of the sample should be cleaned from the inlet port before subsequent chromatographic runs. This is to prevent broadening of peaks and poor resolution for analytes of interest.

2.4.2.6 Column

The column is the most significant component of gas chromatography system because an efficient separation is dependent on the quality of the column. Packed columns that are 1-5 m long and have internal diameters of 1-5 mm were used in the early years of GC applications. However, resolution of this column is affected by pressure decrease on the resistance to gas flow. Capillary columns solved the resolution the problems of the packed column. Its inner surface was coated with a thin film (wall coated open tubular) or coated with a thin film on a solid material (support coated open tubular) [166].

Several column materials have been used up to now, including copper, nickel, stainless steel, nylon tubing and glass columns. Fused-silica columns invented by Dandeneau and Zerenner are quite flexible, stable and chemically inert [167]. It is made of purified silica containing very low amounts of metal oxides and polyimide layer for the protection of the column. Many compounds except for low-molecular weight compounds can be analyzed with the fused-silica column [166].

2.4.2.7 Carrier gas

Mobile gaseous phase in GC systems should be an inert gas such as helium, argon, nitrogen and sometimes hydrogen. Its flow rate is regulated by electronic pressure controllers. Flow rates of 25-150 mL min^{-1} and 1-25 mL min^{-1} can be selected for packed columns and open tubular capillary columns, respectively. Inlet pressure of the carrier gas (10-50 psi) should be above room pressure [168].

2.4.2.8 Detectors

One of the significant components in GC systems is the detector which considerably impacts sensitivity and selectivity of the system. A detector should possess some properties like good sensitivity and reproducibility, robust and reliable outputs, fast and easy to operate and also cost efficient. There are different sorts of detectors utilized for different purposes in GC systems. Some of the common detectors in GC systems are thermal conductivity detector (TCD), flame ionization detector (FID), electron capture detector (ECD), atomic emission detector (AED), infrared spectroscopic detector (IR) and mass spectrometer detector (MS)[169].

- **Mass spectrometer (MS)**

Mass spectrometer is used to ionize the eluted analytes from the GC column in a vacuum region and measure their mass/charge (m/z) ratios. The major ions in the GC-MS system have only one charge but aromatic compounds ionized with electron impact ionization (EI) have double-charged ions. It is stated that m/z ratios of the ions are the mass of the ion when the single charge are the most abundant [170], [171]. A mass spectrometer generally consists of an ion source, a mass analyzer and a detector [172].

- **Ion Sources**

The MS is an essential tool in terms of the ionization of different compounds. Mass spectrum of an analyte is basically based on ion fragments formation. Ion sources currently available include electron impact (EI), chemical ionization (CI), fast atom bombardment (FAB), matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) [158].

The EI source is a common ion source in which works by employing high energy electrons for the bombardment of molecules. Positively charged ions produced after the electron bombardment are passed through the mass analyzer via electrostatic repulsion [168].

CI introduced by Munson and Field [173] depends on the ionization of a reagent gas and its subsequent reaction analyte molecules in the gas phase to attain ion/molecule species [174].

MALDI is a desorption ion source commonly coupled with ESI and employed in the protein sequencing and proteomic studies [175]. In MALDI, the solid sample dissolved in an appropriate solvent is treated with excess amount of matrix and placed on MALDI plate or air-dried. After co-crystallization with the matrix, it is brought into a gaseous phase by a laser beam. The sample with the matrix absorb the laser beam that leads to desorption and ionization of the sample [176].

ESI is applied to nonvolatile, thermally instable inorganic species and complex biological compounds [177]. The sample dissolved in a polar solvent is nebulized into the ion source with a thin needle which has an electrical potential of 3-4 kV. Droplets charged in the needle are vaporized with a neutral gas (usually nitrogen). The repulsive forces between the charged ions result in the desorption of gases into the gas phase [178], [179].

- **Mass Analyzers**

The mass analyzer in MS systems distinguish ions with regard to their m/z ratios. Magnetic sectors or electrically driven analyzer are made use of in the ion separation process. Several mass analyzers are being employed and these include quadrupole (Q), time of flight (TOF), ion cyclotrone resonance (ICR) and ion trap. Mass analyzers vary their responses to different mass ranges and their efficiencies with tandem mass spectrometry (MS/MS) [176], [180].

Quadrupole mass analyzers introduced by Paul Wolfgang [181] are mass filters that are easily coupled with the GC system. There are four cylindrical rods as illustrated in Figure 2.11. Two rods are connected to direct current and the other two to radio frequency voltage. Ions pass through the analyzer through the transducer with proper voltages. Positively charged ions are driven in the direction of negatively

charged rods. The mass spectrum is obtained by observing the voltages on the rods [176], [182].

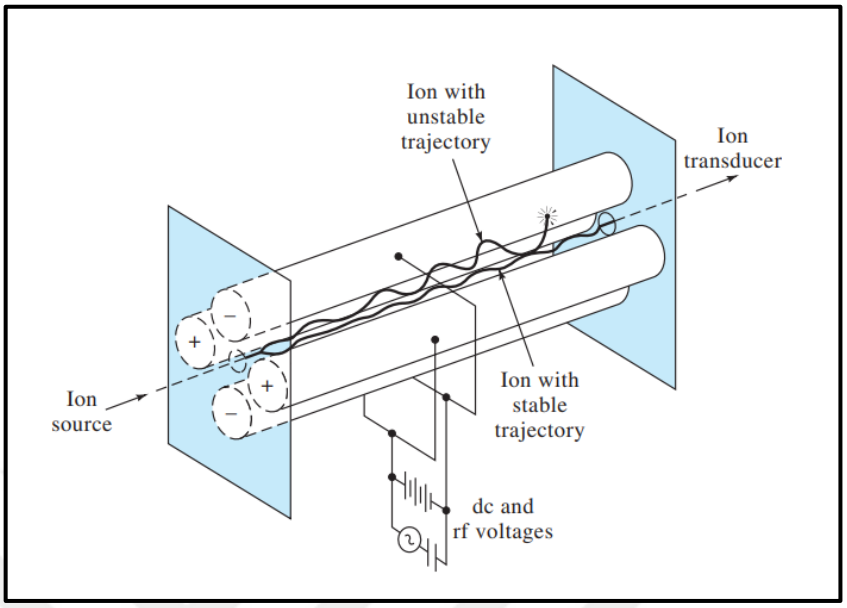

Figure 2.11 A schematic presentation of quadrupole mass analyzer [168]

In TOF analyzers, ions produced in the ionization chamber fly freely in a tube of 1–2 m in length [176], [183]. Ions with high m/z ratios reach the detector slower than those with low m/z ratios. High mass range can be analyzed with TOF in a short time, but it has drawbacks regarding resolution and sensitivity [168].

Ion cyclotron resonance uses a magnetic field and its name defines the frequency of an ion's circular motion within a magnetic field. Ions with high m/z ratios have low cyclotron frequency and in an analogous way, ions with low m/z ratios have high cyclotron frequency [182].

2.5 Experimental Design

Optimizing is known as the steps taken to increase the performance of a process, a system or a product. Optimization is a term generally used in analytical chemistry to find optimum conditions for high responses [184]. The general aspect for optimization is one variable at a time, where the effect of a factor on response is studied by fixing the other factors to constant values. However, this approach does not show the interaction effects between the factors and it needs excess workload to obtain a representative experimental data [185].

Scientists have sought for more information with less experimental work, chemical usage and cost. Experimental design techniques are utilized to obtain high outputs in scientific researches [186]. It actually dates back to the late of 1980s, where Deming and Schoenmakers used experimental design in their chromatographic studies [187], [188]. Experimental design approaches can be simplified as linear of quadratic equations which give some information about the corresponding system.

Some statistical terms are described below in order to make the experimental design more understandable.

Response is a measurement or a quantity which can be peak areas for chromatographic studies.

Factor is effect on the response and it is separated as controlled and uncontrolled factors. Variable, parameter or predictor are synonyms for factor.

Level refers to values that are studied for a factor. It can be different temperature values for temperature factor. Some types of designs are named according to the level of factors, like two level design and three level design.

Response surface is the relationship between the response and the factor(s) [189].

Model is the equation which shows the relationship between the response and the factors.

Effect is the coefficient of a term in the model equation [185].

Residue is defined as the difference between calculated and experimental data for a specific condition. A fitted model to experimental results should have a low residual level [190].

In an experimental design, the result is dependent on the experimental conditions, thus, it can be written as a function of the experimental data:

$$y = f(x) \tag{2.1}$$

The simplest function is the linear model, in which linear relations between the experimental data and the response are determined. For instance, when x_1 and x_2 are the factors, the linear model will be written as:

$$y = b_0 + b_1 x_1 + b_2 x_2 + residual \tag{2.2}$$

However, the interactions between the factors are not presented in the Equation (2.2). A second order interaction model have these interaction terms in its equation as:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + residual \quad (2.3)$$

These two models described above are usually used for robustness tests and screening studies. Another mathematical model is the quadratic model that highlights not only linear interactions but also non-linear interactions between the experimental data and the response. The quadratic model for two factors is as follows:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 + residual \quad (2.4)$$

This equation has some unknown coefficients (b_0, b_1, b_2 …) that are to be calculated. This model can be varied according to different designs. Quadratic models can be employed in order to find an optimum value [185].

There are several kinds of multivariate approaches in analytical chemistry. Response surface methodology is one of the most common technique which refers to a sum of mathematical and statistical approaches on the basis of the fit of polynomial equation to experimental data [190].

2.5.1 Factorial Designs

Factorial design is the experimental design technique used to investigate all combinations between factors and their levels. When k number of factors with 2 levels are studied, the factorial design will be 2^k number of experiments. Figure 2.12 demonstrates full factorial design with examples of two factors two levels and two factors and three levels designs [185]. Full factorial designs are usually used in chromatographic and isolation studies [190], [191].

Fractional factorial designs have less combinations than full factorial designs because of excess experiments with high factors and levels [185]. Fractionation can provide the scientist with information about the main effects using less amount of experiments. 2^{k-p} number of experiments are necessary to perform the fractional factorial design, where k is the factor number and p is the size of fraction.

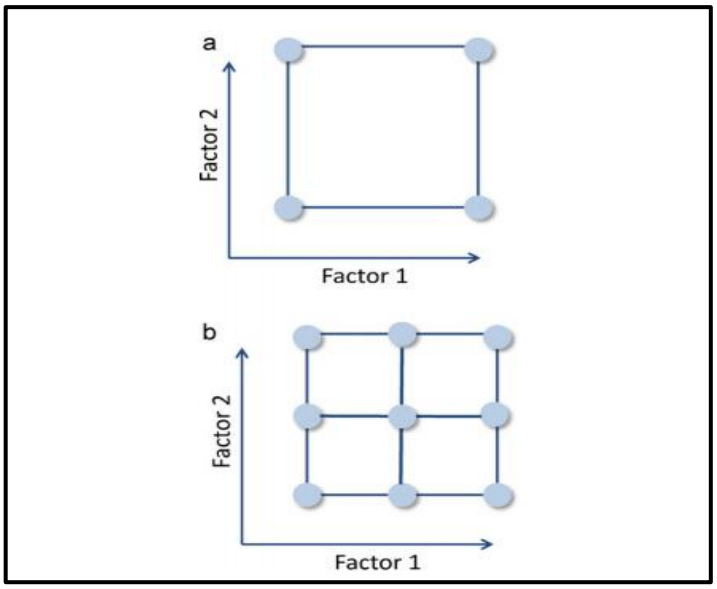

Figure 2.12 Schematic representation of full factorial designs a) Two factors two levels design and b) Two factors three levels design [186]

2.5.2 Plackett-Burman Design

Plackett-Burman design, proposed in 1946 by Plackett and Burman is based on two levels designs with maximum 4n-1 factors [192] . This design can be used to conduct robustness tests because a base level for each factor should be determined for the application of this design. For example, if the base level and base plus level are denoted as – and +, the design gives an estimation of the response between – and + levels [186].

2.5.3 Central Composite Design

The two levels designs can allow linear interactions and linear response but not to obtain non-linear interactions and maximum points (maxima). On the other hand, full factorial designs result in excess number of experiments if the levels of factors are increased to more than two levels. Central composite design makes it possible to investigate higher factor levels via the combination of two-level design, axial and center points. The axial and factorial points have equal distance from the center point, as can be seen in Figure 2.13 For this design, $L^k + Lk + n_c$ number of experiments where *L*, k and n_c are the levels, the factors and the replication number of center points are needed [186]. Besides, extreme points are examined in the central composite design [191].

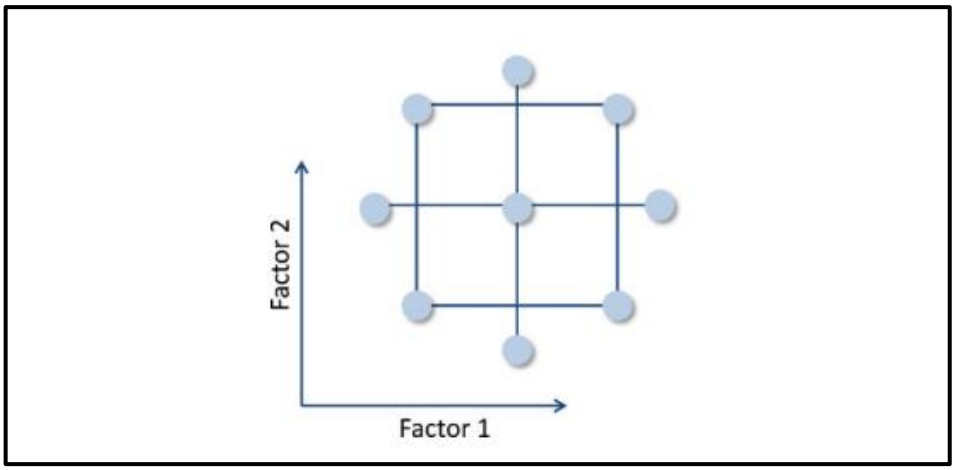

Figure 2.13 Central composite design for two factors [186]

2.5.4 Box-Behnken Design (BBD)

Box Behnken Design is a response surface model in which factors are investigated with factor combinations at the mid-edge of the variable space and at the center point [193]. Three or more factors with three levels can be optimized with Box-Behnken Design [194]. It contains a specific domain of 3^k factorial design. The experimental points are placed into a hypersphere that all points have the same distance from the central point [195]. $2k\ (k-1)+ n_c$ points are needed to carry out this type of design. It includes lower experiment number than central composite design but the same number with Doehlert design [186]. However, this design does not show rotatable property like central composite design and cannot be applied to more than three levels.

BBD is a low cost and useful design for evaluation of interactions between factors without performing extreme points. For example, the extreme points could be high temperature and pressure in a process which cannot be applied [191].

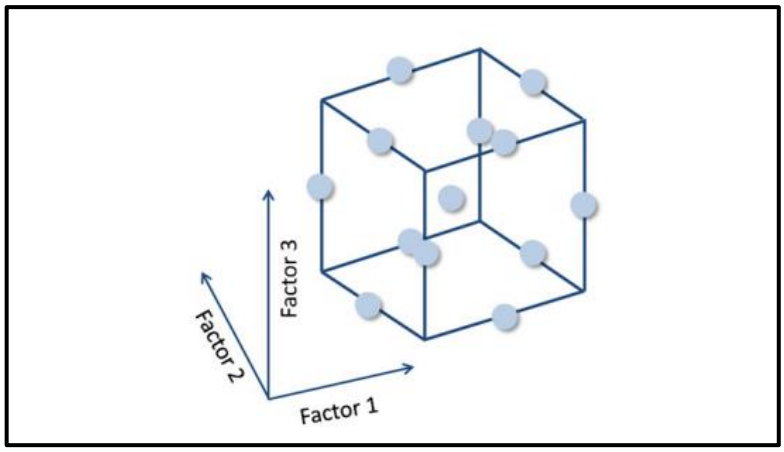

Figure 2.14 Box-Behnken Design for three factors [186]

2.6 Isotope Dilution Strategies

Isotope dilution is a popular calibration technique when mass spectrometry is used as detection system [196]. Today, isotope dilution is an absolute analytical method with traceable outputs according to the International System Units. It is used during the certification of reference materials, development of reference procedures, and validation of analytical methods [196]. All isotopes of one element possess identical chemical behavior with different masses. Mass spectrometry allows detection of these mass differences between isotopes of the same element. Furthermore, compounds consisting of the element or its other isotope can be differentiated by mass spectrometry. For example, hydrogen, carbon, nitrogen, oxygen and sulfur are polyisotopic elements and their isotopes bound to the same compound and yielding mass differences. This concept is utilized in a relatively new measurement technique known as isotope dilution [197].

Isotope dilution mass spectrometry (IDMS) is dependent on the measurement of a mixture that consists of a known amount of isotopically labelled/enriched material and a sample (sample blend) or a blank (calibration blend). The ratio of natural and labelled material is determined by a mass spectrometer after complete equilibration is reached in the blend, so the analyte in the sample is detected with good accuracy [198]. A basic demonstration for the concept of IDMS is given in Figure 2.15.

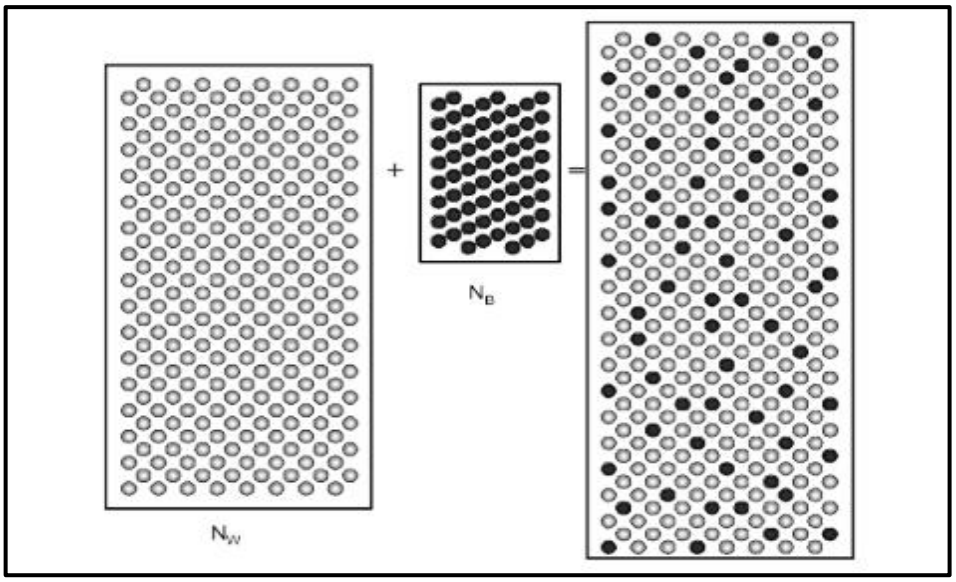

Figure 2.15 The concept of IDMS given by white and black marbles [196]

White marbles in the first box can be found if a known number of black marbles in the second box are added and mixed with the first box. It should be noted that both White and Black marbles are of the same size and weight but not their colors, and the number of black marbles is exactly known. A portion of marbles is sampled from the third box (mixture) and white marbles and black marbles are counted to find the total number of white marbles in the first box (N_W). A simple Equation (2.5) can be obtained as:

$$N_W = N_B \frac{W}{B} \qquad (2.5)$$

The main advantage in this experiment is that, every portion from the mixture has the same white marbles/black marbles ratio when the mixture is well admixed. Hence, the loss of white marbles is not a problem after they are mixed with the black marbles [196].

IDMS strategies are used for both organic [199], [200] and inorganic [201], [202] analyte determinations in several kinds of matrices with high accuracy. For this reason, National Metrology Institutes (NMIs) often use these methods in making comparison with other NMIs for certification of reference materials [203].

IDMS was first developed for elemental analyses in the 1950s and for organic analyses in the 1970s. This method is recognized as a definitive method thanks to

its high accuracy, sensitivity and interference-free measurements [204]. Chronological advancement of isotope dilution is shown in Table 2.2.

Table 2.2 Chronological advancement of isotope dilution [205]

Year	Name	Study
1923	Hahn	Used ^{231}Pa to find the yield of the isolation of ^{234}Pa
1925	Ehrenberg	Used ^{212}Pb to measure the fraction of $PbCO_3$ precipitated by known amounts of CO_3^{2-}. It is a radio reagent method, not isotope dilution.
1932	Hevesy and Hobbie	Used ^{210}Pb to find the yield of separation of Pb from minerals
1933	Starik	Independently used ^{210}Pb for the same purpose
1934	Hevesy and Hofer	Used $2H_2O$ for the determination of water content of the human body.
1940	Rittenberg and Foster	First applied the term 'isotope dilution' analyzed amino and fatty acids in biological media with tracers containing 2H.
1946	Keston	Introduce derivative isotope dilution
1946	Henriques and Margnetti	Reverse isotope dilution in biology.
1948	Bloch and Anker	Introduce the method of double dilution.
1950	Keston	First application of double isotope derivative dilution.
1957	Bojesen	The first application of superior double isotope derivative dilution.
1958-1960	Ruzicka; Suzuki; Zimakov and Rozhavskii	Introduced independently the principle of sub-stoichiometry in isotope dilution.
1960	Ekins and Sgherzi; Yalow and Berson	Used a combination of isotope dilution with saturation analysis.
1961	Stary	Developed a theory of sub-stoichiometry
1964	Landgrebe McClendon and DeVoe ; Krys	Proposed the combination between isotope dilution and the concentration dependent distribution method in inorganic analysis.
1965	Suzuki and Kudo	Introduced isotope dilution after neutron activation
1967	Adamek and Obrusnik; Braun and Ladanyi	Independently introduced displacement sub-stoichiometric analysis.

Isotope dilution is basically based on a mixture consisting of a known amount of isotopically labelled material (D) and sample (N). The analyte concentration in the sample (n_N) can be calculated when the amount and isotopic composition are known as presented in the equations below [206];

$$n_N = n_D \cdot \frac{(R_D - R_{ND})}{(R_{ND} - R_N)} \cdot \frac{\sum R_N}{\sum R_D} \quad (2.6)$$

$$w_N = w_D \cdot \frac{(R_D - R_{ND})}{(R_{ND} - R_N)} \cdot \frac{\sum R_N}{\sum R_D} \cdot \frac{m_{D(ND)}}{m_{N(ND)}} \cdot \frac{M_N}{M_D} \quad (2.7)$$

where; w_N and $m_{N(ND),aq}$ are the mass fraction and the mass of the analyte (N) in the sample, respectively. In this sense, instrumental bias should be considered to reduce uncertainties. A mass bias calibration factor is used to alleviate these problems [207];

$$R = K.r \quad (2.8)$$

where; r is the response of isotope amount ratio, and R is the certified value (true amount). For ICP-MS measurements, mass bias is a physical effect that heavier isotopes are easily transported than lighter isotopes [197].

In literature, there are four different strategies for isotope dilution analyses. These are single, double (reverse), triple and quadrupole isotope dilution methods.

2.6.1 Single Isotope Dilution Mass Spectrometry (IDMS)

Single isotope dilution mass spectrometry requires the measurement of isotope ratio of ND blends (r_{ND}) if other variables are exactly known. The equations for which the K factor is eliminated from Equation (2.7) are given by:

$$w_N = w_D \frac{R_D - K.r_{ND}}{K.r_{ND} - R_N} \cdot \frac{m_{D(ND),aq}}{m_{N(ND),aq}} \cdot \frac{\sum R_N}{\sum R_D} \cdot \frac{M_N}{M_D} \quad (2.9)$$

$$w_N = w_D \cdot \frac{(r_D - r_{ND})}{(r_{ND} - r_N)} \cdot \frac{\sum r_N}{\sum r_D} \cdot \frac{m_{D(ND)}}{m_{N(ND)}} \cdot \frac{M_N}{M_D} \quad (2.10)$$

The mass bias can be deleted from the Equation (2.9) considering all blends have similar mass bias on the instrumental signal. Equation (2.10) refers to the fundamental of isotope dilution strategies but it needs some improvements in terms of the excess number of variables such as w_D, r_D, r_N, $\sum R_N$, $\sum R_D$, M_N and M_D. For this

reason, a second blend containing standard solution of analyte (N*) and known amount of isotopic labelled material (D) was introduced to isotope dilution strategies [208].

2.6.2 Double Isotope Dilution Mass Spectrometry (ID²MS)

Isotopic labelled or enriched materials are usually released to the chemical market in small amounts and this causes difficulties in their weighing processes. However, mass fraction of D (w_D) in any blend is a dependent variable for IDMS strategy and it should be known precisely for accurate measurements to be made. To eliminate this variable, an additional blend can be prepared with standard solution of analyte (N*) and D. This new blend is known as calibration blend in the isotope dilution strategies [208].

Equation (2.7) can be written for N*D blends to screen mass fraction of D (w_D) as following:

$$w_D = w_{N^*} \cdot \frac{R_{N^*} - R_{N^*D}}{R_{N^*D} - R_D} \cdot \frac{m_{N^*(N^*D),aq}}{m_{D(N^*D),aq}} \cdot \frac{\sum R_D}{\sum R_{N^*}} \cdot \frac{M_D}{M_{N^*}} \qquad (2.11)$$

The above equation is derived from Equation (2.7) and w_D, $\sum R_D$ and M_D can be deleted to give:

$$w_N = w_{N^*} \cdot \frac{R_{N^*} - R_{N^*D}}{R_{N^*D} - R_D} \cdot \frac{R_D - R_{ND}}{R_{ND} - R_N} \cdot \frac{m_{N^*(N^*D),aq}}{m_{D(N^*D),aq}}$$

$$\cdot \frac{m_{D(ND),aq}}{m_{N(ND),aq}} \cdot \frac{\sum R_N}{\sum R_{ND^*}} \cdot \frac{M_N}{M_{N^*}} \qquad (2.12)$$

At this point, analyte in the sample solution and standard solution has identical isotopic composition and this eliminates M_N, M_{N^*}, R_N and R_{N^*} variables. This approach may not be valid for metal determinations. The sample may have different isotopic composition, for example, $^{207}Pb/^{206}Pb$ isotope ratio can be varied for some materials in a 10% range. Therefore, these differences should be considered in metal determinations with double isotope dilution strategies [208].

$$w_N = w_{N^*} \cdot \frac{R_N - R_{N^*D}}{R_{N^*D} - R_D} \cdot \frac{R_D - R_{ND}}{R_{ND} - R_N} \cdot \frac{m_{N^*(N^*D),aq}}{m_{D(N^*D),aq}} \cdot \frac{m_{D(ND),aq}}{m_{N(ND),aq}} \qquad (2.13)$$

If the mass bias factor is applied for Equation (2.13), it is established as:

$$w_N = w_{N*} \cdot \frac{(R_N - R_{N*D})}{(R_{N*D} - R_D)} \cdot \frac{(R_D - R_{ND})}{(R_{ND} - R_N)} \cdot \frac{m_{N*(N*D)}}{m_{D(N*D)}} \cdot \frac{m_{D(ND)}}{m_{N(ND)}} \quad (2.14)$$

A different approach to ID^{2}MS is known as exact matching method, used to make the isotope measurements irrelevant to the mass fraction of the sample (w_N) [209]. It can be achieved by preparing ND and N*D blends with equal amounts of sample/standard solution and isotopic labelled material (r_{ND}=r_{N*D}). This approach is expressed as:

$$\frac{m_{N^*(N^*D),aq}}{m_{D(N^*D),aq}} \cdot \frac{m_{D(ND),aq}}{m_{N(ND),aq}} = \left(\frac{m_{N^*(N^*D),aq}}{m_{N(ND),aq}} \cdot \frac{m_{D(ND),aq}}{m_{D(N^*D),aq}} \right) \approx 1 \quad (2.15)$$

$$w_N \approx w_{N^*} \quad (2.16)$$

Finally, r_D is not a variable for ID^{2}MS analysis [208].

2.6.3 Triple Isotope Dilution Mass Spectrometry (ID^{3}MS)

Triple isotope dilution mass spectrometry is an alternative method to double dilution and exact matching methods. It can eliminate direct measurement of r_D by adding a new calibration blend [210], [211]. In other words, there are two double dilution equations in this approach which obviates the r_D value as shown in the equations below:

$$w_N = w_{N*-1} \cdot \frac{(r_N - r_{N*D-1})}{(r_{N*D-1} - r_D)} \cdot \frac{(r_D - r_{ND})}{(r_{ND} - r_N)} \cdot \frac{m_{N*(N*D-1)}}{m_{D(N*D-1)}} \cdot \frac{m_{D(ND)}}{m_{N(ND)}} \quad (2.17)$$

$$w_N = w_{N*-2} \cdot \frac{(r_N - r_{N*D-2})}{(r_{N*D-2} - r_D)} \cdot \frac{(r_D - r_{ND})}{(r_{ND} - r_N)} \cdot \frac{m_{N*(N*D-2)}}{m_{i(N*D-2)}} \cdot \frac{m_{D(ND)}}{m_{N(ND)}} \quad (2.18)$$

$$w_N = \frac{m_{N^*-2} r_1 + m_{N^*-1} r_2}{r_3} \cdot \frac{m_{D(ND),aq}}{m_{N(ND),aq}} \quad (2.19)$$

$$m_{N^*-i} = w_{N^*-i} \frac{m_{N^*(N^*D-i),aq}}{m_{D(N^*D-i),aq}} \quad (2.20)$$

$$r_1 = (r_{ND} - r_{N^*D-1})(r_N - r_{N^*D-2}) \quad (2.21)$$

$$r_2 = (r_{ND} - r_{N^*D-2})(r_{N^*D-1} - r_N) \quad (2.22)$$

$$r_3 = (r_{ND} - r_N)(r_{N^*D-1} - r_{N^*D-2}) \quad (2.23)$$

Equation (2.19) is generated by considering one sample blend (ND) and two calibration blends (N*D-1 and N*D-2). Another approach to conduct ID^{3}MS is to prepare two sample blends (ND-1 and ND-2) and one calibration blend (N*D) as given by [208]:

$$w_N = \frac{r_{3^*}}{m_{N-2}r_1^* + m_{N^*-1}r_2^*} \cdot \frac{m_{N^*(N^*D),aq}}{m_{D(N^*D),aq}} \tag{2.24}$$

$$m_{N-i} = \frac{1}{w_{N^*}} \frac{m_{N(ND-i),aq}}{m_{D(ND-i),aq}} \tag{2.25}$$

$$r_{1^*} = (r_{N^*D} - r_{ND-1})(r_N - r_{ND-2}) \tag{2.26}$$

$$r_{2^*} = (r_{N^*D} - r_{ND-2})(r_{ND-1} - r_N) \tag{2.27}$$

$$r_{3^*} = (r_{N^*D} - r_N)(r_{ND-1} - r_{ND-2}) \tag{2.28}$$

Exact matching method can be applied to ID^{3}MS analysis. Although ID^{2}MS and ID^{3}MS have the same approach for exact matching modelling, there is a conceptual difference in that, ID^{2}MS-exact matching eliminates the numerical value of r_B while ID^{3}MS-exact matching cancels out r_D, analytically [208].

$$w_N \approx w_{N^*-1} \tag{2.29}$$

2.6.4 Quadrupole Isotope Dilution Mass Spectrometry (ID^{4}MS)

When a third calibration blend (N*D-3) is added to ID^{3}MS, it is termed quadrupole isotope dilution mass spectrometry (ID^{4}MS). This approach was introduced to remove sample-related problems. Two sample blends (ND) and two calibration blends (N*D), or three sample blends (ND) and one calibration blend (N*D), or one sample blend (ND) and three calibration blends (N*D) are three designs for ID^{4}MS analysis. The third design is expressed as follows [208]:

$$w_N = -\frac{m_{N*-2}\cdot m_{N*-3}\cdot r_4 + m_{N*-1}\cdot m_{N*-3}\cdot r_5 + m_{N*-1}\cdot m_{N*-2}\cdot r_6}{m_{N*-1}\cdot r_4 + m_{N*-2}\cdot r_5 + m_{N*-2}\cdot r_6} \cdot \frac{m_{D(ND),aq}}{m_{N(ND),aq}} \tag{2.30}$$

$$m_{N*-x} = w_{A*-x} \cdot \frac{m_{N*(N*B-x)}}{m_{B(N*\dot{I}-x)}} \tag{2.31}$$

$$r_4 = (r_{N\dot{1}} - r_{N*D-1}) \cdot (r_{N*D-2} - r_{N*D-3}) \quad (2.32)$$

$$r_5 = (r_{ND} - r_{N*D-2}) \cdot (r_{N*D-3} - r_{N*D-1}) \quad (2.33)$$

$$r_6 = (r_{ND} - r_{N*D-3}) \cdot (r_{N*D-1} - r_{N*D-2}) \quad (2.34)$$

The exact matching method ($r_{ND}=r_{N*D-1}$) is also incorporated in ID^{4}MS strategy. Hence, there is no direct measurement of isotopes, only mixtures are analyzed by a mass spectrometer [208].

2.6.5 Standard Addition Isotope Dilution Mass Spectrometry (SA-IDMS)

Isotope dilution has great accuracy and precision for the determination of both organic and inorganic species. Mass spectrometry is coupled to this method in order to measure the isotopic composition of sample and calibration blends. It is assumed that the measured isotopic ratios are not affected by sample matrix. However, there is an influence on the measured isotopic ratios because of matrix components [212]. Examples for this problem include double spike calibration in geoscience [213], measurements on ICP-MS [214] and ESI-MS [215]. The disadvantages of sample matrix can be expressed as the Achilles heel of quantitative analysis [215]. In literature, Pagliano and Meija proposed a new isotope dilution strategy in order to deal with matrix effects on the measured isotope ratios [212].

2.6.5.1 Single Standard Addition Isotope Dilution Mass Spectrometry (SA-IDMS)

Single standard addition isotope dilution mass spectrometry is started with amount-balanced equation as follows:

$$n_{i,N,N*,D} = \sum_{E=N,N*,D} x_{i,E}\, w_E m_E M_E^{-1} \quad (2.35)$$

This equation forms the following equation for a ternary mixture containing N, N* and D. It also creates the fundamentals of the combination of standard addition and isotope dilution method.

$$R_{N,N*,D} = \frac{\sum_{E=N,N*,D} R_E x_{k,E}\, w_E m_E M_E^{-1}}{\sum_{E=N,N*,D} x_{k,E}\, w_E m_E M_E^{-1}} \quad (2.36)$$

In classical isotope dilution strategies, binary mixtures (ND or N*D mixtures) are employed but in SA-IDMS, ternary mixtures (NN*D) are used and this is analogous with the classical standard addition calibration method. The equation written below represents the SA-IDMS strategy.

$$w_N = -w_{N*} \cdot \frac{m_{N*-1}}{m_{N-1}} + w_D \cdot \frac{m_{D-1} \cdot (R_D - R_1)}{m_{N-1} \cdot (R_1 - R_{N)}} \cdot \frac{M_N}{M_D} \tag{2.37}$$

However, w_D cannot be exactly known just as in IDMS strategy. The solution to eliminate the w_D term is to apply double isotope dilution strategy [212].

2.6.5.2 Double Standard Addition Isotope Dilution Mass Spectrometry (SA-ID²MS)

In order to cancel the mass fraction of isotopic labelled material (w_D), the equation below for double standard addition isotope dilution mass spectrometry (SA-ID²MS) can be used.

$$w_N = w_{N*} \cdot \frac{\begin{matrix} -m_{N*-1} \cdot m_{D-2} \cdot (R_1 - R_{N*}) \cdot (R_D - R_2) \\ -m_{D-1} \cdot m_{N*-2} \cdot (R_D - R_1) \cdot (R_{N*} - R_2) \\ +m_{D-1} \cdot m_{N-2} \cdot (R_D - R_1) \cdot (R_N - R_2) \end{matrix}}{m_{N-1} \cdot m_{D-2} \cdot (R_1 - N) \cdot (R_D - R_2)} \cdot \frac{M_N}{M_{N*}} \tag{2.38}$$

On the other hand, isotopic composition of D is still a problem which also seen in ID²MS strategy. This term can be eliminated by using an isotopic material which do not contain any traces of natural isotope ($R_D \approx 0$). This method may be impossible for elemental species, it can be done for organic ones. It is an example that trisily-[$^{13}C_3$]-cholesterol that used generally in cholesterol quantification has R_D value as approximately 1 x 10^{-6}. Another strategy to cancel R_D term is to use SA-ID³MS strategy [212].

2.6.5.3 Triple Standard Addition Isotope Dilution Mass Spectrometry (SA-ID³MS)

In triple standard addition isotope dilution mass spectrometry, three blends are measured to find the analyte concentration in a sample solution. All blends have equal amounts of sample matrix, so the matrix effect is the same in each blend [212].

$$w_N = w_{N*} \cdot \left(\frac{\begin{matrix}-m_{N*-1}\cdot m_{D-2}\cdot m_{D-3}\cdot(R_2-R_3)\\+m_{N*-2}\cdot m_{D-1}\cdot m_{D-3}\cdot(R_1-R_3)\\-m_{N*-3}\cdot m_{D-1}\cdot m_{D-2}\cdot(R_1-R_2)\end{matrix}}{\begin{matrix}+m_{N*-1}\cdot m_{D-2}\cdot m_{D-3}\cdot(R_2-R_3)\\-m_{N*-2}\cdot m_{D-1}\cdot m_{D-3}\cdot(R_1-R_3)\\+m_{N*-3}\cdot m_{D-1}\cdot m_{D-2}\cdot(R_1-R_2)\end{matrix}}\right) \quad (2.39)$$

N=N* can eliminate molecular weight of the N and N* but this approach should be carefully applied for species that have varieties in nature. For instance, boron isotopic composition is different from its commercial standard in seawater [216]. For this reason, boron determination in seawater by this approach may lead to some errors.

2.6.6 Advantages and Disadvantages of Isotope Dilution Methods

If isotopes in sample/calibration blends attain equilibrium, isotope dilution method provides many advantages. Firstly, it provides superior accuracy and precision in measurement outputs. Further, the amount of analyte in a sample solution can be determined via measuring the analyte and their isotope ratio, thereby the analyte does not need to be totally recovered. Another advantage is that analyte transformation during sample preparation steps can be compensated with the addition of different isotopes of the analyte, like internal standard principle. Isotope dilution can also save time and give more accurate results than conventional standard addition method. Lastly, isotope dilution strategies help to obtain very low uncertainty values.

On the other hand, there are some disadvantages for isotope dilution. One of the most critical point is the cost and small amount of isotopic labelled or enriched materials. Secondly, mass spectrometer should be used to measure isotope ratios, but this instrument is not a cheap instrument [198].

2.7 Literature Survey

One study for the quantitation of bisphenol A, 4-*n*-nonyphenol and 4-tert-octylphenol was proposed by Cai *et al*. 4-*n*-nonylphenol was extracted by an SPE method which used a multiwalled carbon nanotubes-packed cartridge for the adsorption processes and methanol for the desorption processes. Instrumental detection was done by an HPLC system with fluorometric detector. The LOD value

was lowered to 0.024 ng/mL in that study. The presented method was implemented to river water, tap water, sea water and wastewater samples and their percent recoveries were between 90.1 and 98.6% [217].

In 2003, an analytical procedure was suggested for the determination of 4-*n*-nonylphenol and bisphenol A in human milk samples. The samples were extracted by ethanolic KOH and diethyl ether under mild alkaline conditions. N,O Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was used as an derivatizing reagent for the analytes. LOD and LOQ values for 4-*n*-nonylphenol were calculated as 0.50 and 0.91 ng/g, respectively. Recoveries for 10 and 300 ng/g spiked human milk samples were found as 97.0±13.0% and 89.0±3.0%, respectively [218].

DLLME integrated with magnetic nanoparticle-based dispersive solid-phase microextraction was developed for 4-*n*-nonyphenol determination in water samples by HPLC system with a fluorometric detector. After applying the DLLME procedure, LOD and LOQ values for the study were found as 13.9 and 44.4 ng L^{-1}, respectively. Method accuracy was checked by spiking experiments and percent recoveries for drinking water and secondary wastewater were in the range of 96 and 100% [219].

Temperature-controlled ionic liquid dispersive liquid-phase microextraction was used to preconcentrate 4-*n*-nonylphenol, bisphenol A and 4-tert-octylphenol from water samples. 1-oxyl-3-methylimidazolium hexafluorophosphate was used as an ionic liquid in the study. An LOD value of 0.35 µg L^{-1}, with 0.9981 correlation coefficient value was obtained in the study for 4-*n*-nonyphenol. Spiking experiments were done with drainage, wastewater, melted snow water and river water samples. The recovery results were found to be close to 100% with low standard deviations [220].

4-*n*-Nonylphenol was also determined by HPLC system with fluorometric detection after solid phase microextraction method. A carbowax/templated resin (CW/TPR) coating fiber was used to preconcentrate the analyte. Under the optimum conditions, a linear calibration plot was achieved between 2.0- 200 ng/mL with 0.29 ng/mL as LOD. Recoveries from wastewater, tap and river samples were in the range of 95 and 106% [221].

Chormey *et al*. combined ID^4MS and DLLME techniques in order to achieve high accuracy and precision at trace levels for parathion methyl determination in water

samples. Isotope dilution strategies are limited by detection limits of the selected instrumental detection method. For this reason, DLLME was coupled to ID^{4}MS in order to achieve low detection limits. A percent recovery result for the developed method was calculated as 99.92 ± 0.33% at 40.03 μg kg^{-1}. This indicated that high accuracy and precision at trace levels can be acquired by isotope dilution strategies coupled to DLLME method [10].

Pagliano and Mester performed ID^{4}MS for the simultaneous determination of nitrite and nitrate by headspace GC-MS system. ID^{4}MS was studied using ^{18}O-nitrite and ^{15}N-nitrate labelled isotopes. Triethyloxonium tetrafluoroborate was used to derivatize nitrite and nitrate to obtain gas chromatographic separation. After developing the derivatization and ID^{4}MS system, a seawater reference material for nutrients (MOOS-2) was evaluated for its nitrite and nitrate contents. Certified values for nitrite and nitrate were 0.1480±0.0040 and 1.301±0.031 μg g^{-1}, respectively, and the respective experimental results gained by the presented method were 0.1483±0.0011 and 1.3268 ±0.0027 μg g^{-1}. Furthermore, the study also provided nitrite and nitrate determination at low levels with 10-20% uncertainties [222].

In 2006, ID^{2}MS strategy was used to certify a new selenized yeast reference material (SELM-1) in terms of total selenium content for methionine, selenomethionine and total selenium. GC-MS and LC-MS were operated to quantify methionine and selenomethionine by using their ^{13}C labelled isotopes. For the total selenium content, ^{82}Se was employed as enriched isotope material in ID^{2}MS strategy for its determination in inductively coupled plasma mass spectrometry (ICP-MS) system. The study was performed by obtaining experimental results from two National Metrology Institutes and five university/government laboratories. Selenomethionine and total selenium were found to be within the certified ranges but methionine was not determined in the certified ranges [223].

MBT, DBT and TBT were determined in water and sediment samples by GC-FID system after sodium tetraethyl borate derivatization and solid phase microextraction methods. Poly dimethylsiloxane fiber was used to retain the analytes. After optimizing some parameters such as mixing conditions, extraction period and temperature, LOD values for MBT, DBT and TBT were calculated as 1.0,

1.2 and 0.9 μg L^{-1} as Sn, respectively. A good agreement between certified reference sediment (PACS-2) and the determined values by the proposed method was observed for DBT and TBT compound. However, MBT (0.80±0.13 μg g^{-1}) recorded a higher value than the certified reference (0.3 μg g^{-1}) and this was attributed to indicative value of MBT in the PACS-2 material [224].

One study conducted by Japanese scientists was aimed at measuring butyltin and phenyltin accumulation in the Port of Osaka and Yodo River by a gas chromatograph integrated with a flame photometric detector. The concentration of TBT and triphenyltin detected in muscle of fish species was in the range of 0.011–0.182 mg/kg. LOD values for MBT, DBT and TBT 0.6, 0.5 and 0.8 ng/g for muscles of fish samples, respectively. TBT and triphenyltin were detected in the fish species more than the other butyltin and phenyltin compounds [225].

A hyphenated system, LC-ICP-MS was used to determine MBT, DBT, TBT and some phenyltin compounds. LOD values for the spiked sediment samples fell in a range of 0.7 and 2.0 ng/g. In the recovery studies, sediment samples obtained from French harbors and channels were spiked with 1.0 μg g^{-1} of MBT, DBT and TBT, and 0.50 μg g^{-1} of monophenyltin, diphenyltin, triphenyltin compounds. The percent recoveries calculated were in the range of 72 and 102% [226].

Another hyphenated technique (HPLC system coupled to ESI-MS system) was used for the determination of TBT, tripheyltin and triethyltin in textile and plastic materials. TBT, triphenyltin and triethyltin had LOD values 0.4, 20 and 4.0 ng/mL, respectively. Recovery results for spiked textile and plastic materials were between 90 and 99% at 0.5 and 4.0 μg g^{-1} [227].

A simultaneous hydride generation–LLE and GC–MS system was applied to determine MBT, DBT and TBT. LOD values for the hydride generation LLE-GC-MS method were 0.5, 0.4 and 0.7 μg L^{-1} with tripropyl tin as internal standard. Recovery experiments were done by spiking seawater samples to 25 μg L^{-1} of the analytes. Percent recoveries were recorded as 105, 94 and 104% for MBT, DBT and TBT, respectively [228].

3

Materials and Methods

3.1 Chemicals and Reagents

For the determination of 4-*n*-nonylphenol, all chemicals used, their purities and brands are presented in Table 3.1. Analytical grade chemicals were utilized throughout this study.

Table 3.1 Chemicals used for the quantification of 4-*n*-nonylphenol

Chemical Name	**Purity**	**Brand**
4-*n*-nonylphenol	99.9%	Dr Ehrenstorfer GmbH, Germany
4-*n*-nonylphenol-d_8	98.0%	Dr Ehrenstorfer GmbH, Germany
Acetone (ACT)	≥99.8%	Merck, Germany
1,2-Dichloroethane (DCE)	≥99.8%	Merck, Germany
Isopropyl alcohol (IPA)	≥99.8%	Merck, Germany
Acetonitrile (ACN)	≥99.9%	Merck, Germany
Chloroform (CHL)	≥99%	Merck, Germany
Dichloromethane (DCM)	≥99.9%	Merck, Germany
Ethanol (EtOH)	96%	Merck, Germany
Methanol (MeOH)	≥99.9%	Merck, Germany
Carbon tetrachloride	99.9%	Merck, Germany
N,N-Dimethylbenzylamine (DMBA)	≥98%	Merck, Germany
Sodium hydroxide (NaOH)	≥97.0%	Merck, Germany

Standard stock solutions of 4-*n*-nonylphenol (1000 mg/kg) and 4-*n*-nonylphenol-d_8 (10 mg/kg) were prepared in acetone. All solutions for ID^{4}MS and SS-LPME-ID^{4}MS studies were prepared gravimetrically with an OHAUS analytical balance. Solid carbon dioxide (dry ice) was bought from a local company in İstanbul, Turkey. Distilled water was obtained from a Milli Q-system with a conductivity of 18 MΩ.

For the determination of selected butyltin compounds, chemical names, their purity and brand information are listed in Table 3.2. In a same manner, all chemicals used in this study were of analytical grade.

Table 3.2 Chemicals used for the determination of the selected butyltin compounds

Chemical Name	**Purity**	**Brand**
Monobutyltin trichloride ($C_4H_9Cl_3Sn$)	95%	Sigma Aldrich, Germany
Dibutyltin dichloride ($C_8H_{18}Cl_2Sn$),	96%	Sigma Aldrich, Germany
Tributyltin chloride ($C_{12}H_{27}ClSn$)	96%	Sigma Aldrich, Germany
Sodium tetraethylborate ($NaBEt_4$)	97%	Acros Organics, Belgium
Tetrahydrofurane	≥99.8%	Merck, Germany
Acetic acid	96%	Merck, Germany
Dichloromethane	≥99%	Sigma Aldrich, Germany
Ethanol	96%	Merck, Germany
Methanol	≥99.9%	Merck, Germany
Carbon tetrachloride	99.9%	Merck, Germany
Sodium acetate	≥99.0 %	Merck, Germany
Acetone	≥99.8%	Merck, Germany
1,2-dichloroethane	≥99.8%	Merck, Germany
Isopropyl alcohol	≥99.8%	Merck, Germany
Acetonitrile	≥99.9%	Merck, Germany

Chloroform	≥99%	Merck, Germany

Each butyltin compound was dissolved in methanol to prepare their stock solutions (1000 mg/kg as Sn). A mixed standard solution containing all butyltin compounds (250 mg/kg as Sn) was prepared in methanol by diluting appropriate aliquots from the 1000 mg/kg stock solution of each analyte. Sodium tetraethylborate (97%) was dissolved in tetrahydrofuran (THF) to prepare 9.7% (w/v) of $NaBEt_4$ solution. pH 5.0 buffer solution was prepared with 1.0 M of sodium acetate solution using concentrated acetic acid solution for pH adjustment.

3.2 Instrumentation

3.2.1 4-*n*-nonylphenol and 4-*n*-nonylphenol-d_8

The determination of 4-*n*-nonylphenol and 4-*n*-nonylphenol-d_8 was performed using an Agilent gas chromatograph (HP 6890 series) and a mass spectrometer 5973 as detector. GC-MS conditions for 4-*n*-nonylphenol and its deuterated form are given in Table 3.3. The quantifier/qualifier ions used for 4-*n*-nonylphenol and 4-*n*-nonylphenol-d_8 were 107/220 and 113/228 (m/z), respectively.

Table 3.3 GC-MS conditions for 4-*n*-nonylphenol and 4-*n*-nonylphenol-d_8

Parameter	**Value**
Column	HP-5MS capillary column (30 m × 250 µm i.d. × 0.25 µm film thickness)
Carrier gas	Helium, 1.1 mL/min.
Injection mode	Splitless, 1.0 µL
Temperature program	120 ºC (held for 1.0 min), to 300 °C at 30 °C/min.
MS Source temperature	230 °C
MS Quad temperature	150 °C
Transfer line temperature	280 °C

3.2.2 Butyltin Compounds

The same GC-MS system described in Section 3.2.1 was used for the separation, identification and quantification of butyltin compounds according to the parameters given in Table 3.4.

Table 3.4 GC-MS conditions for the selected butyltin compounds

Parameter	Value
Column	HP-5MS capillary column (30 m × 250 µm i.d. × 0.25 µm film thickness)
Carrier gas	Helium, 1.1 mL/min
Injection mode	Splitless, 1.0 µL
Injection port temperature	250 °C
Temperature program	60 °C, to 130 °C at 20 °C/min, to 160 °C at 30 °C/min, to 300 °C at 50 °C/min
MS Source temperature	230 °C
MS quad temperature	150 °C
Transfer line temperature	280 °C

The quantifier/qualifier ions used were 179/235 for derivatized MBT, 149/263 for derivatized DBT and 263/207 for derivatized TBT.

3.3 Experimental Procedures

3.3.1 SS-LPME combined with ID^{4}MS for 4-*n*-nonylphenol

3.3.1.1 Procedure of SS-LPME for 4-*n*-nonylphenol

Sample and calibration blends were fixed to 8.0 g with the aim of obtaining the same preconcentration factors. Firstly, 2.0 mL of protonated DMBA was admixed with 8.0 g of these blends and a homogeneous mixture was observed for each blend. To reverse back the protonated DMBA into DMBA, 1.50 mL of 1.0 M NaOH was added to the homogeneous mixture, resulting in a cloudy solution. Vortexing for 20 s was applied to the mixture for a proper distribution of DMBA phase throughout the

aqueous solution. Centrifugation for 2.0 min at 3461 *g* was employed in order to separate DMBA phase from the aqueous solution. The final step was to withdraw the DMBA phase and analyze by GC-MS system.

3.3.1.2 Synthesis of Switchable Solvent

To synthesize a switchable solvent, carbon dioxide, a tertiary amine, and distilled water can be employed. Miscibility property of the amine is changed by the addition of carbon dioxide and water, and transforms to miscible form in aqueous phase. This miscible form can be reversed into its immiscible form if a stimulus like sodium hydroxide is added [229]. The synthesis procedure is given by the reactions below [230];

$$NR_3 + H_2O + CO_2 \longleftrightarrow NR_3H^+ + HCO_3^-$$

$$NR_3H^+ + HCO_3^- + NaOH \longleftrightarrow NR_3 + H_2O$$

In this study, DMBA was employed as a tertiary amine. DMBA and distilled water (1:1, v/v) were mixed in an Erlenmeyer flask and small pieces of dry ice were carefully added to this solution. The synthesis was continued with the addition of dry ice to the flask on a magnetic stirrer at 300 rpm. A homogeneous phase, protonated DMBA, was observed at the end of the reaction. The protonated DMBA was kept in the refrigerator and covered with aluminum foil to protect it from sunlight and air.

3.3.1.3 Procedure of ID^{4}MS and SS-LPME–ID^{4}MS for 4-*n*-nonylphenol

At the beginning of the ID^{4}MS studies, all stock solutions (4-*n*-nonylphenol and 4-*n*-nonylphenol-*d*8) were gravimetrically prepared in acetone and stored at 4.0 °C. Then, 3.0 mg/kg of sample solution (N), standard solution of 4-*n*-nonylphenol (N*) and isotopically labelled material 4-*n*-nonylphenol-*d*8 (D) were prepared in deionized water. The sample (ND) and calibration blends (N*D-1, N*D-2, N*D-3) were prepared according to three points calibration strategy established by Pagliano *et al.* In order to get exact matching method, the isotope ratio of ND blends was fixed to the same ratio as the N*D-2 blend. All blends preparation and their isotope ratios are summarized in Table 3.5.

Table 3.5 Sample and calibration blends for the determination of 4-*n*-nonylphenol by ID^{4}MS strategy

Sample/Calibration Blends	Mass of N or N*, g	Mass of D, g	Isotope Ratios
ND	0.375	0.400	1.011
N*D-1	0.200	0.400	0.549
N*D-2	0.375	0.400	1.011
N*D-3	0.750	0.400	2.002

For the SS-LPME-ID^{4}MS method, sample and calibration blends had to be fixed to the same final mass because of different preconcentration factors. All blends were fixed to 8.0 g by considering both their isotope ratios and final masses as shown in Table 3.6. The *Solver tool* in *Microsoft Excel program* was used to calculate these masses given in Table 3.6. The working concentration for SS-LPME-ID^{4}MS method was 40 ng/g. This was determined according to signal to noise ratio of 4-*n*-nonylphenol and 4-*n*-nonylphenol-*d*8 after the SS-LPME-GC-MS method.

Table 3.6 Sample and calibration blends for the determination of 4-n-nonylphenol by SS-LPME-ID^{4}MS strategy

Sample/Calibration Blends	Mass of N or N*, g	Mass of D, g	Isotope Ratios
ND	3.871	4.129	1.011
N*D-1	2.667	5.333	0.549
N*D-2	3.871	4.129	1.011
N*D-3	5.217	2.783	2.002

3.3.2 BS-LPME combined with SA-ID^{3}MS for 4-*n*-nonylphenol

3.3.2.1 Procedure of BS-LPME for 4-*n*-nonylphenol

The sample/standard solution (8.0 g) was weighed into a 15 mL centrifuge tube. Then, a mixture containing 125 µL of dichloromethane and 125 µL of 1,2-dichloroethane as binary extraction solvents were mixed with 1.85 mL of ethanol as dispersive solvent and injected into the sample/standard solution. A cloudy solution was observed, indicating the distribution of binary organic solvent throughout the aqueous phase. The bottom phase was transferred into a vial and sent to the GC-MS system after performing vortex assisted mixing for 15 s and centrifugation at 3460 *g* for 120 s.

3.3.2.2 Procedure of SA-ID^{3}MS and BS-LPME-SA-ID^{3}MS for 4-*n*-nonylphenol

Gravimetric standard/sample preparation was applied to all blends. 4-*n*-nonylphenol and 4-*n*-nonyphenol-d8 were dissolved in acetone and diluted to working concentrations with ultrapure water. Sample (N), natural standard (N*) and isotopically labelled material (D) were mixed according to the SA-ID^{3}MS approach proposed by Pagliano and Meija [212], as given in Table 3.7. All blends have the same total amount and same sample amount in order to make the matrix effects identical for all blends.

Table 3.7 Preparation of blends for SA-ID^{3}MS and BS-LPME- SA-ID^{3}MS

	SA-ID^{3}MS			**BSME-SA-ID^{3}MS**			
Blend	**m_N, g**	**m_{N^*}, g**	**m_D, g**	**m_N, g**	**m_{N^*}, g**	**m_D, g**	**Target isotope ratio**
NN*D-1	0.30	0.00	0.30	2.00	0.00	2.00	1.08
NN*D-2	0.30	0.60	0.30	2.00	4.00	2.00	3.19
NN*D-3	0.30	0.30	0.60	2.00	2.00	4.00	1.08

The amount of blends prepared in SA-ID^{3}MS and BS-LPME-SA-ID^{3}MS studies were 1.2 g and 8.0 g, respectively. When preparing NN*D-1 blends, de-ionized water was used to fix the total amount of sample to 1.2 or 8.0 g.

3.3.2.3 Procedure of ID^{2}MS for 4-*n*-nonylphenol

ID^{2}MS was applied to the wastewater samples. Sample blend consisted a mixture of 0.30 g of D and 0.30 g of N while calibration blend consisted of 0.30 g of D and 0.30 g of N*.

3.3.3 DLLME-GC-MS for Butyltin Compounds

3.3.3.1 Procedures of DLLME for Butyltin Compounds

For the derivatization of butyltin compounds, 0.25 mL of pH 5.0 acetate buffer solution and 47.34 µL of 9.7% (w/v) $NaBEt_4$ were added to 8.0 mL of standard/sample solution. Next, the solution was left for 30 minutes in order to complete the derivatization reaction. In another tube, 300 µL dichloromethane and 1.64 mL ethanol were mixed and then injected into the derivatized butyltin solution. Mixing by vortex was applied for 15 s. To facilitate settling down of the dichloromethane phase, centrifugation at 6000 rpm was applied for 120 s. The dichloromethane phase was then put into a vial and sent to the GC-MS system.

3.4 Samples

3.4.1 Preparation of Wastewater Samples for SS-LPME-ID^{4}MS and BS-LPME-SA-ID^{3}MS studies

In the SS-LPME-ID^{4}MS studies, municipal wastewater was sampled into a polypropylene bottle from Atatürk University, Erzurum – Turkey. The sample was kept at 4.0 °C throughout all experimental steps. Before the wastewater sample was analyzed by GC-MS, it was firstly filtered with 125 mm (diameter) filter paper and 0.45 µm syringe filter, and then diluted 10 folds.

In BS-LPME-SA-ID^{3}MS studies, wastewater sample was taken from an Advanced Biological Wastewater Treatment Plant, İstanbul, Turkey, and stored in a polycarbonate bottle at 4.0 °C. Filtration was also performed with a 125 mm (diameter) filter paper and a 0.45 µm regenerated cellulose syringe filter.

3.4.2 Preparation of Infant Formula Samples for BS-LPME-SA-ID^{3}MS study

One brand of infant formula was purchased from a supermarket located in İstanbul, Turkey. Approximately 1.0 g of infant formula was weighed, and 8.0 g of simulated

gastric juice was added to the sample. The solution was maintained at 37 °C for 30 min and then centrifuged for 3.0 min in order to settle solid particles from the supernatant. It was also filtered through a 0.45 μm RC syringe filter and diluted to approximately 50 g with ultrapure water. Simulated gastric juice was prepared according to the method proposed by Unutkan *et al*. [231], where 0.10 g of NaCl, 0.16 g of pepsin and 0.35 mL of 37% (w/w) HCl were mixed in a 50 mL volumetric flask.

3.4.3 Preparation of Fish and Mussel Samples for Organotin Compounds by DLLME-GC-MS

Anchovy (fish) and mytilus galloprovincialis (mussel) samples were purchased from a fish market in Sarıyer, a coastal region of İstanbul, Turkey. Samples were cleaned and weighed to determine its water content. Lyophilization was carried out on a Telstar freeze at -50 °C for three days to dry the samples. After the lyophilization process, the samples were weighed again and the water contents for two fish samples were found to be between 68-74%, and between 80-84% for the two mussel samples.

The lyophilized samples were grinded into small pieces and about 0.60 g of each grinded sample was weighed into a 50 mL polypropylene tube. Ultrapure water (20 g) was added to the samples and followed by vortex mixing for 2.0 min. The resulting sample-water mixture was filtered through 110 mm Sartorius black filter papers (wide pore) and the extract was completed to 120 g with deionized water. 25 g of this solution was used in the recovery experiments with 5.0 μg kg^{-1} spike concentration of each analyte. After these sample pretreatments, dilution factor of the samples was calculated as approximately 240 folds.

4

Results and Discussion

4.1 Chromatographic Separation and Quantification

4-*n*-nonylphenol was eluted through an HP5-MS column (5% Phenyl 95% dimethylpolysiloxane) with the temperature program given in Table 3.3 and 4-*n*-nonylphenol-d_8 was also eluted with the same temperature program. Mass fragments selected for 4-*n*-nonylphenol were 107, 220 and 77 m/z. For its deuterium form, 113, 228 and 41 m/z were selected as major ion fragments. Mass spectroscopic data for 4-*n*-nonylphenol was confirmed by ChemStation mass spectrum database. In order to identify 4-*n*-nonylphenol-d_8, different concentrations were overlaid and they overlapped at the same retention time for 113, 228 and 41 m/z ratios. Chromatograms and mass spectrum of 4-n-nonylphenol and 4-*n*-nonylphenol-d_8 can be seen in Figures 4.1 – 4.4.

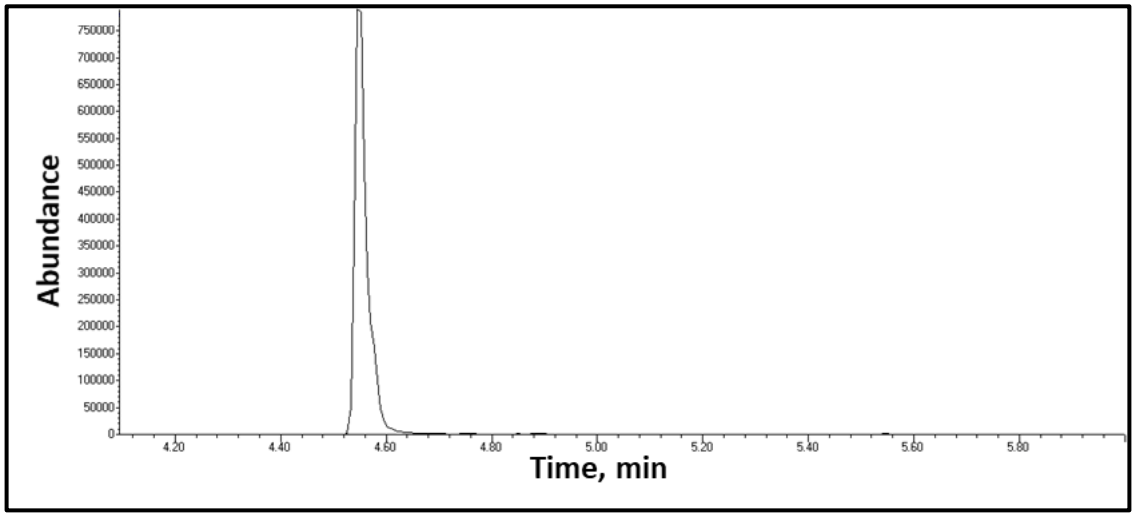

Figure 4.1 Chromatogram (SIM mode, m/z = 107) for 25 mg/kg of 4-*n*-nonylphenol analyzed by GC-MS system

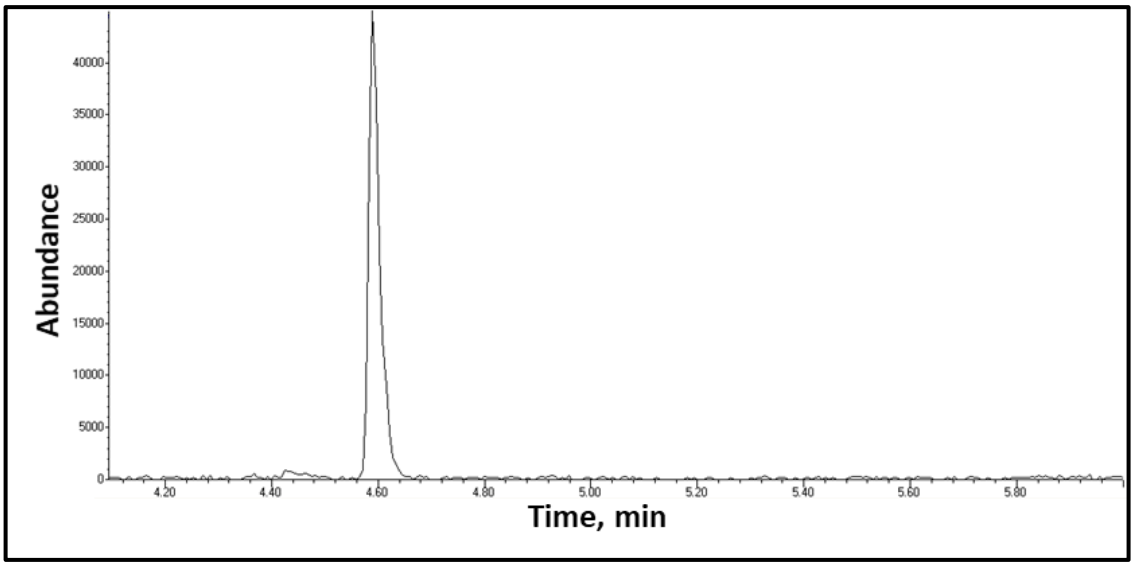

Figure 4.2 Chromatogram (SIM mode, m/z = 113) for 2.0 mg/kg of 4-n-nonylphenol-d_8 analyzed by GC-MS system

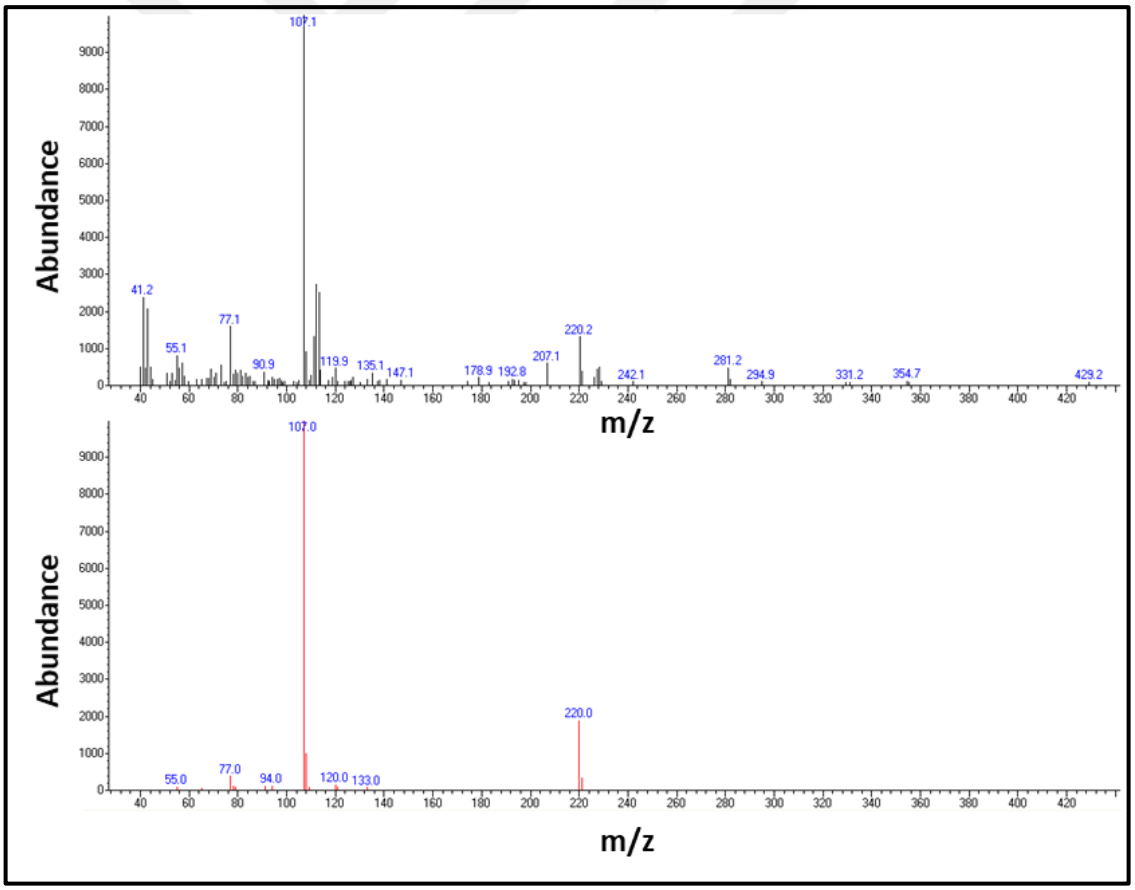

Figure 4.3 Mass spectrum for 4-*n*-nonylphenol obtained from standard solution (up) and ChemStation MS library (down)

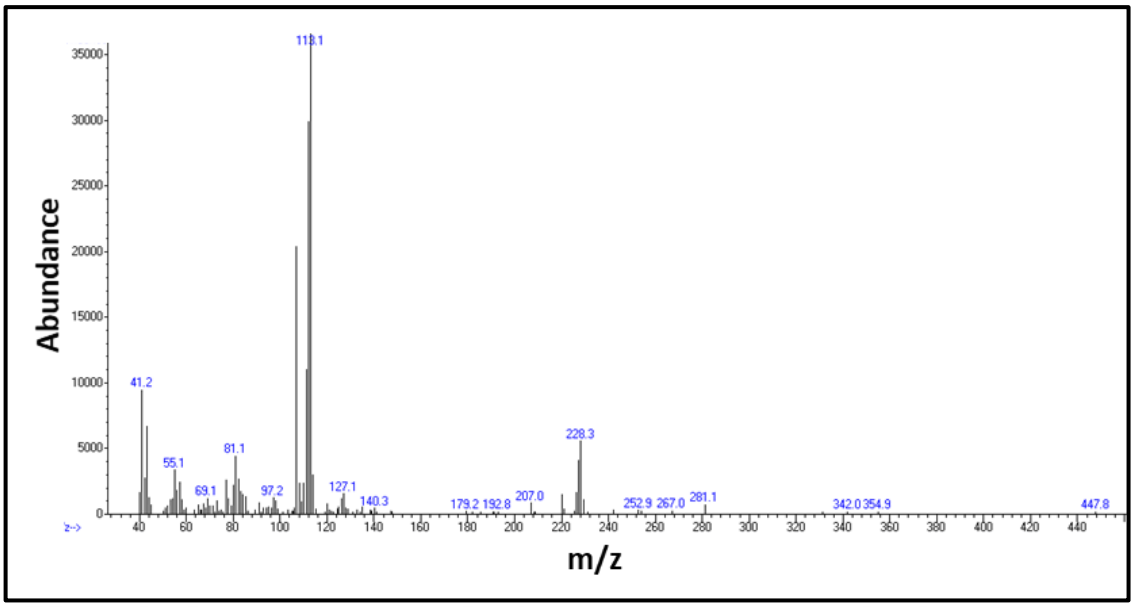

Figure 4.4 Mass spectrum for 4-*n*-nonylphenol-d_8 attained from GC-MS system

Derivatized MBT, DBT and TBT standard solutions were sent separately to the GC-MS system and eluted with the temperature program given in Table 3.4. Retention times and mass fragments were found to be 4.5 min and 179 m/z for MBT, 5.3 min and 149 m/z for DBT, 5.9 min and 263 m/z for TBT. A mix standard solution containing MBT, DBT and TBT were then prepared and analyzed by GC-MS system. An overlay chromatogram can be seen in Figure 4.5.

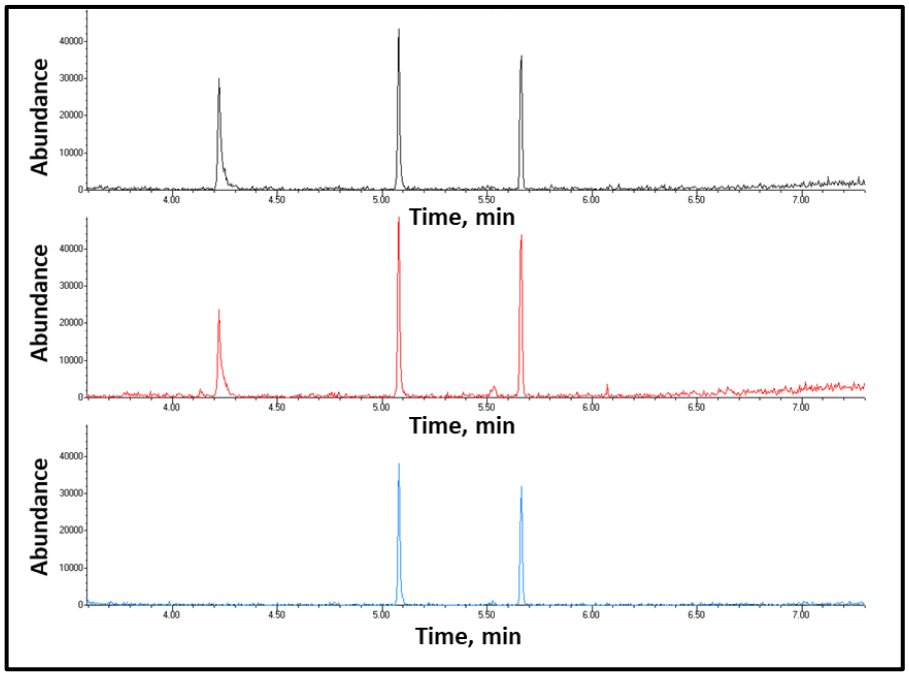

Figure 4.5 Overlay chromatogram (SIM mode, m/z = 179, 149 and 263) for mix standard solution of MBT, DBT and TBT analyzed using GC-MS system

MBT, DBT and TBT had the same m/z ratios but their retention times were different from each other. Mass spectra for derivatized compounds were compared to the ChemStation mass spectrum database as shown in Figures 4.6 – 4.8.

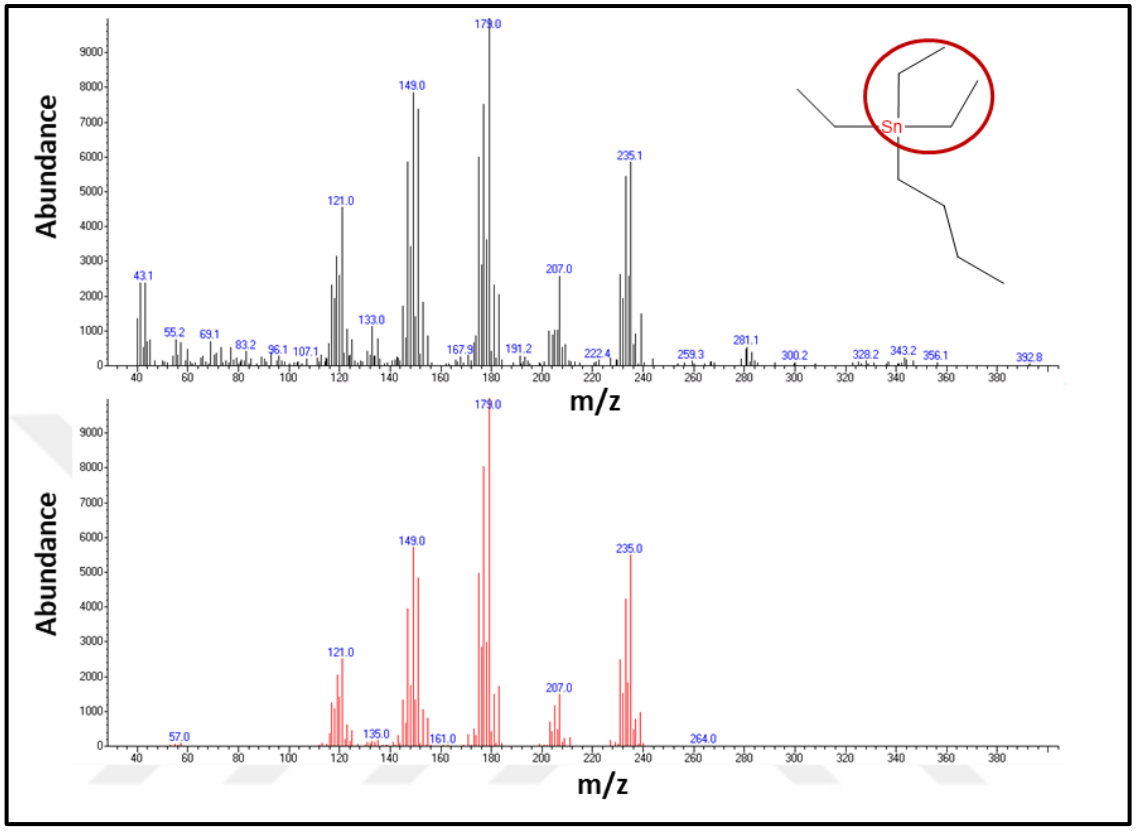

Figure 4.6 Mass spectrum of measured MBT acquired from GC-MS system (up) and MS library (down)

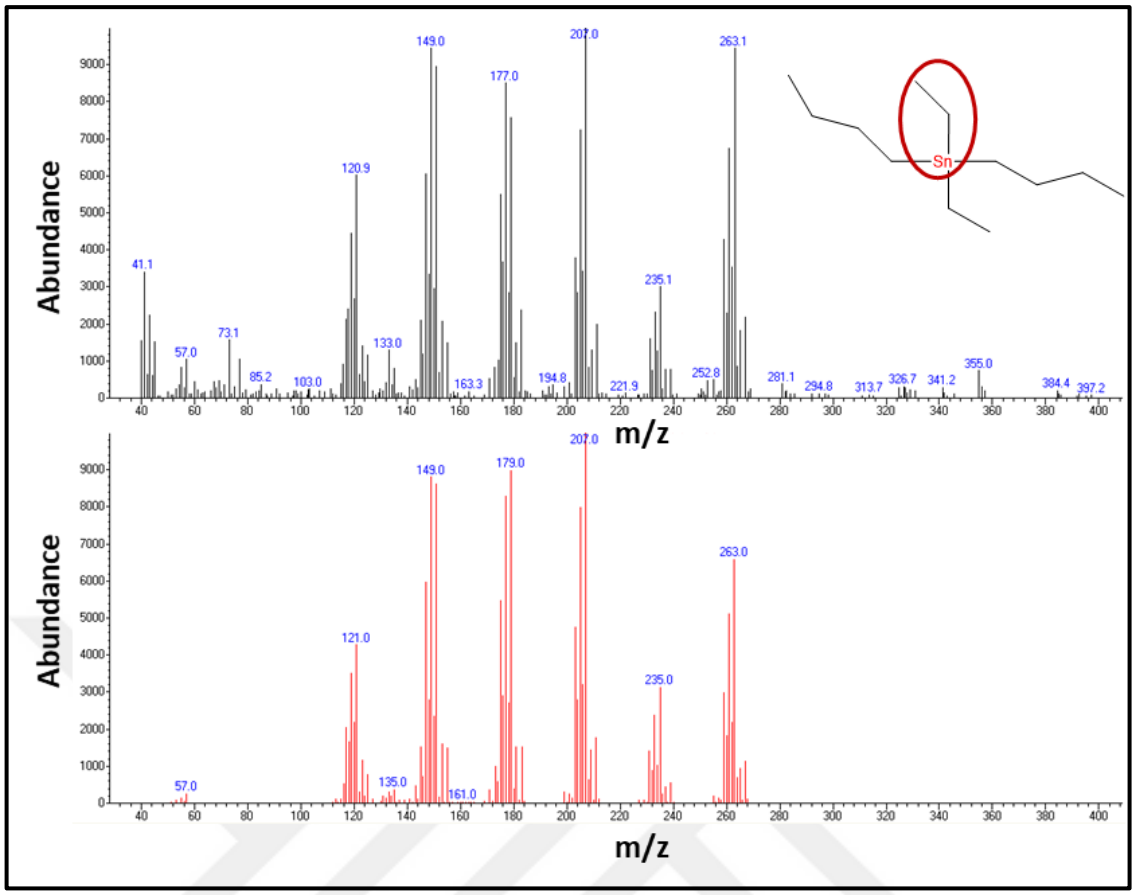

Figure 4.7 Mass spectrum of measured DBT obtained from GC-MS system (up) and MS library (down)

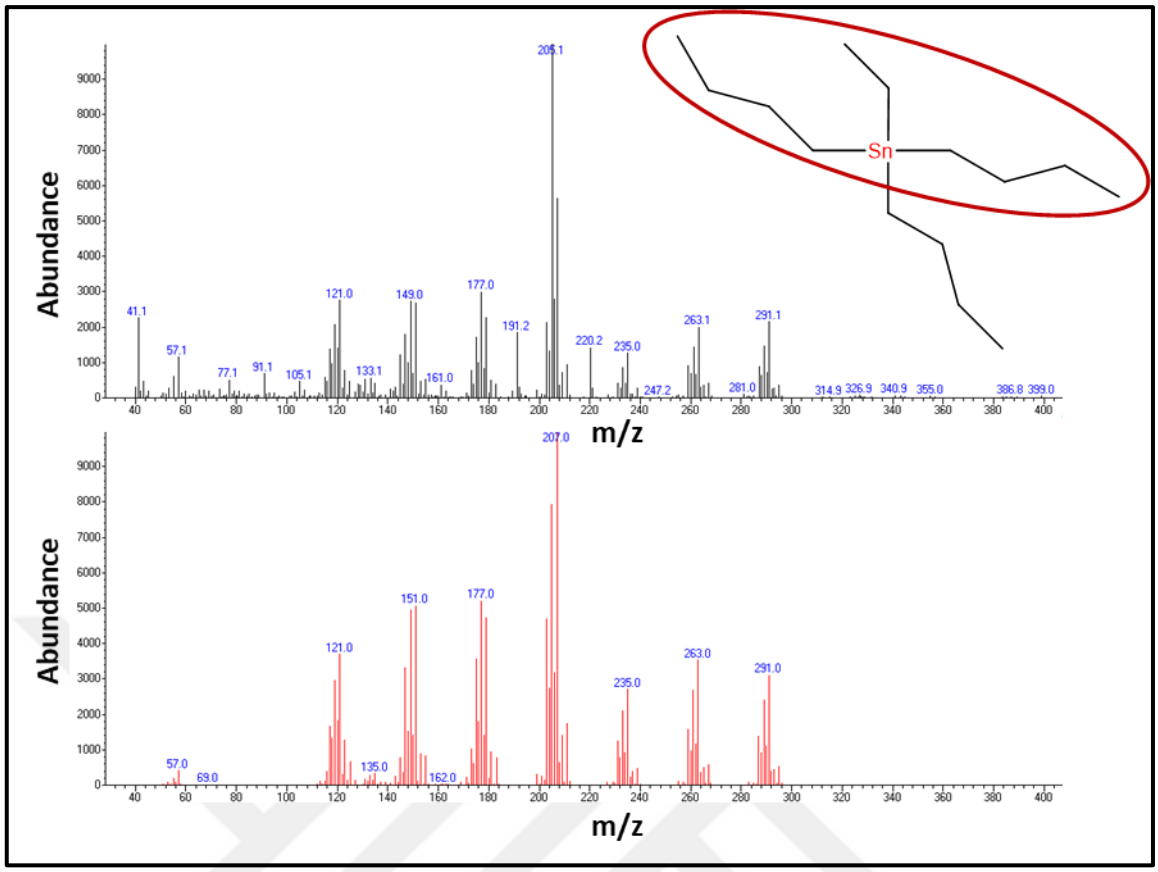

Figure 4.8 Mass spectrum of measured TBT attained from GC-MS system (up) and MS library (down)

4.2 Optimization and Validation of SS-LPME- ID⁴MS for Determination of 4-*n*-nonylphenol

4.2.1 Experimental Design of SS-LPME Method

BBD was used to determine optimum parameters of the SS-LPME method. This type of experimental design contains three levels for each parameter [232]. For this reason, the volume of protonated DMBA (X), concentration of NaOH (Y), the volume of NaOH (Z) and vortex period (T) were evaluated at three levels, given in Table 4.1.

Table 4.1 Minimum (-1), mean (0) and maximum (+1) values for BBD parameters

Parameters/Levels	**-1**	**0**	**+1**
Volume of protonated DMBA, mL (X)	1.0	2.0	3.0
Concentration of NaOH, M (Y)	1.0	2.0	3.0
Volume of NaOH, mL (Z)	1.0	1.50	2.0
Vortex period, s (T)	15	30	45

The chemometric experiments were performed with 75 ng/mL 4-*n*-nonylphenol aqueous standard solutions. The BBD consisted of 29 runs with 5 center points. Each run was performed in triplicates to get better precision. The following quadratic equation showed an approximation model according to the measurements:

$$R = 425400 - 135800X - 383700Y - 216500Z + +259000YZ + 253700Y^2 + 158500Z^2 \quad (4.1)$$

Analysis of variance (ANOVA) was used to provide values of effects and interaction factors that were modeled with Trial *Design Expert 7.0.0* software. A summary of ANOVA results is given in Table 4.2.

Table 4.2 ANOVA results for significant parameters and their interactions

Source	Sum of squares	Degree of freedom	Mean squares	F-value	P-value
Model	3.37×10^{12}	6	5.62×10^{11}	24.19	< 0.0001
X	2.21×10^{11}	1	2.21×10^{11}	9.53	0.0054
Y	1.77×10^{12}	1	1.77×10^{12}	76.07	< 0.0001
Z	5.62×10^{11}	1	5.62×10^{11}	24.22	< 0.0001
YZ	2.69×10^{11}	1	2.69×10^{11}	11.56	0.0026
Y^2	4.44×10^{11}	1	4.44×10^{11}	19.12	0.0002
Z^2	1.73×10^{11}	1	1.73×10^{11}	7.47	0.0122
Residual	5.11×10^{11}	22	2.32×10^{10}		
Lack of fit	5.03×10^{11}	18	2.79×10^{10}	14.03	0.0101
Pure error	7.96×10^{9}	4	1.99×10^{9}		
Cor. total	3.88×10^{12}	28			
R^2	0.8684				
R^2-Adjusted	0.8325				

X: the volume of protonated DMBA, Y: NaOH concentration, Z: the volume of NaOH, T: Vortex period.

When the p-value of the model is lower than 0.05, the model is statistically important. The R^2 value demonstrates the fitness of the model equation [233]. The R^2 value of the proposed model (0.8684) proved good agreement between the predicted and the experimental values.

Lack of fit is a test in ANOVA to calculate the failure of the model in which the points are not appended (included) in regression analysis. The p-value of the lack of fit was calculated as 0.0101 and this suggested that the model was fitted with the response variables.

Normal probability plot for the model depicted in Figure 4.9 demonstrated the normal distribution of experimental data and this also confirmed the agreement between the predicted and the experimental values.

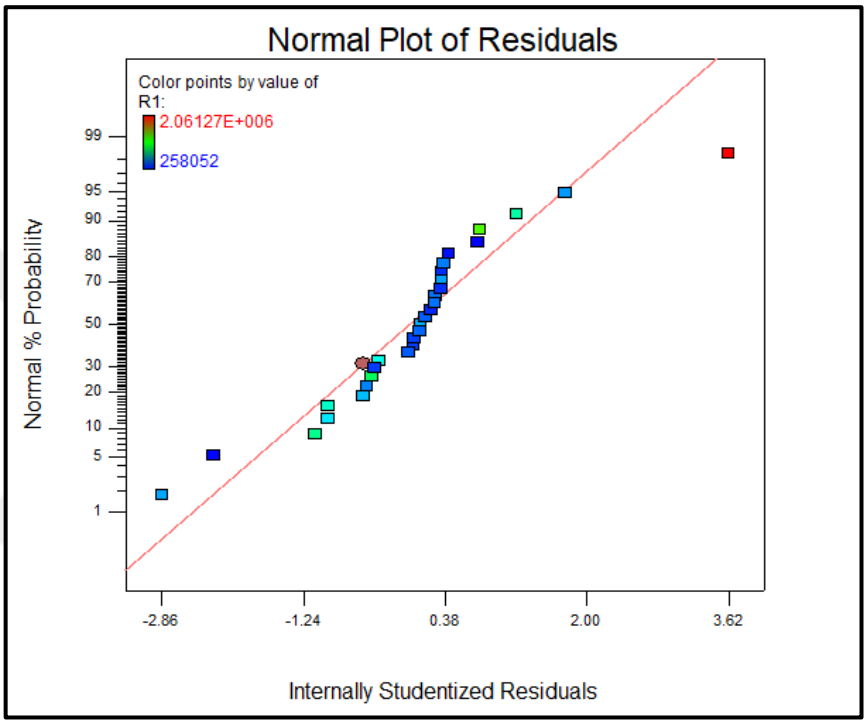

Figure 4.9 Distribution graph of internally studentized residuals - normal probability

In addition, the three-dimensional response surface plot given in Figure 4.10 was useful to understand the interactions of the Y and Z parameters on the response values. The other factors, X and T were kept constant at 2.0 mL and 30 s, respectively. A better response was obtained when the concentration and amount of NaOH were decreased from 1.0 to 3.0 M and 1.0 to 2.0 mL, respectively. Other parameters important to the model were volume of protonated DMBA (X), and the quadratic effects of concentration and volume of NaOH (Y^2 and Z^2).

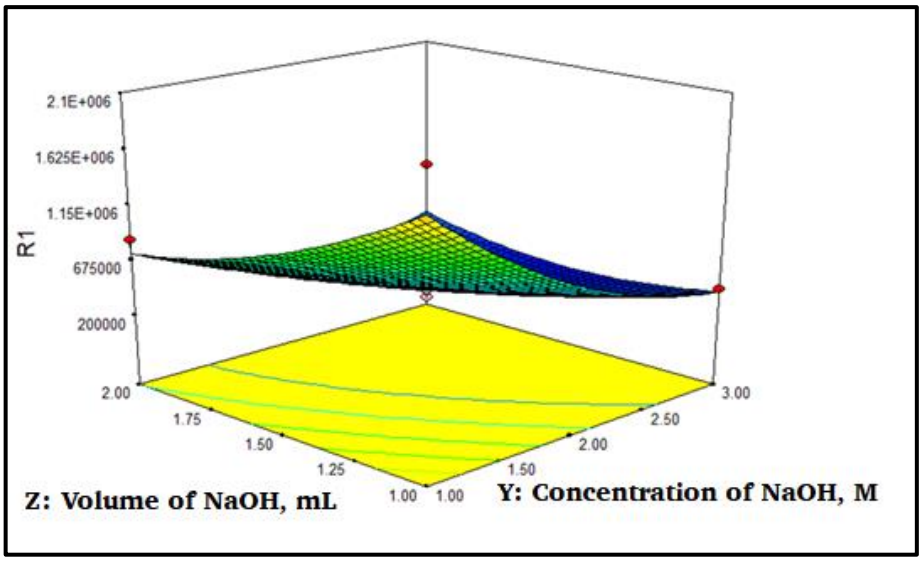

Figure 4.10 Three-dimensional response surface of chemometric experiment

According to these evaluations, the chemometric studies predicted optimum SS-LPME parameters as 2.0 mL of protonated DMBA, 1.0 M of 1.50 mL NaOH and 20 s vortex period. As expected, increasing the amount of protonated DMBA led to analyte dilution and this caused low preconcentration factors but decreasing amounts were not efficient to collect the analyte from aqueous solution. The dilution problem was also observed for increasing amount of NaOH. The lowest level of the NaOH concentration was enough to reverse the protonated DMBA back into the DMBA form.

4.2.2 Analytical Figures of Merit for SS-LPME-GC-MS

A series of standard solutions of 4-*n*-nonylphenol were prepared to construct a calibration plot of the analyte after GC-MS measurements. After the analyses, a linear calibration plot for the determination of 4-*n*-nonylphenol by GC-MS system was drawn between 0.16 and 80.6 mg/kg as shown in Figure 4.11. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 0.04 and 0.12 mg/kg, respectively. The formulas used to calculate LOD and LOQ were as follows;

$$LOD = \frac{3s}{m} \tag{4.2}$$

$$LOQ = \frac{10s}{m} \tag{4.3}$$

where s was the standard deviation of the lowest concentration in the calibration plot and m was the slope of the calibration plot.

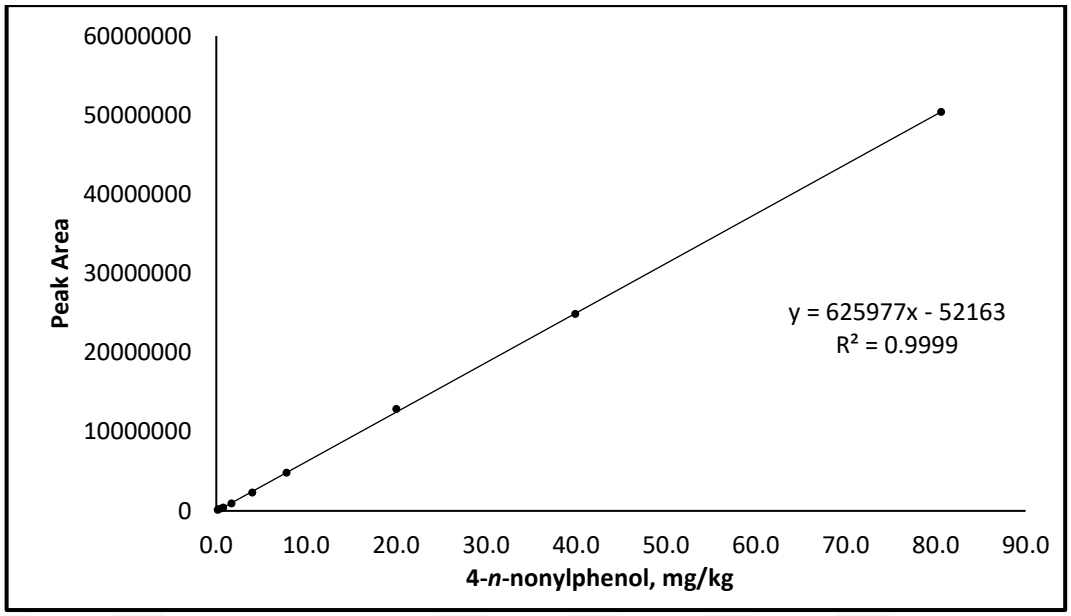

Figure 4.11 GC-MS calibration plot of 4-*n*-nonylphenol

In the validation of SS-LPME-GC-MS system, standard solutions in the range of 1.0-250 ng/mL were treated with the optimized SS-LPME method. A good linearity was observed between 1.0 and 50 ng/mL as shown in the calibration plot of Figure 4.12. Eight replicates of the lowest concentration were used in the calculation of LOD and LOQ values which were found to be 0.32 and 1.05 ng/mL, respectively. All analytical figures of merit are illustrated in Table 4.3.

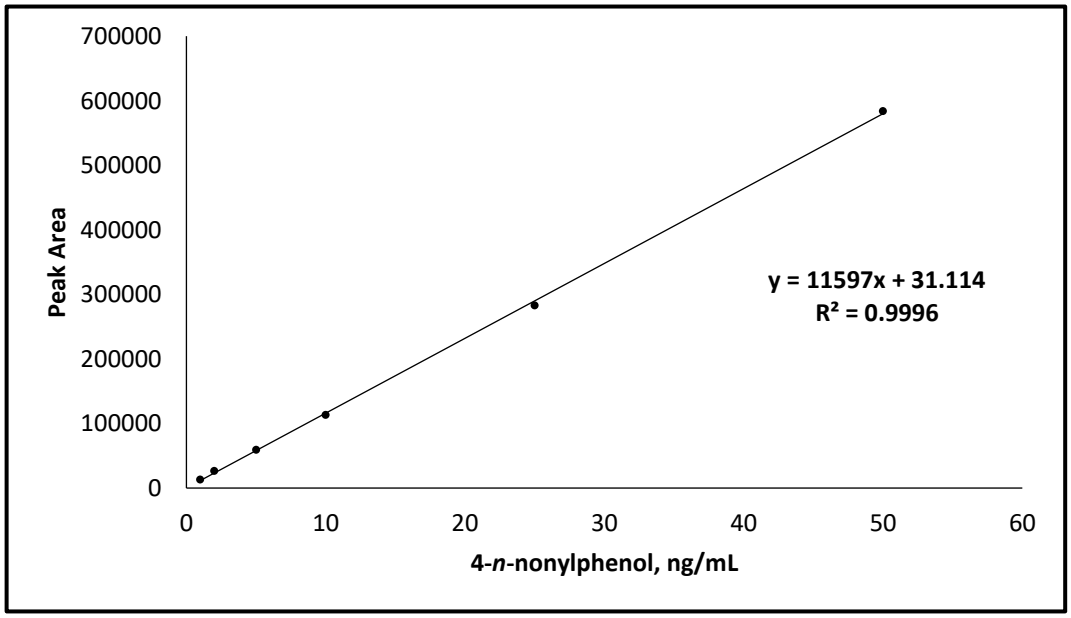

Figure 4.12 SS-LPME-GC-MS calibration plot of 4-*n*-nonylphenol

Table 4.3 Comparison of GC-MS and SS-LPME-GC-MS systems

Validation parameters	GC-MS	SS-LPME-GC-MS
LOD, ng/g	37	0.32
LOQ, ng/g	122	1.05
Linear Range, ng/g	163-80603	1.0-50
Expression of dynamic range	y = 625977x - 52163	y=11597x+31.114
Correlation coefficient, R^2	0.9999	0.9996
Enhancement in detection power		115

4.2.3 Recovery Studies for SS-LPME-GC-MS

The applicability of the developed preconcentration method was verified with spiking experiments. For this purpose, municipal wastewater samples were spiked to 20, 30 and 40 ng/g and preconcentrated with the SS-LPME method. 4-*n*-nonylphenol was not detected in the sample according to blank analysis. The percent recovery results with their percent relative standard deviations were calculated as 114.9 ± 1.3, 90.2 ± 6.9 and 107.4 ± 4.5% for 20, 30 and 40 ng/g spiked samples, respectively. The results obtained though satisfactory showed effects of sample matrix components on 4-*n*-nonylphenol recovery by SS-LPME-GC-MS method, but the accuracy and precision can be improved by a superior method like an isotope dilution strategy.

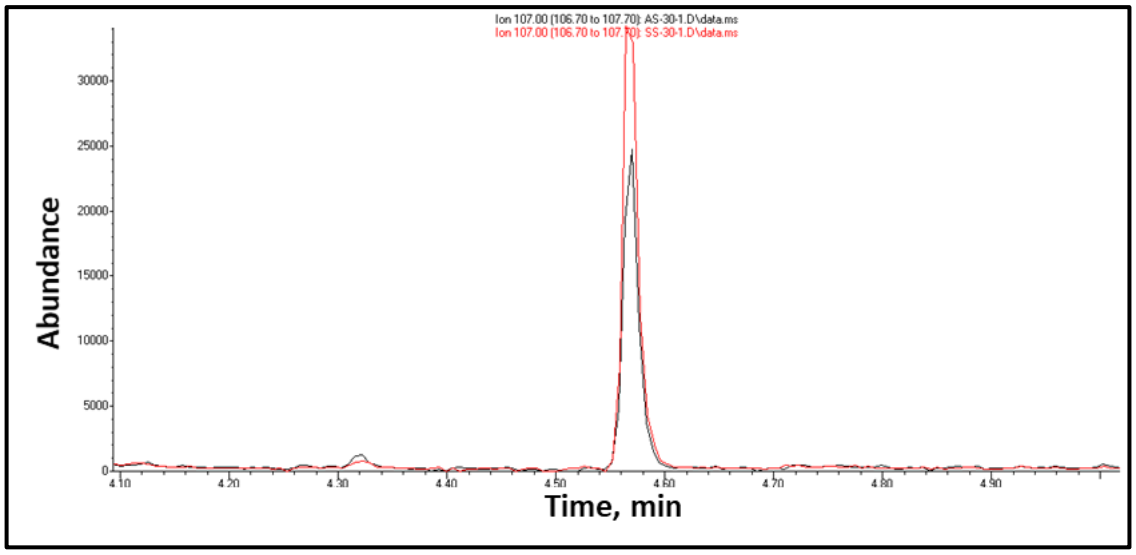

Figure 4.13 Overlay chromatograms of standard (red) and municipal wastewater (black) spiked to 30 ng/mL and preconcentration with SS-LPME technique

4.2.4 Equilibrium Period

In all types of IDMS strategies, it is essential to attain a homogeneous mixture regarding isotopes of the analyte in the blends. If not, isotope measurements for all blends are non-accurate and changeable. Thus, the equilibrium period that is a period to reach a homogenous mixture in the blends should be determined via observing isotope ratios at certain periods [206]. For the blends containing 4-*n*-nonylphenol and 4-*n*-nonylphenol-d_8, 2.0 hours were enough to reach equilibrium between the isotopes, as can be seen in Figure 4.14.

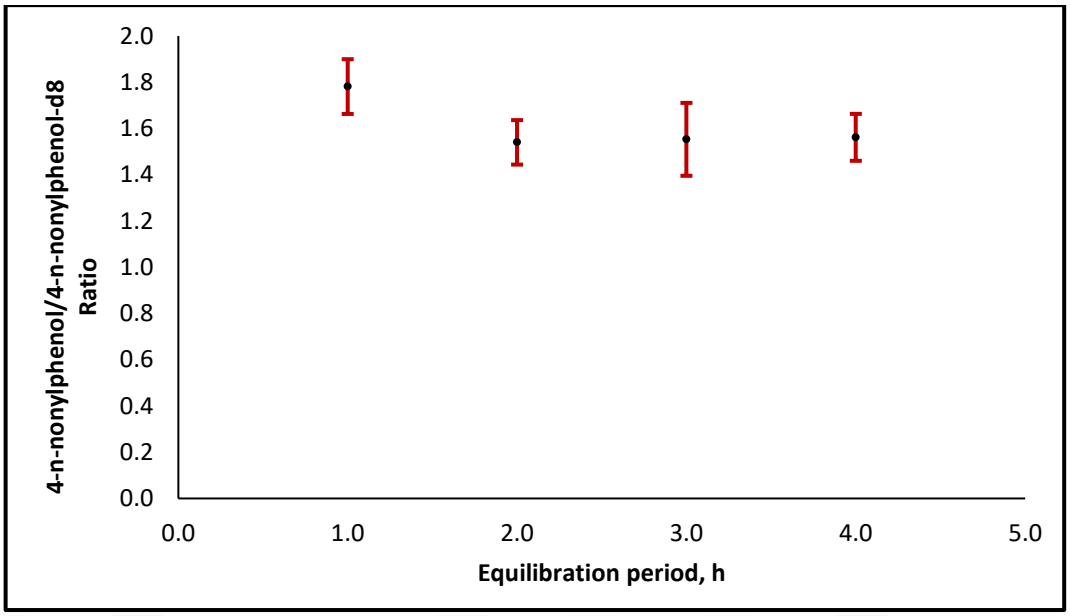

Figure 4.14 Optimization of equilibration period for isotope homogeneity

4.2.5 ID⁴MS

Blank measurement for the municipal wastewater sample was done to determine the presence or absence of 4-*n*-nonylphenol. There was no detectable 4-*n*-nonylphenol signal in the sample blank, therefore it was spiked to 3.0 mg/kg and analyzed by ID⁴MS. Sample (ND) and calibration blends (N*D-1, N*D-2, N*D-3) were prepared according to the protocol explained in Section 3.3.3. 4-n-nonylphenol standard solution (N*) and 4-*n*-nonylphenol-d_8 standard solution (D) used in the preparation of all blends were fixed to 3.0 mg/kg to have an appreciable signal to noise ratio by the GC-MS system. The blend compositions and ID⁴MS results are shown in Table 4.4. The theoretical amount of 4-*n*-nonylphenol in the spiked sample was 2.9184 mg/kg and the experimental results belonging to the sample blends were 2.9243, 2.9147 and 2.9154 mg/kg. The percent recovery result was found to be 99.9 ± 0.54% with the ID⁴MS system.

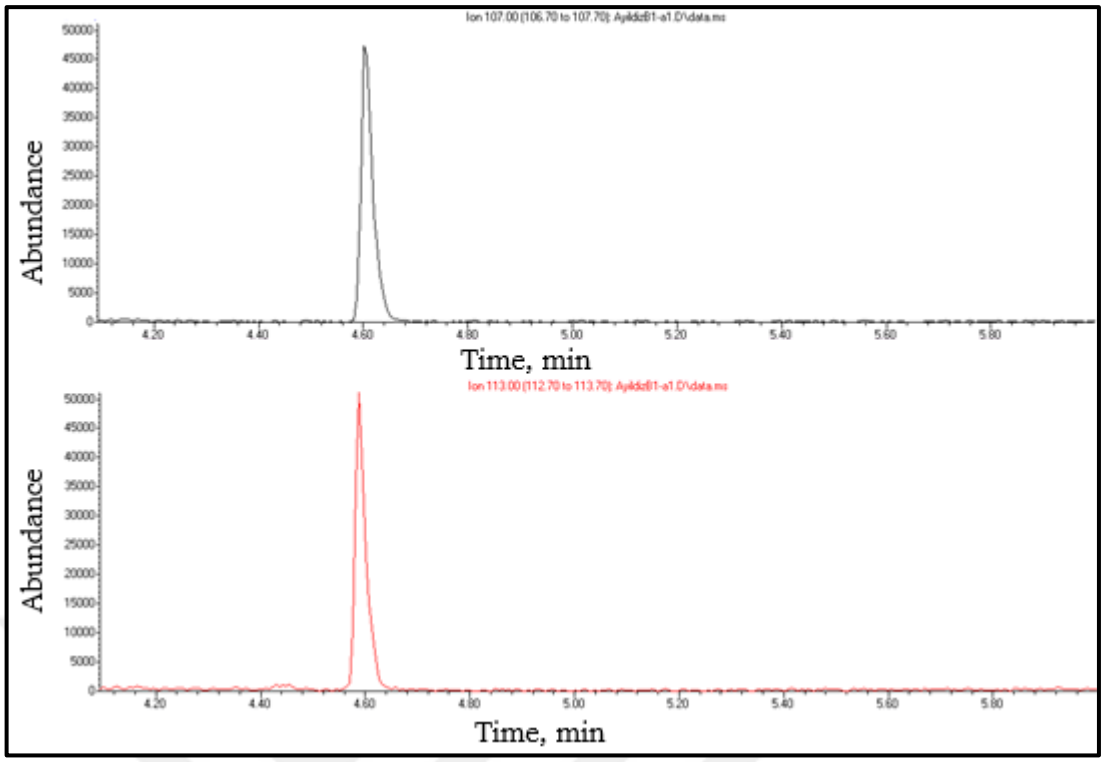

Figure 4.15 Extract ion chromatograms (SIM mode) of 4-*n*-nonylphenol (m/z = 107) and 4-*n*-nonylphenol-d_8 (m/z = 113) in the N*D-1 blend

Table 4.4 Composition of blends and the response obtained for the blends after applying ID^{4}MS

Blend	$m_{N/N^*(N^*D)}$/g	$m_{D(ND/N^*D)}$/g	r_{N/N^*D}/(V/V)	w_{N/N^*}/mg/kg
N*D-1	0.2009	0.4039	1.0891	2.9726
N*D-2	0.3756	0.4007	2.0180	
N*D-3	0.7485	0.4004	4.1224	
ND-1-1	0.3766	0.4005	1.9912	2.9243
ND-1-2	0.3775	0.4015	1.9845	2.9147
ND-1-3	0.3743	0.4063	1.9450	2.9154

4.2.6 SS-LPME-ID^{4}MS

The ID^{4}MS experiments gave remarkable results in terms of accuracy and precision, however, this system can only be applied to concentrations which are in a dynamic range of the instrument used in the isotope measurements [10]. Hence, isotope

dilution was combined with a preconcentration method to enable determination of the analyte (s) at trace levels with high accuracy and precision. In this study, the developed SS-LPME method was coupled to ID^{4}MS to serve the purpose mention earlier. Here, it should be noted that the initial amount of sample in the SS-LPME method was 8.0 g but the total amount of the blends in the ID^{4}MS system are different from each other. In combining these two methods, the final amounts of the blends were fixed to 8.0 g in order to avoid different preconcentration factors. The *Solver tool* in Microsoft Excel was employed to adjust the final masses to 8.0 g with the selected isotope ratios. After preparing 8.0 g of each sample and calibration blend, the blends were held for 2.0 hours to obtain a homogeneous mixture between the isotopes. Then, 4-*n*-nonylphenol and 4-*n*-nonylphenol-d_8 were extracted from the aqueous solution based on the optimized SS-LPME method. The extracts were sent to the GC-MS system and the results obtained are presented in Table 4.5.

Table 4.5 For SS-LPME-GC-MS system, the composition of the blends and their results after applied ID^{4}MS

Blend	$\mathbf{m_{N/N^*(N^*D)}/g}$	$\mathbf{m_{D(ND/N^*D)}/g}$	$\mathbf{r_{N/N^*D}/(V/V)}$	$\mathbf{w_{N/N^*}/mg/kg}$
N*D-1	2.6702	5.3331	0.6670	0.0394
N*D-2	3.8687	4.1340	1.3445	
N*D-3	5.2212	2.7827	2.3258	
ND-1-1	3.8714	4.1309	1.3543	0.0396
ND-1-2	3.8745	4.1259	1.3626	0.0398
ND-1-3	3.8718	4.1240	1.3660	0.0399

As stated earlier, the absence of 4-*n*-nonylphenol in wastewater was proved by blank analysis. Accordingly, the sample was spiked to 39.2 ng/g of 4-*n*-nonylphenol and analyzed by the SS-LPME-ID^4-GC-MS system. The experimental amounts for each sample were 39.6, 39.8 and 39.9 ng/g, as can be seen from Table 4.5. The percent recovery with its relative standard deviation for the municipal wastewater at the spiking level of 39.2 ng/g was calculated as 101.54 ± 0.38%. When all recovery results in this study are compared to each other as presented in Table 4.6, the SS-LPME-ID^4-GC-MS shows superiority thanks to its high recovery result, accuracy and

precision. SS-LPME mitigates the sensitivity limitation of GC-MS to allow detection and quantification of analytes at trace levels. ID^{4}MS was utilized for its high accuracy and precision, and its combination with SS-LPME method provided a more superior technique.

Table 4.6 Comparison of recovery results for all systems studied

Method	Spiked Concentration, ng/g	Percent Recovery Results (±%RSD)
SS-LPME-GC-MS	20	114.9 ± 1.3
SS-LPME-GC-MS	30	90.2 ± 6.7
SS-LPME-GC-MS	40	107.4 ± 4.5
ID^{4}MS	2918.4	99.9 ± 0.5
SS-LPME-ID^4-GC-MS	39.2	101.5±0.4

4.3 Optimization and Validation of BS-LPME-SA-ID^{3}MS for Determination of 4-*n*-nonylphenol

4.3.1 Experimental Design for BS-LPME

BS-LPME was employed as a preconcentration method for trace determination of 4-*n*-nonylphenol. This microextraction technique was optimized by Box-Behnken experimental design after selecting the most influential parameters by univariate approach. Firstly, the type of extraction solvent type was examined by testing chloroform, carbon tetrachloride, dichloromethane and 1,2-dichloroethane (300 µL of each solvent) and their binary combinations (150 µL + 150 µL). The extraction solvents were mixed with 3.0 mL ethanol as dispersive solvent. As shown in Figure 4.16, the binary mixture of dichloromethane and 1,2-dichloroethane had the highest peak area with low standard deviation and it was therefore selected as optimum extraction solvent in this study.

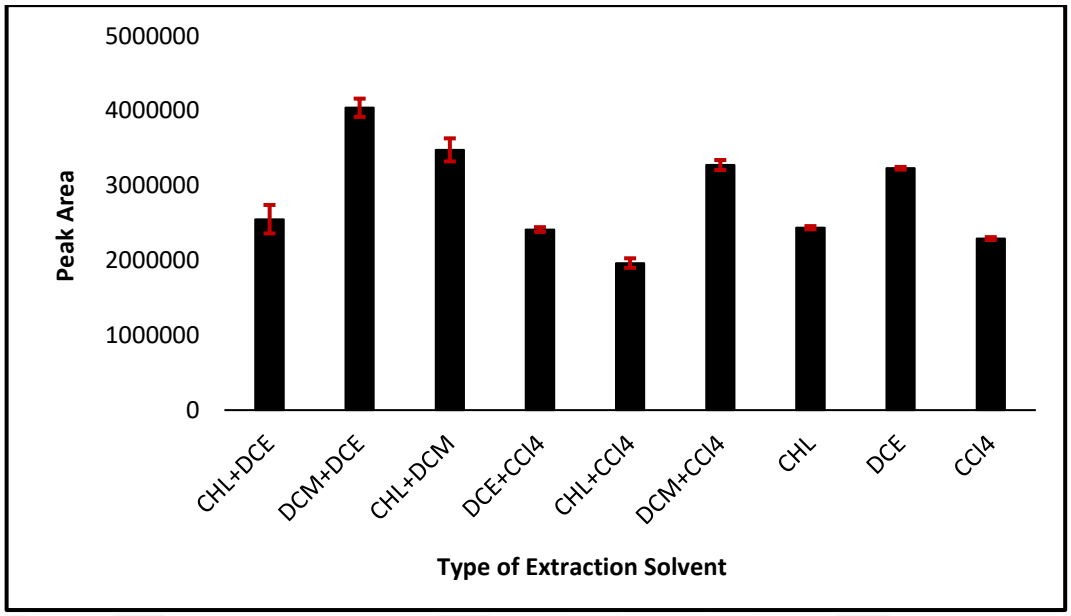

Figure 4.16 Optimization results of extraction solvent type; CHL: chloroform, DCE: 1,2 dichloroethane, DCM: dichloromethane, CCl4: carbon tetrachloride

Another substantial parameter was the type of dispersive solvent which helps to increase the interaction between extraction solvent and aqueous phase. For this reason, acetone, ethanol, acetonitrile and isopropyl alcohol were studied to evaluate their effects on extraction yields. Among these water miscible solvents, ethanol gave a considerable enhancement of peak areas, thus, subsequent experiments were continued with ethanol as dispersant solvent.

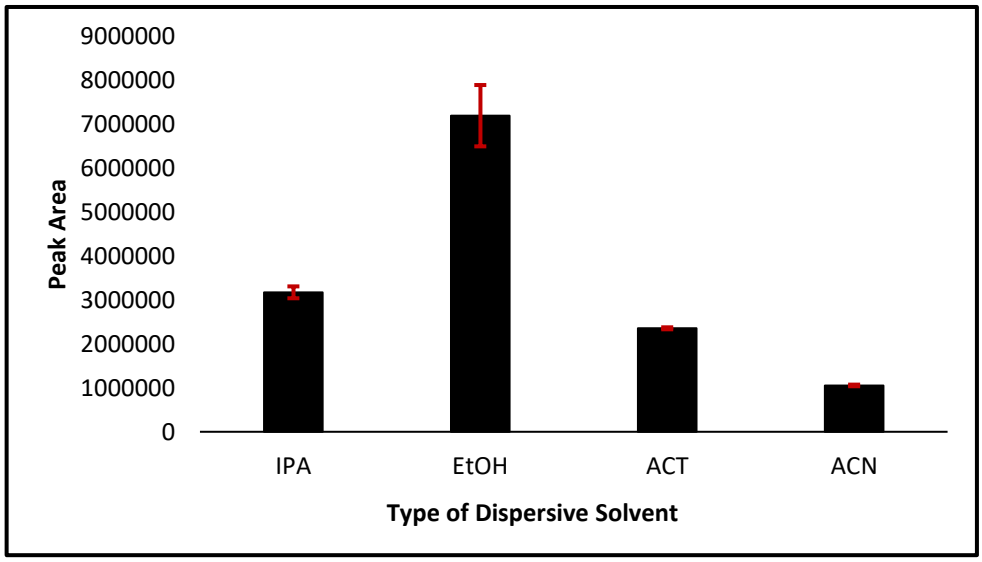

Figure 4.17 Dispersive solvent optimization results; IPA: isopropyl alcohol, EtOH: ethanol, ACT: acetone, ACN: acetonitrile

After the univariate optimizations, Bayesian ANOVA which provides the strength of evidence for the studied variables was applied to the optimum parameters. All variables and their interactions were determined as strong factors according to Bayesian ANOVA results, given in Table 4.7.

Table 4.7 Important variables for the optimized parameters with respect to Bayesian ANOVA

Effects	**P(incl)**	**P(incl|data)**	**BFInclusion**
DCM+DCE	0.737	1.000	3.334e +6
Ethanol	0.737	0.896	3.081
Vortex	0.737	0.602	0.540
DCM+DCE ✻Ethanol	0.316	0.635	3.765
DCM+DCE ✻ Vortex	0.316	0.302	0.936
Ethanol ✻ Vortex	0.316	0.285	0.864
DCM+DCE ✻Ethanol ✻ Vortex	0.053	0.129	2.671

Interactions between variables should be taken into consideration to develop the best model to determine optimum conditions [234]. The volume of dichloromethane + 1,2-dichloroethane (α), the volume of ethanol (β) and vortex period (γ) were evaluated at three levels (low: −1, medium: 0 and high +1) as 200 – 400 µL, 1.0 – 2.5 mL and 15 – 45 s for α, β and γ parameters, respectively. These levels were determined with a pre-study for phase separation, extraction efficiency, peak areas and time.

Box-Behnken Design experiments needs $2k(k-1) + n_c$ points where k and n_c are the number of parameters and the number of central point [194]. For three factors with five central points, a total of 17 combination runs is attained as given in Table 4.8. Each combination was repeated three times utilizing 200 ng/ mL standard solution.

Table 4.8 Combinations for the chemometric optimization of BS-LPME according to Box-Behnken experimental design

STD	RUN	α, µL	β, mL	γ, s
3	1	200	3	30
5	2	200	2	15
2	3	400	1	30
9	4	300	1	15
10	5	300	3	15
7	6	200	2	45
17	7	300	2	30
12	8	300	3	45
14	9	300	2	30
8	10	400	2	45
15	11	300	2	30
13	12	300	2	30
6	13	400	2	15
11	14	300	1	45
1	15	200	1	30
16	16	300	2	30
4	17	400	3	30

After the chemometric experiments, a quadratic model was obtained as follows:

$$R = 3522000 - 4626000\,\alpha + 891800\,\beta - 1084000\,\alpha\beta + 3106000\alpha^2 \qquad (4.4)$$

Table 4.9 The ANOVA results of applied chemometric experiments

Source	Sum of squares	Degree of freedom	Mean squares	F-value	P-value
Model	2.231×10^{14}	4	5.578×10^{13}	197.15	<0.0001
α	1.712×10^{14}	1	1.712×10^{14}	605.10	<0.0001
β	6.362×10^{12}	1	6.362×10^{12}	22.49	0.0005
αβ	4.698×10^{12}	1	4.698×10^{12}	16.61	0.0015
α^2	4.086×10^{13}	1	4.086×10^{13}	144.41	<0.0001
Residual	3.395×10^{12}	12	2.829×10^{11}		
Lack of fit	2.986×10^{12}	8	3.732×10^{11}	3.65	0.1134
Pure error	4.094×10^{11}	4	1.023×10^{11}		
Cor. total	2.265×10^{14}	16			
R^2	0.9542				
R^2-Adjusted	0.9800				

α: The volume of dichloromethane+1,2-dichloroethane, β: The volume of ethanol, γ: Vortex period.

Design-Expert 7.0.0 software was used in all chemometric analysis and fitting. The analysis of variance (ANOVA) demonstrated in Table 4.9 confirms that the F-value is greater than F critic and p-values are less than 0.05, thus the model is significant. The lack of fit of p value showed no statistical significance because p value was higher than 0.05. In addition, the small difference between R^2 and adjusted R^2 was enough for the model's suitability. It can be said that predicted values were compatible with the experimental values thanks to normal distribution of the experimental data, as shown in Figure 4.18.

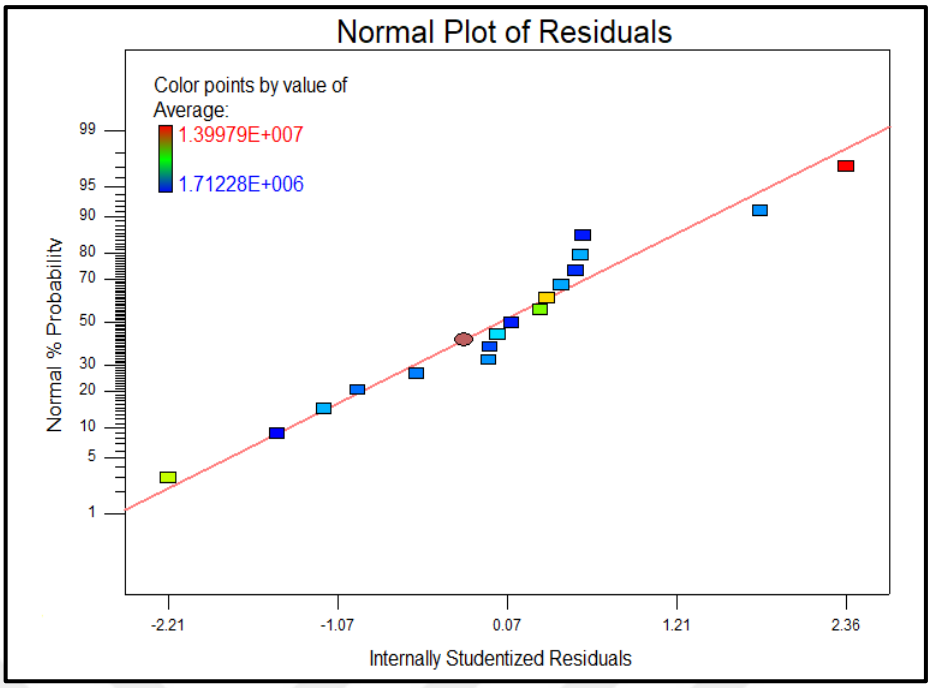

Figure 4.18 Normal distribution plot for the experimental data

The α and αβ terms affected the model negatively because increasing the volume of binary solvent leads to over-dilution of the standard/sample and low preconcentration factors. However, β term had a positive trend on the peak areas. Higher volumes of the dispersive solvent make an over-dispersion of the extraction solvent but for lower volumes had an inadequate dispersion through the aqueous solution. Hence, β term should be adjusted to an optimum value. γ term was not a significant variable according to the attained model. The optimum conditions were recorded as 250 µL of dichloromethane + 1,2-dichloroethane (1:1, v/v), 1.85 mL of ethanol and 15 s vortex period with respect to results of chemometric experiments.

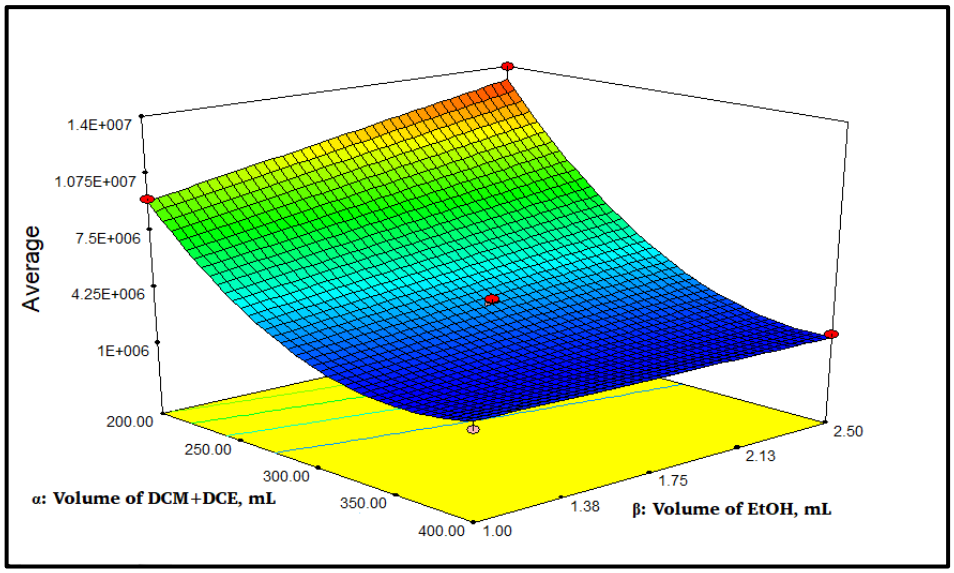

Figure 4.19 The three-dimensional response surface plots showing the effect of binary solvent amount and ethanol amount

Three-dimensional response surface plot shows graphical representations of the regression model. It is obtained as a function of volume of dichloromethane+1,2-dichloroethane and ethanol at 30 s fixed vortex period.

4.3.2 Analytical Figures of Merit for BS-LPME-GC-MS

BS-LPME was used to decrease the detection limit for the determination of 4-*n*-nonylphenol by GC-MS. In order to make a comparison between BS-LPME-GC-MS and GC-MS systems, different concentrations of the analyte standard solutions were injected into GC-MS system. A linear calibration plot was obtained in the range of 1.0 and 25.0 mg/kg, with 0.29 mg/kg of LOD and 0.98 mg/kg of LOQ.

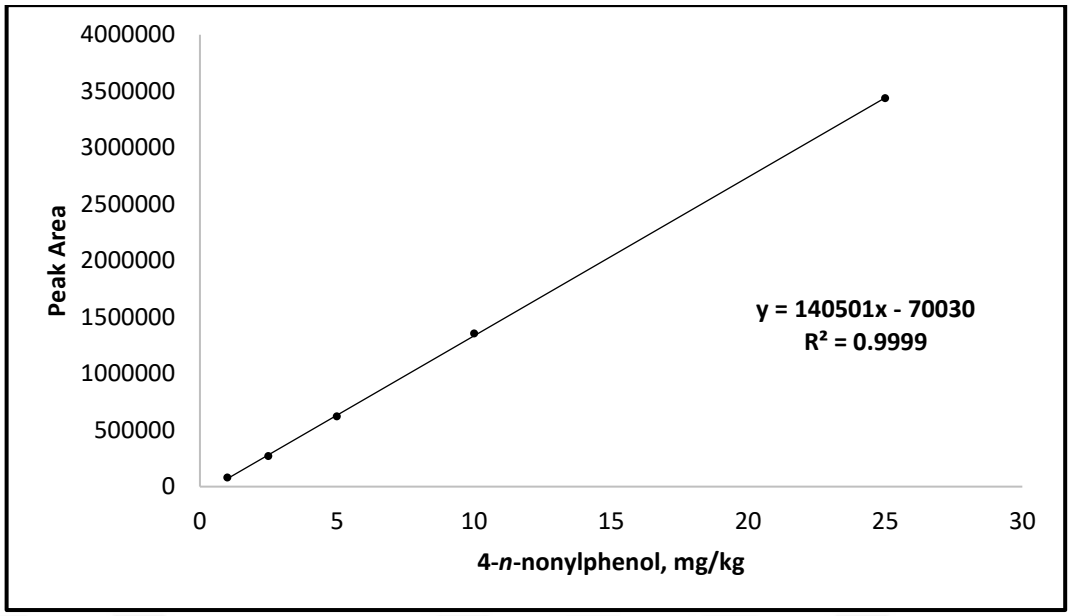

Figure 4.20 GC-MS calibration plot for 4-*n*-nonylphenol

The next step was to validate the BS-LPME-GC-MS method, where the optimum BS-LPME conditions were applied to different concentrations of the analyte. The first and the last points of the calibration plot were determined as 2.0 and 100 ng/g, as shown in Figure 4.21.

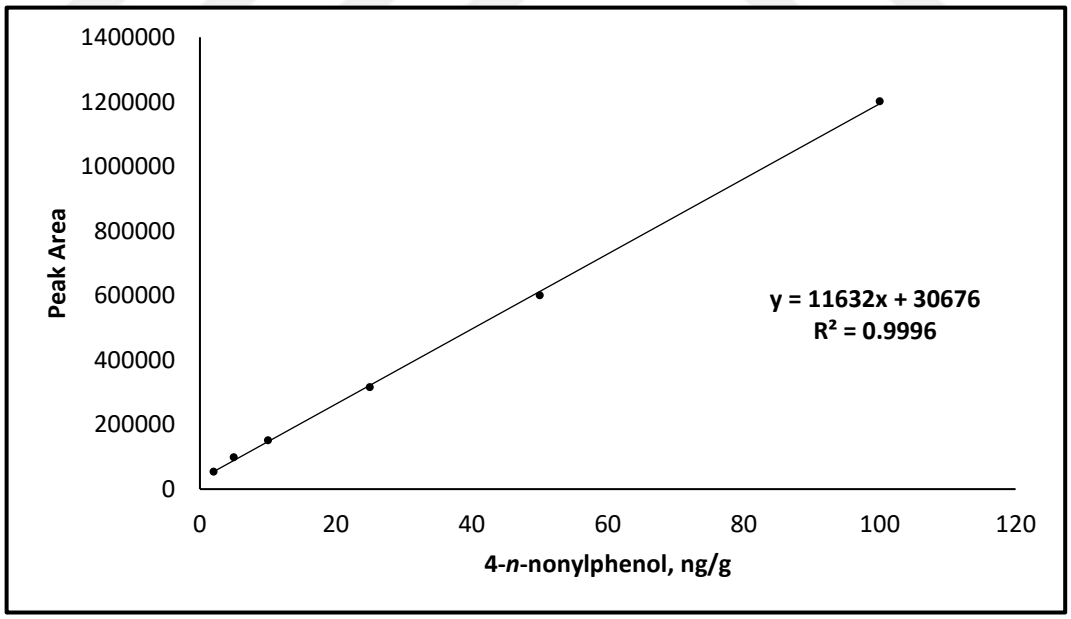

Figure 4.21 BS-LPME-GC-MS calibration plot of 4-*n*-nonylphenol

LOD and LOQ values for the BS-LPME-GC-MS method were recorded as 0.6 and 2.0 ng/g, respectively. The coefficient of variation for the lowest concentration in the calibration plot was calculated as 4.3%, for six replicates of the same concentration.

Enhancement in detection power was found to be 495.4 times, by taking the ratio of GC-MS LOD to BS-LPME-GC-MS LOD.

4.3.3 Recovery Studies for BS-LPME-GC-MS

Two different samples, wastewater and infant formula were selected for recovery studies. The wastewater sample was analyzed by the developed BS-LPME-GC-MS method but there was no detectable 4-*n*-nonylphenol in the sample. After that, it was spiked to 20, 40 and 80 ng/g levels. Their recovery results were calculated as 213±3.0, 181±7.0 and 152±2.0%, respectively. The calculation was made by comparing peak areas of the spiked samples with peak areas of standard solution at the same concentrations.

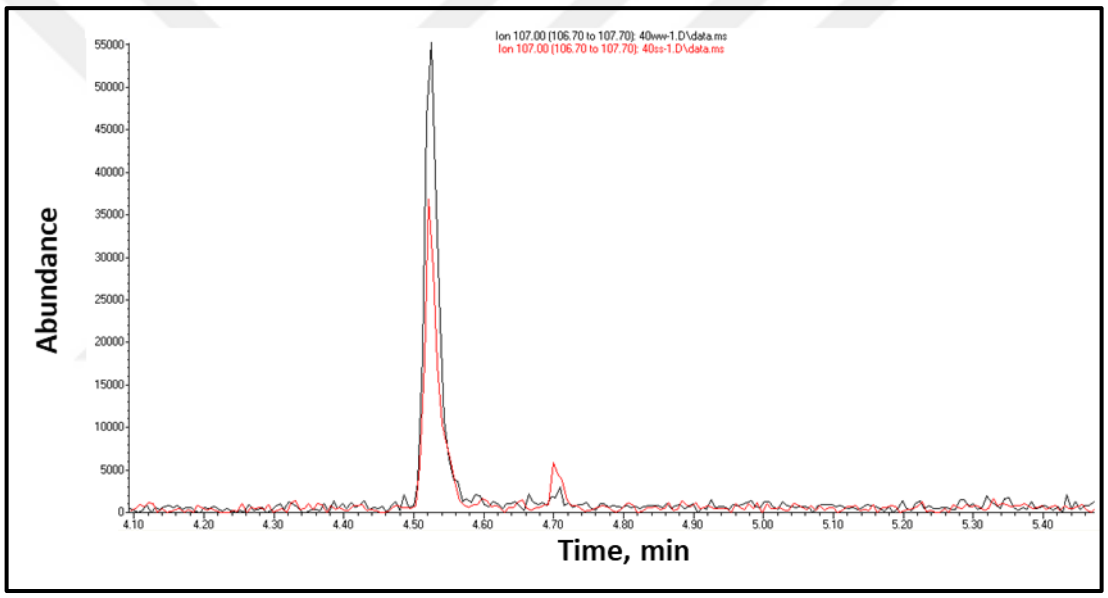

Figure 4.22 Comparison of 40 ng/g of wastewater sample (black) and standard solution (red) chromatograms with each other

Meanwhile, 1.0 g of infant formula sample was weighed, and 8.0 g simulated gastric juice was added into the sample. The spiking step was done to obtain 20, 40 and 80 ng/g of the analyte in the sample solution, and the developed BSME-GC-MS was applied to the spiked sample solutions. The respective recovery results recorded for 20, 40 and 80 ng/g spiked sample solutions were 160±5.0, 179±9.0 and 120.0±0.3%. All recovery results for wastewater spiked at different concentrations indicated that there were matrix interferences on the target analyte. For this reason, a new

analytical method was needed in order to minimize the interferences arising from complex matrices.

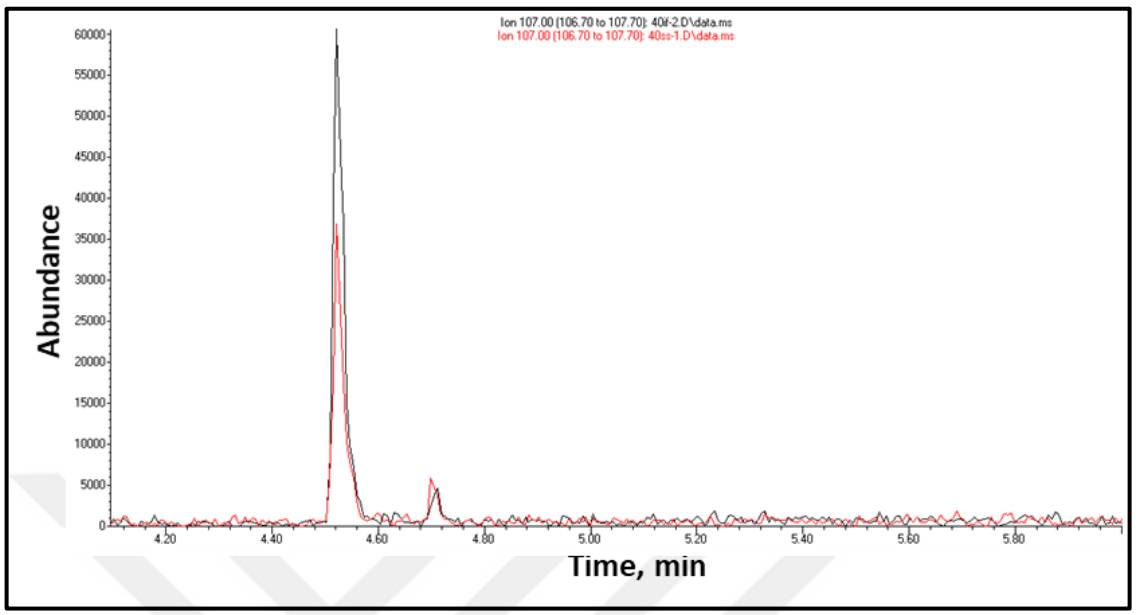

Figure 4.23 Chromatograms of infant formula (black, spiked 40 ng/g) and 40 ng/g of standard solution (red)

4.3.4 Direct SA-ID^{3}MS Method

The SA-ID^{3}MS method was proposed to mitigate matrix effects on instrumental measurements [212]. Only wastewater sample was analyzed by this method at approximately 4.0 mg/kg level. Neither dilution nor preconcentration was performed in this study. The wastewater sample was filtered and directly spiked to the desired concentration. All blends were prepared according to the masses given in Table 4.10. Triplicates of each blend were then held for 2.0 hours to obtain homogeneity in terms of the isotopes. Isotope ratios measured by GC-MS are written in Table 4.10.

Table 4.10 Weight of isotope solutions in each blends and measured isotopes ratios by GC-MS system

Blend	$m_{N(NN^*D)}$/g	$m_{N^*(NN^*D)}$/g	$m_{D(NN^*D)}$/g	r_{NN^*D}/(V/V)	w_{N/N^*}/mg/kg
NN*D-1	0.2957	0.0000	0.2987	2.4997	
NN*D-2	0.3004	0.5985	0.2970	7.6505	3.9999
NN*D-3	0.2963	0.2985	0.5989	2.5054	
NN*D-1	0.3012	0.0000	0.3006	2.5562	
NN*D-2	0.2958	0.5979	0.2983	7.3478	3.9970
NN*D-3	0.3006	0.2961	0.6016	2.5305	
NN*D-1	0.2997	0.0000	0.2930	2.5533	
NN*D-2	0.3008	0.5991	0.3002	7.5343	3.9944
NN*D-3	0.2981	0.2962	0.5982	2.4793	

Theoretical and experimental amount of 4-*n*-nonylphenol were calculated to be 3.992 and 3.997 mg/kg, respectively. A low %RSD value of 2.6% was achieved in the experiment. This result establishes high recovery of 100.1% with low standard deviation for wastewater matrix. It can be easily expressed that the SA-ID^{3}MS method can be used to overcome matrix effects of wastewater for the determination of 4-*n*-nonylphenol.

4.3.5 Comparison of ID^{2}MS and SA-ID^{3}MS Methods

A comparison between ID^{2}MS and SA-ID^{3}MS methods was also investigated to show the differences between the two methods with regard to accuracy and precision. In the ID^{2}MS studies, calibration and sample blends were prepared by using 4.0 mg/kg 4-*n*-nonylphenol and 4-*n*-nonylphenol-d_8. Isotope ratios were the same for both blends as 1.077. Theoretical and experimental recovery results for this study were 3.9789 and 3.9431 mg/kg, respectively, resulting in 99.1±1.9% recovery result. Similar results were also obtained by SA-ID^{3}MS method with recovery results as 100.1±2.6%. On the other hand, SA-ID^{3}MS has a great advantage regarding isotope ratio of D (r_D) which does not need to be exactly known for SA-ID^{3}MS strategies. For ID^{2}MS calculations, r_D is a variable in the equations and does not focus on the matrix interferences. Hence, SA-ID^{3}MS can achieve higher recoveries than ID^{2}MS for complex matrices like wastewater.

4.3.6 Integration of BS-LPME and SA-ID^{3}MS Methods

Detection limits of isotope dilution techniques are limited by the sensitivity of mass spectroscopic techniques. For this reason, a preconcentration technique can be integrated into isotope dilution strategies by a proper blend preparation. This integration can make it possible to perform trace level determinations with considerably high accuracy and precision [10].

In this study, all blends had the same mass of sample but different mass of 4-*n*-nonylphenol and its labelled compound. In addition, all blends were fixed to a total mass of 8.0 g because different masses of the blends can lead to different preconcentration factors. After preparation of all blends as presented in Table 3.7, equilibration was set at 2.0 hours. Then, the optimized BS-LPME method was employed to preconcentrate and separate 4-*n*-nonylphenol from wastewater sample and infant formula. All masses of blends for wastewater and infant formula are presented in Table 4.11 and 4.12. For wastewater sample, 49.0 ng/g theoretical and 49.2 ng/g experimental concentrations were calculated, while for infant formula, 50.7 ng/g of theoretical concentration and 50.4 ng/g of experimental concentration were found. Recovery results for wastewater and infant formula were found to be 100.3±1.6% and 99.4±1.7%, respectively. It was proved that BS-LPME-SA-ID^{3}MS can mitigate matrix effects of wastewater and infant formula at trace levels of the target analyte and provides high recoveries, accuracy and precision when compared to the BS-LPME-GC-MS system.

Table 4.11 Masses of isotopes in the blends and BS-LPME-SA-ID^3-GC-MS for wastewater sample

Blend	$m_{N(NN^*D)}$/g	$m_{N^*(NN^*D)}$/g	$m_{D(NN^*D)}$/g	r_{NN^*D}/(V/V)	w_{N/N^*}/mg/kg
NN*D-1	1.9970	0.0000	1.9961	1.3000	
NN*D-2	1.9950	4.0175	1.9970	1.8719	0.0494
NN*D-3	2.0012	1.9967	4.0048	1.2989	
NN*D-1	2.0059	0.0000	2.0090	1.3205	
NN*D-2	2.0020	4.0173	2.0043	2.9933	0.0492
NN*D-3	1.9995	2.0177	3.9985	1.3256	
NN*D-1	1.9986	0.0000	2.0729	1.3070	
NN*D-2	2.0019	4.0020	1.9965	2.3726	0.0488
NN*D-3	1.9995	2.0177	3.9985	1.3300	

Table 4.12 BS-LPME- SA-ID^3-GC-MS results and masses of isotope solutions in the blends for infant formula sample

Blend	$m_{N(NN^*D)}$/g	$m_{N^*(NN^*D)}$/g	$m_{D(NN^*D)}$/g	r_{NN^*D}/(V/V)	w_{N/N^*}/mg/kg
NN*D-1	1.9975	0.0000	1.9990	0.8069	
NN*D-2	2.0002	4.0076	2.0019	1.9951	0.0511
NN*D-3	2.0026	2.0027	4.0086	0.7956	
NN*D-1	2.0021	0.0000	1.9962	0.8126	
NN*D-2	1.9973	4.0078	2.0024	2.0230	0.0498
NN*D-3	2.0017	2.0064	3.9937	0.8093	
NN*D-1	2.0007	0.0000	1.9992	0.8061	
NN*D-2	1.9997	4.0083	2.0017	2.0805	0.0502
NN*D-3	1.9990	2.0055	4.0007	0.8000	

4.4 Optimization and Validation of DLLME for Determination of Selected Organotin Compounds

Parameters of ethyl borate derivatization reaction and DLLME for the determination of butyltin compounds were optimized to have lower detection limits. Some parameters were optimized by a univariate approach while the rest were optimized

by chemometric approach (Box Behnken Design). In the course of optimizations, 150 and 50 µg/kg mixed standard solutions were used. Optimum parameters were selected based on three replicates of each experiment for univariate optimizations and mathematical calculations by Design Expert 7.0 BBD for chemometric optimizations.

4.4.1 Univariate Optimizations

4.4.1.1 Selection of extraction solvent

Extraction output mainly depends on the ability of an extraction solvent to gather the analyte of interest from aqueous solution. Dichloromethane, chloroform, 1,2-dichloroethane, carbon tetrachloride and their binary mixtures in the 1:1 (v/v) ratio were tried to achieve the highest extraction efficiency. For the extraction of organotin compounds, hexane has been widely used in literature but its lower density than water makes it settle as upper phase and this could create volatility problems for hexane phase and volatile analytes [235], [236].

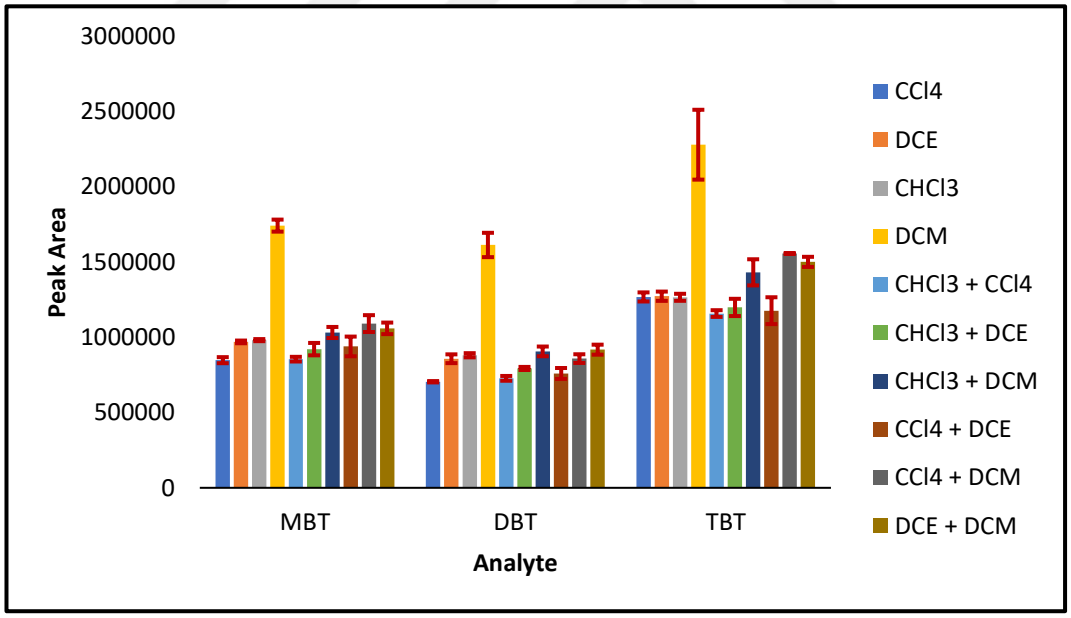

Figure 4.24 Optimization of extraction solvent type (n=3 error bars)

As given in Figure 4.24, dichloromethane had the highest peak area for each analyte than the other solvents and binary mixtures. Hence, dichloromethane was used for further optimizations.

4.4.1.2 Selection of dispersive solvent

In the DLLME technique, dispersive solvents play a vital role in the distribution of extraction solvent throughout the aqueous solution. This distribution provides more interactions between droplets of the extraction solvent and aqueous solution. Methanol, isopropyl alcohol, acetone, ethanol and acetonitrile were tried for their dispersion efficiency on dichloromethane. Methanol and ethanol gave nearly the same results, but ethanol had the highest signal for MBT and TBT seen in Figure 4.25. For this reason, ethanol was selected as dispersive solvent for the DLLME procedure.

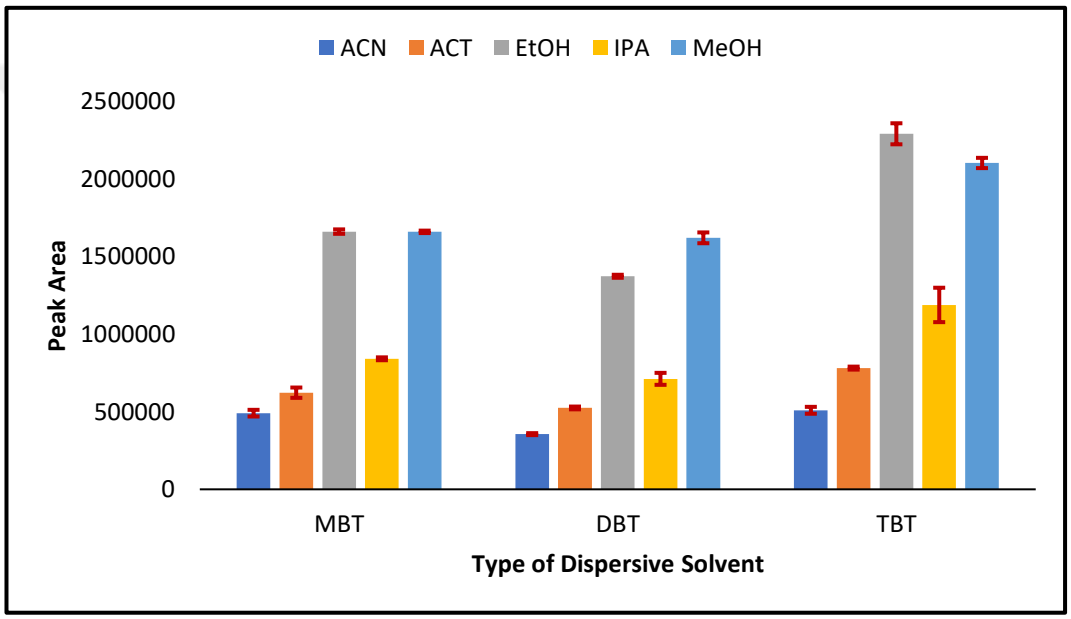

Figure 4.25 Optimization of dispersant solvent type (n=3 error bars)

4.4.1.3 Volume of acetate buffer solution

Sodium tetraethylborate ($NaBEt_4$) is also applied for the derivatization of metal and organometallic species in aqueous solution [237]. Its general reaction can be seen in Figure 4.26 for the derivatization of organotin compounds. As an example, pesticidal and nonpesticidal organotin compounds were detected in water matrices by gas chromatography pulsed flame photometric detection (GC-PFPD). $NaBEt_4$ was used as an ethylating agent in that study[238].

$$3NaB(Et)_4 + RSn^{3+} \rightarrow (Et)_3SnR + 3(Et)_3B + 3Na^+$$

Figure 4.26 An example of ethylation reaction for organotin compounds [237]

Derivatization of butyltin compounds by $NaBEt_4$ reagent was investigated by de la Calle Guntinas *et al*. by considering the terms: pH, $NaBEt_4$ concentration and reaction period. The highest derivatization yield was obtained between 4.5 and 5.0 pH values [239].

In this study, pH 5.0 acetate buffer solution was prepared for the derivatization reaction, but its volume was optimized by testing 0.10, 0.25, 0.50, 1.0 and 1.50 mL of the buffer solution. As shown in Figure 4.27, 0.25 mL of the buffer solution gave the highest signal for MBT, DBT and TBT. Increasing volumes above 0.25 mL may cause excess dilution of the aqueous solution leading to decreased extraction outputs. In addition, insufficient pH adjustment was observed for volumes lower than 0.25 mL. An additional experiment was done for extraction with no addition of the buffer solution and this recorded the lowest extraction output.

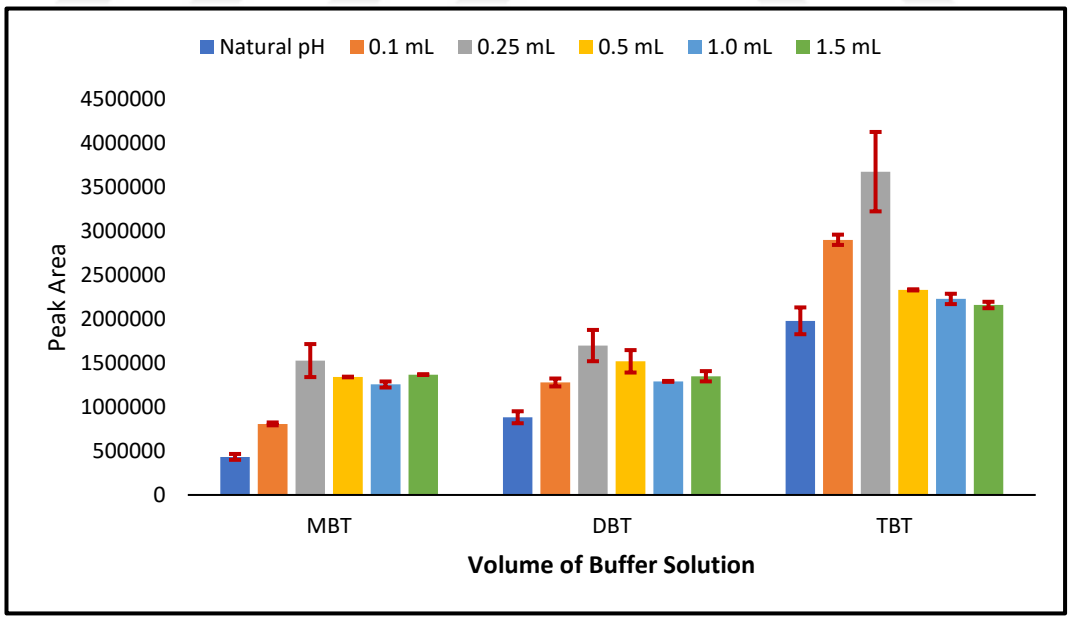

Figure 4.27 Optimization of buffer solution volume (n=3 error bars)

4.4.1.4 Vortex Period

Mixing is an important process for microextraction processes. If interaction between extraction solvent and aqueous phase is enhanced, analytes can be easily collected by the extraction solvent. Mixing is an effective way to improve this interaction[240].

In this study, mixing by vortex was selected for its practical and easy application. Testing of different vortex periods was essential to attain better extraction yields. Different periods including 0.0, 15, 30 and 45 seconds of vortex mixing were tried with three replicates. As can be seen in Figure 4.28, 15 seconds were enough to enhance mass transfer of butyltin compounds into the organic phase. Higher period may lead to analyte transfer from the organic phase into aqueous phase. When compared with no mixing experiments, the 15 seconds period recorded peak area values about sixty times higher than no mixing.

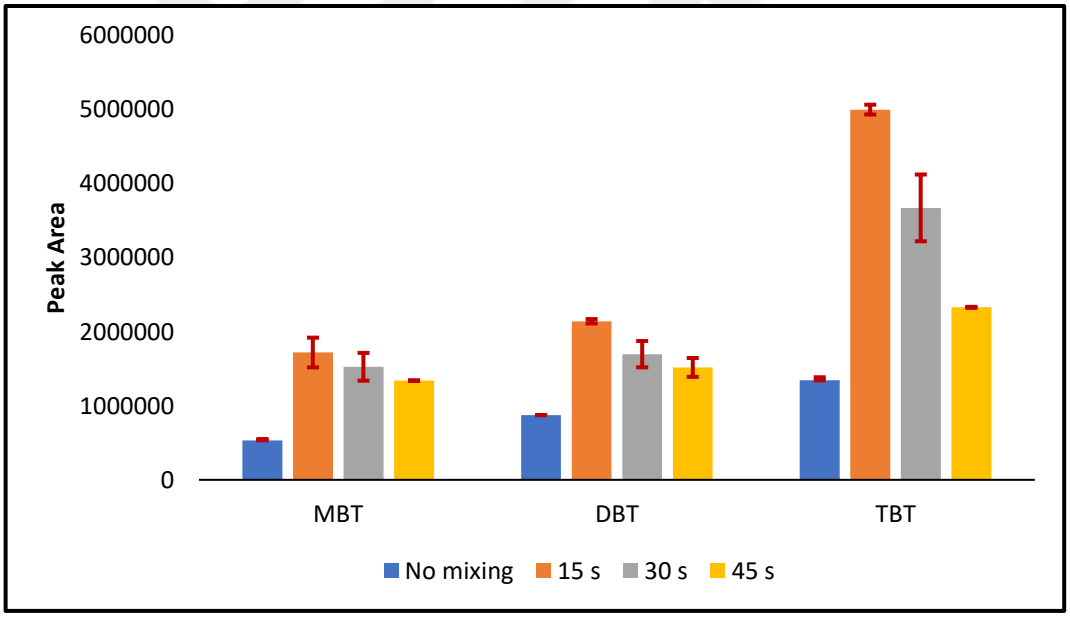

Figure 4.28 Optimization of mixing period (n=3 error bars)

4.4.1.5 Derivatization Period

Ethyl borate derivatization was evaluated in terms of the reaction period. In order to obtain high amounts of derivatized analytes, 5.0, 10.0, 20.0 and 30.0 minutes of derivatization periods were tested after adding pH 5.0 buffer solution and $NaBEt_4$ reagent. Before the selected wait periods, manual shaking in the up-down motion was applied. According to Figure 4.29, an increasing trend with increasing

derivatization period was attained for all analytes. The highest peak area values were obtained with 30.0 minutes for all derivatized analytes. In order not to have a time-consuming method for routine laboratories, wait periods higher than 30.0 minutes were not investigated.

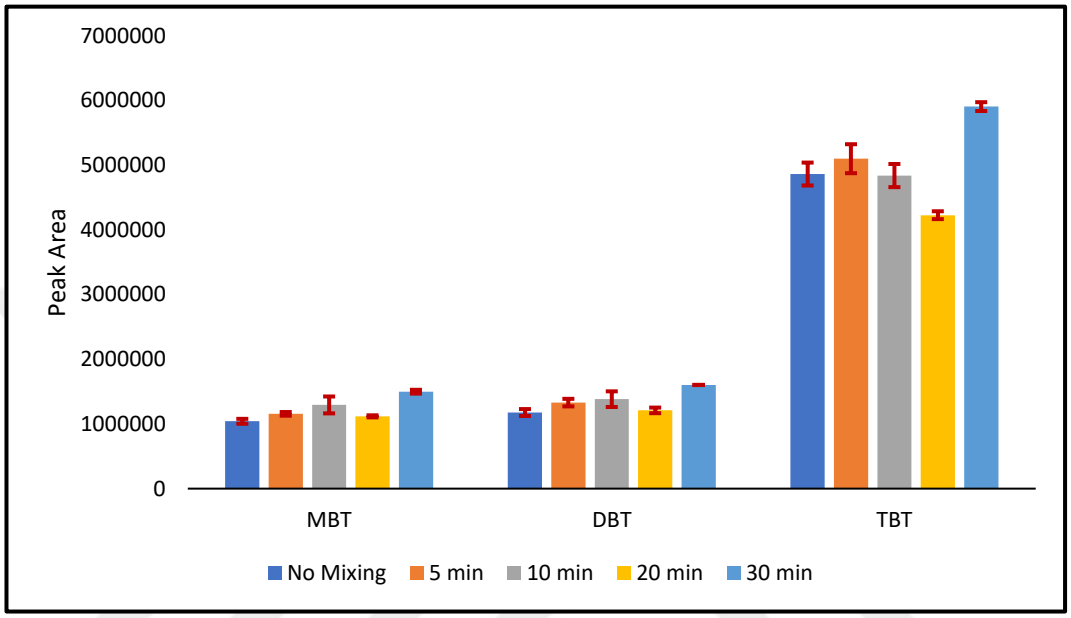

Figure 4.29 Optimization of derivatization period (n=3 error bars)

4.4.2 Chemometric Optimization

Box-Behnken Design (BBD) was used to find optimum conditions for the volume of dichloromethane (A, extraction solvent), volume of ethanol (B, dispersive solvent) and volume of $NaBEt_4$ (C, derivatizing reagent). In this step, 17 experimental runs with five center points were scheduled by Box-Behnken Design (*Design Expert 7.0*) at three levels (low; -1, middle; 0 and high; +1) as given in Table 4.13. Throughout this study, 50.0 ng/mL of mix standard solution was used.

Table 4.13 Combinations of experimental design

Std	Run	A, µL	B, mL	C, µL
2	1	500	1.000	30
5	2	300	1.375	10
1	3	300	1.000	30
3	4	300	1.750	30
6	5	500	1.375	10
15	6	400	1.375	30
8	7	500	1.375	50
14	8	400	1.375	30
12	9	400	1.750	50
13	10	400	1.375	30
4	11	500	1.750	30
9	12	400	1.000	10
17	13	400	1.375	30
11	14	400	1.000	50
7	15	300	1.375	50
10	16	400	1.750	10
16	17	400	1.375	30

The following empirical quadratic equation for A, B and C variables were utilized to fit and predict the optimum conditions for derivatization and extraction of MBT, DBT and TBT compounds:

$$R_{MBT} = 491900 - 256700A + 123700B + 34126.31C - 64826.38BC + 235800\ A^2 \quad (4.5)$$

$$R_{DBT} = 394100 - 300900A + 54425.25B - 63545.71AC + 254500\ A^2 \quad (4.6)$$

$$R_{TBT} = 1243000 - 1113000A + 849100C - 7.42300AC + 879000\ A^2 \quad (4.7)$$

All coefficients of the three quadratic equations were produced by Design Expert 7.0 software. Additionally, all experimental outputs with three replicates were interpreted by analysis of variance (ANOVA) as given in Table 4.14 for each butyltin

compound. When analytes are individually evaluated, P-value was smaller than 0.0001 at 95% confidence level, meaning the P-value was statistically significant. A good correlation between the estimated and experimental results indicated that the design was properly fitted according to R^2 and adjusted R^2 values of 0.9447 and 0.9196, respectively. For DBT and TBT compounds, their P-values were also smaller than 0.0001 which shows the estimated model fitted to the experimental results. R^2 and adjusted-R^2 values for DBT and TBT showed the correlation that offered high accuracy and reliability of the results.

Table 4.14 ANOVA results for MBT, DBT and TBT

Analyte	Source	Sum of squares	Degree of freedom	Mean squares	F-value	P-value
MBT	**Model**	$9.114x10^{11}$	5	$1.823x10^{11}$	37.60	< 0.0001
	A	$5.274x10^{11}$	1	$5.274x10^{11}$	108.79	< 0.0001
	B	$1.224x10^{11}$	1	$1.224x10^{11}$	25.25	0.0004
	C	$9.317x10^{9}$	1	$9.317x10^{9}$	1.92	0.1931
	BC	$1.681x10^{10}$	1	$1.681x10^{10}$	3.47	0.0895
	A^2	$2.355x10^{11}$	1	$2.355x10^{11}$	48.58	< 0.0001
	Residual	$5.332x10^{10}$	11	$4.847x10^{9}$		
	Lack of fit	$4.825x10^{10}$	7	$6.892x10^{9}$	5.43	0.0606
	Pure Error	$5.073x10^{9}$	4	$1.268x10^{9}$		
	Cor. total	$9.647x10^{11}$	16			
	R^2	0.9447				
	R^2-Adjusted	0.9196				
DBT	**Model**	$1.039x10^{12}$	4	$2.596x10^{11}$	79.07	< 0.0001
	A	$7.245x10^{11}$	1	$7.245x10^{11}$	220.63	< 0.0001
	B	$2.370x10^{10}$	1	$2.370x10^{10}$	7.22	0.0198
	AC	$1.615x10^{10}$	1	$1.615x10^{10}$	4.92	0.0466
	A^2	$2.742x10^{11}$	1	$2.742x10^{11}$	83.51	< 0.0001
	Residual	$3.940x10^{10}$	12	$3.284x10^{9}$		
	Lack of fit	$3.161x10^{10}$	8	$3.951x10^{9}$	2.03	0.2582
	Pure error	$7.794x10^{9}$	4	$1.948x10^{9}$		
	Cor. total	$1.078x10^{12}$	16			
	R^2	0.9634				
	R^2-Adjusted	0.9513				

TBT	**Model**	$2.115x10^{13}$	4	$5.287x10^{12}$	69.28	< 0.0001
	A	$9.902x10^{12}$	1	$9.902x10^{12}$	129.77	< 0.0001
	C	$5.768x10^{12}$	1	$5.768x10^{12}$	75.58	< 0.0001
	AC	$2.204x10^{12}$	1	$2.204x10^{12}$	28.88	0.0002
	A^2	$3.273x10^{12}$	1	$3.273x10^{12}$	42.89	< 0.0001
	Residual	$9.157x10^{11}$	12	$7.631x10^{10}$		
	Lack of fit	$8.328x10^{11}$	8	$1.041x10^{11}$	5.02	0.0681
	Pure error	$8.288x10^{10}$	4	$2.072x10^{10}$		
	Cor. total	$2.206x10^{13}$	16			
	R^2	0.9585				
	R^2-Adjusted	0.9447				

A (volume of dichloromethane) and A^2 (quadratic effect of A) terms were found to be significant for all three analytes. The B term was significant for MBT and DBT but C term was important for MBT and TBT. The AC term (interaction between volume of dichloromethane and $NaBEt_4$) was important for DBT and TBT determination while BC (interaction between volume of ethanol and $NaBEt_4$) term was important for MBT compound. Figure 4.30 shows a three-dimensional response surface plots for Equation (4.5), (4.6) and (4.7).

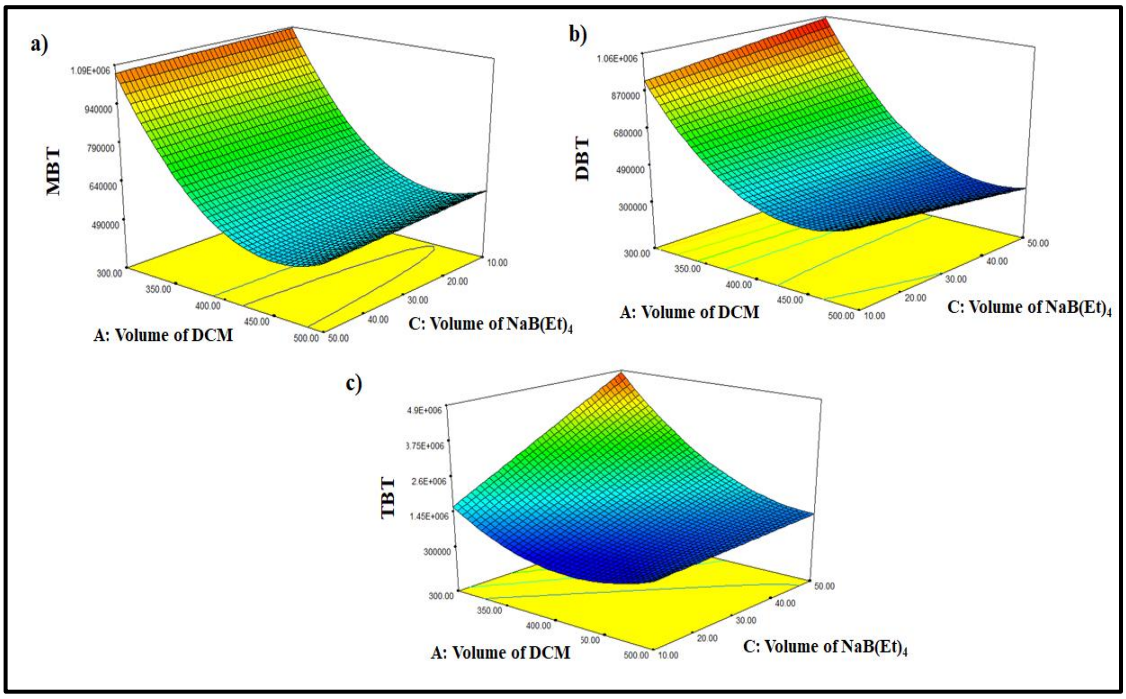

Figure 4.30 The three-dimensional response surface according to results of experimental design for each analyte

Lower volumes of dichloromethane and higher volumes of $NaBEt_4$ gave the highest peak areas for each analyte. Higher volumes of dichloromethane could dilute the analyte after extracting it from the standard/sample solution. The volume of $NaBEt_4$ was also important to obtain derivatized form of the analytes and its low volumes could not be enough to get high signals. Volume of ethanol was also substantial because it increases the surface area between extraction solvent and aqueous solutions, but higher volumes could lead to over dispersion of the extraction solvent in the standard/sample solution.

Model predictor in *Design Expert 7.0 software* proposed 300 µL of DCM, 1.64 mL of EtOH and 47.34 µL of $NaBEt_4$ as the optimum conditions to obtain high outputs for MBT, DBT and TBT compounds.

Al optimum conditions determined by univariate and chemometric approaches are summarized in Table 4.15.

Table 4.15 Optimum conditions for DLLME after univariate and chemometric optimizations

Variable	**Optimum Value**
Extraction solvent and its volume	Dichloromethane, 300 µL
Dispersive solvent and its volume	Ethanol, 1.64 mL
Vortex Period	15.0 s
Volume of $NaBEt_4$ (9.7% in THF)	47.34 µL
Volume of pH 5.0 buffer solution	0.25 mL
Derivatization Period	30 min

4.4.3 Analytical Figures of Merit for DLLME-GC-MS

Analytical figures of merit for the direct determination of butyltin compounds by GC-MS and for the optimized DLLME method are presented in Table 4.16. The parameters evaluated included LOD, LOQ, %RSD and correlation coefficient. The linear ranges for MBT, DBT and TBT in the GC-MS system were 281 – 4875, 281 – 4868 and 86 – 10009 µg/kg, respectively. Linearity of the DLLME-GC-MS method was determined between 2.0 and 44.2 µg/kg for all three analytes. In addition, enhancement in detection power recorded by the DLLME-GC-MS method relative to

direct GC-MS for MBT, DBT and TBT compounds were calculated as 191.5, 83.6 and 70.9 folds, respectively.

Table 4.16 Analytical figures of merit for each analyte and comparison of DLLME-GC-MS and GC-MS systems

Analyte	System	LOD (µg/kg)	LOQ (µg/kg)	%RSD for the lowest concentration in calibration plot	R^2	Enhancement in Detection Powers
MBT	GC-MS	75	250	8.0	1.0000	191.5
	DLLME-GC-MS	0.40	1.3	9.3	0.9999	
DBT	GC-MS	52	172	5.8	0.9999	83.6
	DLLME-GC-MS	0.60	2.1	7.9	0.9994	
TBT	GC-MS	35	116	9.3	0.9998	70.9
	DLLME-GC-MS	0.50	1.6	9.31	0.9997	

As can be seen from Table 4.16, high preconcentration factors with good correlation were achieved by the proposed DLLME-GC-MS method. Low detection limits in the range of 0.40 and 0.60 µg/kg were attained over wide linear ranges of the analytes.

4.4.4 Recovery Studies for DLLME-GC-MS System

With the aim of establishing applicability and accuracy of the proposed method, anchovy and mytilus galloprovincialis samples were spiked with the analytes and analyzed by the developed derivatization and microextraction methods. Four aquatic samples were firstly weighed and lyophilized as detailed in Section 3.4.3. There was no detectable MBT, DBT and TBT in the blank analysis of anchovy and mytilus galloprovincialis samples. Afterwards, one anchovy and one mytilus galloprovincialis samples were spiked to 5.0 µg/kg and analyzed by the DLLME-GC-MS system. Percent recovery results were calculated between 84 and 113% with low standard deviation values as given in Table 4.17. It can be clearly stated that MBT, DBT and TBT compounds can be determined from anchovy and mytilus galloprovincialis samples with high accuracy and repeatability. Figures 4.31 and

4.32 are overlay chromatograms respectively showing the closeness of spiked (5.0 ng/g) fish and mussel samples to 5.0 ng/g standard solution.

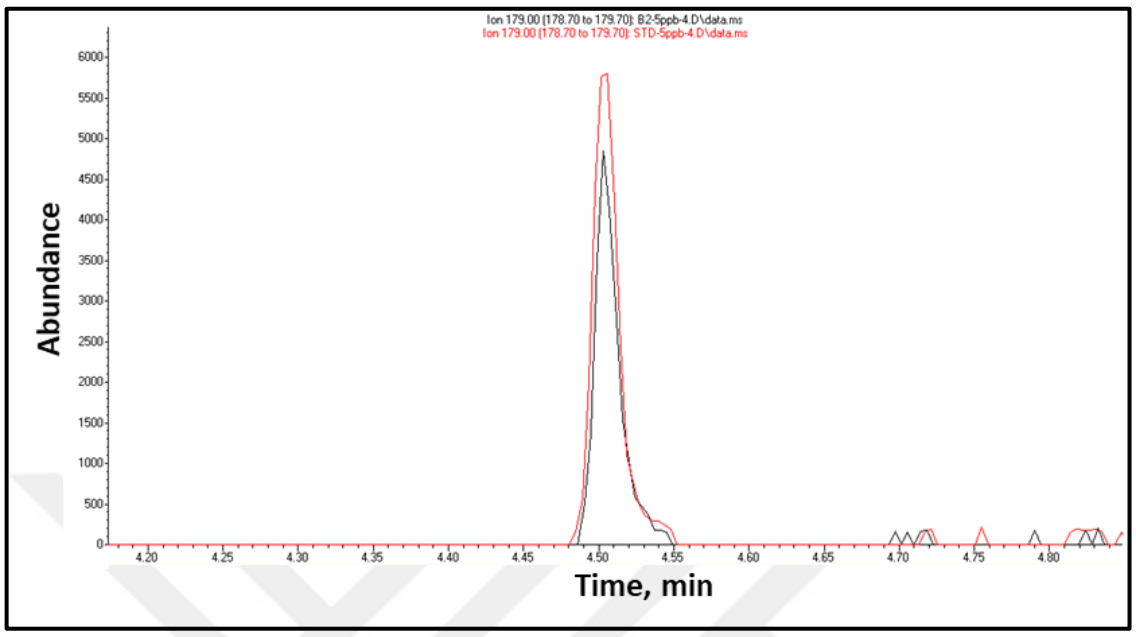

Figure 4.31 Overlay chromatograms of standard solution (red, 5.0 ng/g) and fish sample (black, spiked concentration of 5.0 ng/g) for MBT

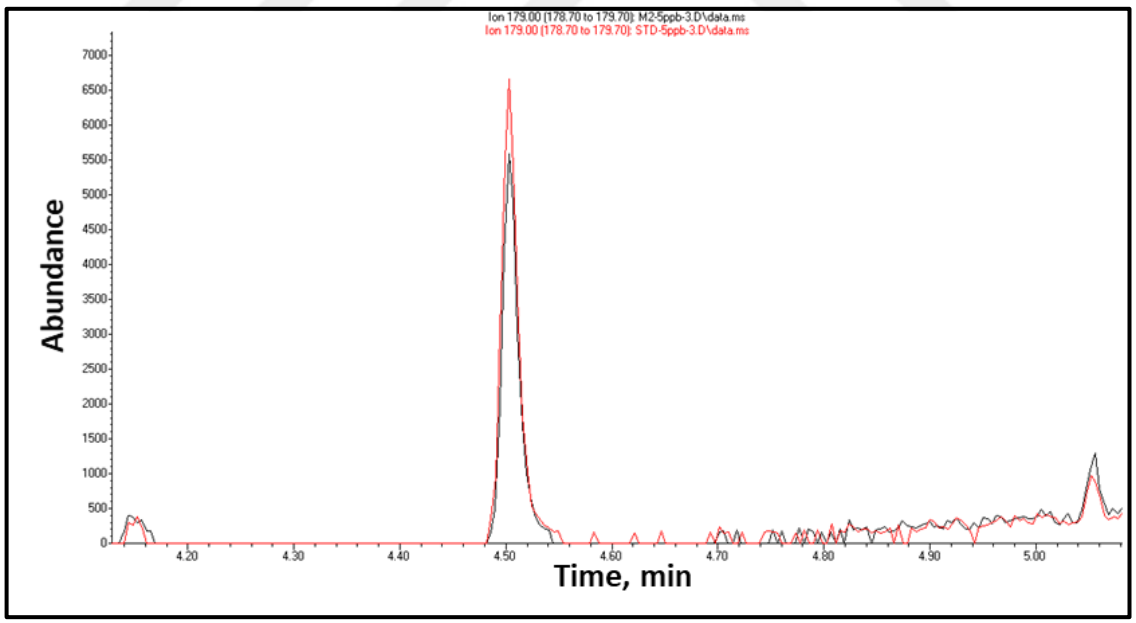

Figure 4.32 Overlay chromatograms of standard solution (red, 5.0 ng/g) and mussel sample (black, spiked concentration of 5.0 ng/g) for MBT

Table 4.17 Recovery results for mussel and fish samples spiked to 5.0 ng/g of the analytes

Analyte	**Anchovy sample (Recovery % ± SD)**	**Mytilus galloprovincialis sample (Recovery % ± SD)**
MBT	84.2 ± 1.2	94.9 ± 4.7
DBT	89.5 ± 1.5	100.2 ± 0.8
TBT	88.6 ± 2.2	112.6 ± 8.2

5

Conclusion

Determination of EDCs at trace levels is an important issue because of their carcinogenic effects, bioaccumulations and persistence in the environment. The main purpose of this thesis was to propose sensitive, accurate and precise analytical methods for the determination of selected analytes by GC-MS system.

The determination of 4-*n*-nonylphenol in municipal wastewater sample was achieved by SS-LPME-GC-MS, ID^{4}MS and SS-LPME-ID^4-GC-MS systems. Optimum conditions for SS-LPME method were determined via Box Behnken experimental design in order to highlight effects of the important parameters and their interactions on the preconcentration method. Limits of detection and quantitation in the GC-MS system were lowered to 0.32 and 1.05 ng/mL with the SS-LPME method. Among isotope dilution strategies, ID^{4}MS has robust and precise results with low uncertainties. These two methods were coupled to each other to detect 4-*n*-nonylphenol at trace levels with high accuracy and precision. Thus, the combination of SS-LPME and ID^{4}MS gave significant and high results when compared to SS-LPME-GC-MS and ID^{4}MS. In addition, the use of switchable solvents for preconcentration made the developed method more efficient and eco-friendlier thanks to its unique physical and chemical properties.

4-*n*-nonylphenol was also determined by BS-LPME-GC-MS, SA-ID^{3}MS and BS-LPME-SA-ID^{3}MS systems. Binary solvent-liquid phase microextraction was used to extract 4-*n*-nonylphenol from aqueous solution using two different extraction solvents. Box-Behnken Design was used to obtain optimum values for volume of binary solvent, volume of dispersive solvent and vortex period. Under the optimum conditions, LOD and LOQ values for BS-LPME-GC-MS were found to be 0.60 and 2.0 ng/g, respectively. Enhancement in detection power for the BS-LPME-GC-MS system was recorded as approximately 500 folds when compared with LOD values of GC-

MS system. However, this method did not overcome matrix effects of wastewater and infant formula samples when recovery studies of these samples were conducted. Thus, standard addition-triple isotope dilution mass spectrometry (SA-ID^{3}MS) was applied to overcome matrix effects rooting from the complex samples. Recoveries for wastewater and infant formula samples were 152-213% and 120-179% for the BS-LPME-GC-MS system, but these results were improved to 100.3±1.6% for wastewater and 99.4±1.7% for infant formula by BS-LPME- SA-ID^{3}MS system.

A new and ecofriendly analytical method for quantitative determination of monobutyltin, dibutyltin and tributyltin compounds by GC-MS system was proposed. Tetraethyl borate derivatization reaction was utilized to make the analytes detectable by the GC-MS system. A well utilized and effective microextraction method, dispersive liquid-liquid microextraction was employed as a preconcentration and separation method of the selected analytes. Optimization studies involved type of extraction and dispersive solvent, volume of pH 5.0 buffer solution, vortex period and derivatization period performed by a univariate approach. On the other hand, optimum volumes of extraction solvent, dispersive solvent and derivatizing reagent were established by a chemometric approach, Box-Behnken Design. After determining analytical figures of merit for GC-MS and DLLME-GC-MS system, 191.5, 83.6 and 70.9 folds enhancement in detection power were recorded for MBT, DBT and TBT compounds, respectively. Following that, recovery experiments were conducted on anchovy and mytilus galloprovincialis samples via spiking to 5.0 µg/kg of the analytes. Percent recovery results were found close to 100%, which expressed applicability and accuracy of the proposed DLLME-GC-MS method.

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Publications from The Thesis

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Papers

1) S. Erarpat, S. Bodur, D. S. Chormey and S. Bakırdere, "Switchable solvent liquid-phase microextraction-gas chromatography-quadrupole isotope dilution mass spectrometry for the determination of 4-*n*-nonylphenol in municipal wastewater," *Microchem. J.,* vol. 144, p. 1-5, 2019.

2) S. Erarpat, S. Bodur, E. Öz and S. Bakırdere, "Determination of butyltin compounds in fish and mussel samples at trace levels by vortex assisted dispersive liquid-liquid microextraction-gas chromatography mass spectrometry," J. Food Compos. Anal., vol. 82, p. 103248, 2019.

Conference Papers

1) S. Erarpat, S. Bodur, E. Öz and S. Bakırdere, "Sensitive and Accurate Determination of Butyltin Compounds in Fish and Mussel Samples by Vortex Assisted Dispersive Liquid-Liquid Microextraction-Gas Chromatography Mass Spectrometry," in 1st International Congress on Analytical and Bioanalytical Chemistry, Antalya, 2019, pp 159.

2) S. Erarpat, S. Bodur, D. S. Chormey and S. Bakırdere,"Switchable Solvent Liquid-Phase Microextraction-Gas Chromatography-Quadrupole Isotope Dilution Mass Spectrometry for the Determination of 4-*n*-Nonylphenol in Municipal Waste Water," in 11th Aegean Analytical Chemistry Days, Crete, 2018, pp. 73.