# DETERMINATION OF PARABENS IN SEAFOODS BY MATRIX SOLID PHASE DISPERSION COUPLED WITH GAS CHROMATOGRAPHY-MASS SPECTROMETRY

**THESIS**

**Untuk Memenuhi Persyaratan**

**Memperoleh Gelar Magister**

**PROGRAM DOUBLE DEGREE**

**UNIVERSITAS BRAWIJAYA**

**NATIONAL CENTRAL UNIVERSITY, TAIWAN**

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**MALANG**

**2016**

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# DETERMINATION OF PARABENS IN SEAFOODS BY MATRIX SOLID PHASE DISPERSION COUPLED WITH GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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## UCAPAN TERIMA KASIH

Puji dan syukur kehadirat Allah SWT yang Maha Pengasih lagi Maha Penyayang atas rahmat dan karunia-Nya sehingga penulis dapat menyelesaikan Tesis dengan judul "Determination of Parabens In Seafoods by Matrix Solid Phase Dispersion Coupled With Gas Chromatography-Mass Spectrometry", sebagai salah satu syarat untuk mendapatkan gelar Magister Sains dalam bidang Kimia, dengan baik. Terwujudnya tesis ini tidak lepas dari dukungan dan bantuan dari berbagai pihak. Oleh karena itu, penulis ingin menyampaikan terima kasih yang setulus-tulusnya kepada :

1. Prof. Wang Hsien-Ding selaku advisor yang telah memberikan dukungan, bantuan, dan arahan kepada penulis dengan tulus untuk menyelesaikan penelitian dan penyusunan tesis.
2. Dr. Hermin Sulistyarti selaku pembimbing yang dengan sabar bersedia meluangkan waktu dan pikiran serta perhatian yang tinggi kepada penulis dalam penyusunan tesis ini.
3. Dr. Sasangka Prasetyawan, MS Ketua Program Studi S2 Kimia Universitas Brawijaya atas semua bantuan dan arahan Beliau sehingga penulis dapat menyelesaikan studi S2 di Universitas Brawijaya dengan lancar.
4. Seluruh dosen pengajar dan staf di Universitas Brawijaya dan di National Central University yang telah memberikan ilmu dan bekal bagi penulis melalui materi-materi kuliah sehingga bermanfaat dalam penyempurnaan penulisan Tesis ini.
5. Orangtua penulis, ayahanda Heru Jatmika dan Ibunda Dwi Ariani yang telah memberikan kasih sayang, doa yang tulus, semangat, serta dukungan sehingga penulis dapat menyelesaikan pendidikan S2 ini dengan baik.
6. Saudara kandung penulis, adik Wiku Seta Djatmika, dan tante Endah S. serta segenap keluarga penulis atas semua dukungan dan doa yang terus mengalir untuk penulis.
7. Teman-teman Lab di National Central University : John, Jimmy, Moon, Ting, Sophi, Kammie, yang telah banyak memotivasi dan membantu penulis dalam menyelesaikan penelitian di Taiwan dengan baik.
8. Sahabat-sahabat terbaikku : Firdausy Amalina Esya, Ratna Juwita, Alda Khairunnisa, Rizka Dwitya Puspitasari, Friska Devi Mella, Laely nur Afida, Novi Anitra, Afidatul M, Redyaksa D.B, Saad Anshari dan sahabat-sahabat yang tak mampu penulis sebutkan satu-persatu, terimakasih atas semua dukungan, doa dan semangat yang senantiasa diberikan kepada penulis.

9. Teman-teman FORMMIT UTADA dan PPI NCU yang telah menjadi keluarga selama di Taiwan dan memberikan dukungan kepada penulis untuk segera menyelesaikan penelitian.
10. Teman-teman Kemuslimahan di Taiwan : Helda Wika Amini, Fivitria Istiqomah, Nurra Keprin, Via Romadhona, Aprilia, dan Indira, terimakasih atas semua ilmu, doa dan semangat selama menjalani hari-hari di Taiwan.
11. Rekan-rekan mahasiswa Universitas Brawijaya Program Studi Kimia S1 angkatan tahun 2010 dan S2 angkatan tahun 2014, terimakasih atas dukungannya kepada penulis untuk dapat menyelesaikan penelitian dan tesis ini.
12. Segala pihak yang telah membantu penulis, baik dalam segi ilmu, meteriil, tenaga, dan pikiran sehingga peulis dapat menyelesaikan penelitian dan tesis ini dengan baik.

Akhirnya dengan iringan do'a semoga Allah SWT memberikan pahala yang berlipat ganda atas segala amal baik yang telah diberikan kepada penulis. Semoga Tesis ini bermanfaat bagi penulis dan bagi semua pihak yang berkepentingan.

Malang, 30 Agustus 2016

Rosalina Djatmika

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Chemistry 系所 104 學年度第 2 學期

取得☑碩士☐博士學位之論文

論文題目：Determination of Parabens in Seafood by Matrix Solid Phase Dispersion Coupled with Gas Chromatography- Mass Spectrometry

指導教授：丁望賢

因以下原因（請勾選，並填寫相關資料）

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## Advisor's Recommendation for Graduate Students

This thesis is by Rosalina Djatmika of the graduate program in Chemistry Department, entitled:
Determination of Parabens in Seafoods by Martrix Solid-Phase Dispersion Coupled with Gas Chromatography-Mass Spectrometry,
which is written under my supervision, and I agree to propose it for examination.

Advisor _____

2016, 5, 27 (YYYY/MM/DD)

# National Central University

## Verification Letter from the Oral Examination Committee

## for Graduate Students

This thesis is by Rosalina djatmika of the graduate program in Chemistry, entitled: Determination of Parabens in Seafoods by Matrix Solid Phase Dispersion Coupled with Gas Chromatography-Mass Spectrometry, who is qualified for master degree through the verification of the committee.

Convener of the degree examination committee_____

members Cheng-Huang Lin

Date: 2016 / 6 / 14

## ABSTRACT

Parabens or esters of p-hydroxybenzoic acid are used as antimicrobial preservatives. Parabens are commonly detected in aquatic systems, including environmental water, sediment and sludge. Previous studies have reported that parabens have potential long-term effects on aquatic organisms and human health. Parabens cause endocrine disruptive which adversely affects the secretion of testosterone and enhance the risk of breast cancer.

An optimized method for determination of parabens in seafoods was presented. Analytes were simultaneously extracted and cleaned-up using the matrix solid-phase dispersion (MSPD) procedure. Several combinations of elution solvent, dispersants and clean-up co-sorbent were investigated in order to get free-lipid extracts and quantitative recoveries of parabens. The parameters affecting the MSPD extraction efficiency was investigated by statistical experimental design and analysis of variance. Under optimized condition, 0.5 gram of fish sample was mixed with 0.5 gram anhydrous sodium sulfate and dispersed with 1.0 gram of Florisil and then transferred to SPE column containing 1.5 gram of silica:C18 (9:1) as clean-up co-sorbent. Analytes were eluted with 12 mL of acetonitrile. The extract was concentrated to dryness under a gentle stream of nitrogen and re-constituted with 50 µL methanol. Then, a fraction of 10 µL was mixed with 1.0 µL acetic anhydride reagent and directly injected to GC-MS system to obtain online derivatization. The optimized method presented good linearity ($R^2 > 0.9994$) for all target analytes over six level concentration in ranged 4-500 ng/g, provided low detection limits less than 0.3 ng/g, obtained a good precision (relative standard deviations less than 8% in inter-and intra- day analysis) and offered good recoveries (in ranged 93%-112%).



**Keywords :** Parabens, MSPD, GC-MS, online derivatization, biota sample

**Abstract in Chinese**

對-羥基苯甲酸酯常被用來當作抗菌的防腐劑，土壤、底泥和水資源中皆有其蹤跡的存在。過往研究顯示對-羥基苯甲酸酯對於人體與水中有機物有潛在的長期影響，可能造成內分泌干擾，干擾睪酮素的分泌以及增加罹患乳癌的風險。

本研究開發出一套檢測對-羥基苯甲酸酯在海鮮中含量的方法，使用基質固相分散法 (Matrix solid-phase dispersion, MSPD) 同時將待測物進行萃取以及淨化，利用不同組合的沖提溶劑、分散劑和吸附劑，達到去除脂肪的效果。影響萃取效率的參數，　使用實驗設計以及變異數分析進行探討，最佳化的萃取條件為：將0.5 g魚肉、0.5 g無水硫酸鈉和1 g矽酸鎂置於震盪機均勻混和，將混和物轉移到含有1.5 g吸附劑 (矽膠:C18 = 9:1) 的玻璃管柱中，接下來使用12 ml的乙腈進行沖提，最後將萃取液旋濃乾燥，以50 μL甲醇回溶，取10 μL與1 μL醋酸酐進行線上衍生，導入GC/MS進行分析。

此方法回收率介於93%-112%之間；在 4-500 ng/g 的線性範圍內，檢量線具有良好線性關係 ($R^2$>0.9994)；偵測極限小於0.3 ng/g。精密度以及準確度，相對標準偏差皆小於8 %，表示此方法具有良好的穩定性以及精密度。

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# CHAPTER I

# Introduction

## 1.1 Origin of the research

Parabens or esters of p-hydroxybenzoic acid that include methyl paraben (MP), ethylparaben (EP), propylparaben (PP) and butylparaben (BP) are extensively used as preservatives in food, pharmaceutical and personal care products due their broad spectrum of action against numerous microorganisms, biodegradability, efficiency in wider pH range, non-volatility, and no color (Azzouz et al., 2016, Kajornkavinkul et al., 2016). Because of their highly lipophilic nature and degradation resistance, parabens were potentially entered in the aquatic food chain and bioaccumulate in aquatic biota. As a matter of fact, parabens are ubiquitous and are among the most commonly detected compounds in aquatic systems, including environmental water, sediment and sludge (Rocío-Bautista et al., 2015, Ferreira et al., 2011). The finding parabens in human urine, blood, breast milk and serum proved that human has been exposed parabens (Azzouz et al., 2016, Hines et al., 2015, Kang et al., 2016, Moos et al., 2015)

Parabens were considered low acute toxicity compounds, but controversy about paraben side effect arose due to their potential long-term effects on aquatic organisms and human health. Previous studies have reported that parabens have an endocrine disruptive effect which adversely affects the secretion of testosterone and the functions of the male reproductive system (Oishi, 2002). Several studies also suggested that parabens affect estrogen levels, potentially impacting female reproductive health and enhance the risk of breast cancer (Shanmugam et al., 2010, Gao et al., 2016). Due to their potential toxicity to aquatic organisms and human beings, it is important to study these pollutants in seafoods that were often consumed by a human.

In consequence of the concentration of parabens in aquatic biota assumed to be very low to pose, lead some difficulties regarding the limitation of methods and instrumentation. The direct analysis only can be used with a relatively high concentration of these analytes, with the result the pretreatment process was needed to enrichment the analytes before determined by instrumentation. There are many attempts to enrichment parabens from the sample. These techniques were systematically reviewed by Ocaña-González et al.(2015). They are DLLME (Dispersive Liquid–Liquid Microextraction), HF-LPME (Hollow Fiber Liquid Phase Microextraction), LE (Liquid Extraction), MSPE (Magnetic Solid-Phase Extraction), SBSE (Stir-Bar Sorptive Extraction), SFVCDME (Solidified Floating Vesicular-Conservative Drop Microextraction), SPME (Solid-Phase Microextraction), MSPD (Matrix Solid Phase Dispersion). Among them, MSPD is the most common technique for pretreatment aquatic biota. Tsai et al. (2014) applied MSPD method for marketed fish pretreatment since it has many advantages, such as extraction and clean-up column are integrated into a single step, thus making the procedure simple, low-cost, and convenient (Tsai et al., 2014). From our previous research, MSPD was successfully applied for determining dechlorane compound, salicylate and benzophenone-type UV-absorbing substance in fishes. Matrix solid-phase dispersion (MSPD) was selected for the simultaneous extraction and purification of target species. In this technique, samples are first blended and dispersed around the particles of a suitable sorbent, in a mortar with a pestle, and then transferred to a solid-phase extraction (SPE) cartridge, which also contains a clean-up co-sorbent (Canosa et al., 2008). The challenge of this pretreatment technique was both to get high efficient in enrichment process and to get the efficient composition of dispersant and co-sorbent to remove the fish lipid. MSPD has also been applied successfully to the determination of various micropollutants in

aquatic biota, animal tissue, and foodstuff samples. Moreover, MSPD coupled with gas chromatography-mass spectrometry (GC-MS) has been developed as a method in proposed to determine trace amount of parabens in a seafoods.

Gas Chromatography-Mass Spectrometry (GC-MS) is well suited for the identification of a large number of potential steroids (include parabens) due to its high chromatographic resolution capacity and reproducible ionization efficiency. GC-MS has been applied to analyze and quantify organic volatile and semi-volatile compound. Otherwise, parabens are semi-polar and semi-volatile compound, so that derivatization of parabens before GC analysis is needed to allow the parabens compound more amenable for GC-MS analysis, by improving volatility, thermal stability, and increasing chromatographic performance (Bowden et al., 2009). Derivatization reactions are frequently performed off-line in reaction vessels which are separated from the GC instrument. However, off-line derivatization needs multi-step reactions. As a result, the procedure was laborious, tedious and time-consuming. Off-line derivatization also uses toxic and harmful reagents, the reaction efficiency is relatively low, need a large amount of derivatization reagent and an organic solvent (Wang et al., 2013). Injection-port derivatization (IPD) or on-line derivatization has developed a method to derivative analytes and enhance the analytical efficiency of organic compounds in short time reaction (Wu et al., 2009). IPD performed derivatization reaction occurred in the hot GC injection port by injecting the sample directly (Wang et al., 2013). GC-MS methods for paraben analysis can be performed based on derivatization by acylation, in this case, we used acetic anhydride as acylation derivatization reagent.

Derivatization by acylation is a type of reaction in which an acyl group is introduced to an organic compound. In the case of parabens, the reaction involved the

introduction of the acyl group, the loss of the hydroxyl group and converted into esters (Bizkarguenaga et al., 2013). Acylation is one of the most widely used derivatization procedures for chromatography since the acylation reduces the polarity of a hydroxyl group and this is able to improve their chromatographic properties. One of the popular derivatizing agents for acylation is acetic anhydrides.

Due to all the reason above, in this study, we optimize MSPD for determination of parabens in seafoods. The parameters affecting MSPD (i.e., the types and amounts of dispersant, clean-up co-sorbent, as well as type and volume of elution solvent) were systematically investigated, and the conditions were optimized. The accuracy and precision of the optimized method were evaluated, and the suitability of the method for the determination the trace levels of the target analytes in seafoods samples was also performed.

**1.2 Purpose of the Study**

The main objective of this study was to develop a rapid and convenient pretreatment procedure to determine parabens in seafoods. In this experiment, Matrix solid-phase dispersion extraction (MSPD) was used to concentrate and purify analytes from complex matrices of seafoods.

This experiment uses a gas chromatograph with ion trap mass spectrometer (Ion-trap mass spectrometry) for the qualitative and quantitative determination of parabens with Injection Port Derivatization (IPD). IPD can reduce the experimental time and derivatization reagent volume.

In this present study, we applied statistical experimental design to optimize extraction conditions, response surface methodology (RSM) was applied to discuss the multi-factor interaction of the whole experiment. The use of Design-Expert software can more accurately find the optimized experimental conditions. Regarding using the

calibration curve, Mandel test, and Lack-of-fit linear regression was applied to test the linear or quadratic regression. In order to test the matrix effects, we calculated using the t-test and F-test. These data can display the best of accuracy and applicability of experiment.

# CHAPTER II

## Literature Review

### 2.1 Emerging contaminants (ECs)

During the last century, large amounts of different chemicals were released to the environment through industrial emission and waste, agricultural practice (including manure and sewage sludge applications) and via wastewater treatment plant effluent discharges. This contamination can have a critical impact on the ecosystem due to their strong activity at low doses. A chemical or material that is characterized by a perceived, potential, real threat to human health or the environment, or by a lack of published health standards, named "emerging contaminant." A contaminant may also be "emerging" because a new source or a new pathway to humans has been discovered or a new detection method or treatment technology has been developed (DoD, 2011).

Emerging contaminants (ECs), including pharmaceuticals and personal care products (PPCPs), are increasingly being detected at low levels in surface water, and there is concern that these compounds may have an impact on aquatic life. The characteristic of some contaminants that they do not need to be persistent to cause negative effects since their high transformation/removal rates is compensated by their continuous introduction into the environment (Barcelo, 2003). For most of the occurring emerging contaminants, and risk assessment data are not available and therefore it is difficult to predict which health effects they may have on humans, terrestrial and aquatic organisms, and ecosystems. Also, the budgets (sources, entry routes, and fate) for environmental pollutants would be of importance. **Table 2.1** lists the classes of ECs in the environmental (Barcelo, 2003). **Table 2.2** summarizes the data regarding the occurrence of several emerging contaminants in the environment.

Many of these emerging contaminants are toxic and are also classified as endocrine-disrupting compounds (EDCs). These compounds disturb the endocrine system by mimicking, blocking or also disrupting the function of the hormone, affecting the health of humans and animals species. Additionally, EDCs are exogenous substances which interfere with the normal hormones at very low concentration in the human body (Zhu, 2015). This implies that although EDCs may be present in effluent at trace concentrations, adverse effects have been found in aquatic biota, and hence they may have health impact to humans (Gomes et al., 2003). The major exposure route both for humans and animals is by ingestion of EDCs via food/drink intake which leads to bioaccumulation and biomagnification, especially towards species at the top level of the food chain. More of the selected EDCs and its health effect to the livings are shown in **Table 2.3**

**Table 2.1 Emerging compound classes**

| Compound class | Examples |
|---|---|
| Pharmaceuticals | |
| Veterinary and human antibiotics | Trimethoprim, erythromycin, lincomycin, sulfamethoxazole |
| Analgesics, anti-inflammatory drugs | Codein, ibuprofene, acetaminophen, acetylsalicilyc acid, diclofenac, fenoprofen |
| Psychiatric drugs | Diazepam |
| Lipid regulators | Bezafibrate, clofibric acid, fenofibric acid |
| β-blockers | Metoprolol, propanolol, timolol |
| X-ray contrasts | Iopromide, iopamidol, diatrizoate |
| Steroids and hormones | Estradiol, estrone, estriol, diethylstilbestrol |
| Personal care products | |
| Fragrances | Nitro, polycyclic and macrocyclic musks |
| Sun-screen agents | Benzophenone, methylbenzylidene camphor |
| Insect repellents | N,N-diethyltoluamide |
| Antiseptics | Triclosan, Chlorophene |
| Surfactants and Surfactant metabolites | Alkylphenol ethoxylates, 4-nonylphenol, 4-octylphenol, alkylphenol carboxylates |
| Flame retardants | Polybrominated diphenyl ethers (PBDEs), Tetrabromo bisphenol A, $C_{10}$-$C_{13}$ chloroalkanes Tris (2-chloroethyl)phosphate |
| Industrial additives and agents | Chelating agents (EDTA), aromatic sulfonates |
| Gasoline additives | Dialkyl ethers, Methyl-*t*-butyl ether (MTBE) |

**Table 2.2 Summary data for selected emerging contaminants**

| Compound | Origin | Persistence Bioaccumulation | Observed in environment | Concentration level |
|---|---|---|---|---|
| Nonylphenol | Degradation product of non ionic surfactants | Medium persistent; Bioaccumulative | Soil;<br>Sediment;<br>Sludge;<br><br>Water | Low mg/kg*<br>Low mg/kg<br>Low-high mg/kg<br>Low µg/L |
| Bisphenol A | Plastics | Not bioaccumulative | Surface water;<br>Groundwater | Low-high ng/L<br>Low-high ng/L |
| Phthalates | Plastics | Low to medium persistent; atmospheric deposition | Water;<br><br>Sediment;<br>Sludge | Low-medium µg/L<br>Low µg/kg<br>Low-medium µg/kg |
| PBDEs | Flame retardant | Persistent/highly accumulative; atmospheric deposition | Sediment;<br><br>Soil;<br><br>Sludge | Low-medium µg/kg<br>Low-high ng/kg*<br>Low-medium µg/kg |
| $C_{10}$-$C_{13}$ chloroalkanes | Flame retardant | Persistent/ bioaccumulative | Surface water | Low-medium µg/L |
| Sulphonamides | Human and veterinary drug | Slightly-very persistent | Groundwater | |
| Tetracyclines | Human and veterinary drug | Moderately-very persistent | Groundwater;<br>Soil;<br>Sludge | |
| Steroid sex hormones | Contraceptives | Moderately persistent | Water;<br>Sediment;<br>Sludge | Low ng/L<br>Low µg/kg<br>Low-medium µg/kg |
| MTBE | Gasoline additive | Persistent;<br>Not bioaccumulative, but ubiquitous in the atmosphere | Groundwater | |

*sludge amended soil

**Table 2.3 Environmental effects of EDCs and PPCPs**

| Endocrine disrupting compounds | Health effects |
|---|---|
| Bisphenol A (BPA), used in epoxy resin and Polycarbonate plastics, used in food and drink packaging | Proven to have estrogenic effects in rats and hormonal effects which increase breast cancer risk in human. Reported to act as anti-androgen that causes feminizing side-effects in men. |
| Butylated Hydroxyanisole (BHA), used as a food antioxidant | Estrogenic to breast cancer cells, rainbow trout estrogen receptor and stimulates human estrogen receptor |
| Alkylphenols (i.e., nonylphenol), used in detergents | Mimicking estrogen and disturbing reproduction by an increasing number of eggs produced by Minnos and vitellogenin levels |
| Phthalates used as plasticizers in plastic, PVC baby toys, flooring | Exposure to high levels reported causing miscarriage and pregnancy complication |
| Pesticides | • Dichloro-diphenyl-trichloroethane (DDT) cause hormonal effects such as thinning of eggshells, damage of male reproductivity, and behavioral changes<br>• Lindane an organochlorine pesticides that shows vitellogenin and zona radiata (eggshell protein) in liver cells of Atlantic Salmon<br>• Penconazole can affect thyroid, prostate and testes weight<br>• Prochloraz can affect pituitary weight<br>• Propiconazole can affect steroid metabolism<br>• Tridemorph can cause cystic ovaries<br>• Epoxyconazole affects sex hormone balance and causes ovarian tumors |
| Polychlorinated biphenyls (PCBs), used in capacitors and transformers. | The metabolites are able to mimic estradiol (female hormone) and cause carcinogenic. Exposure was reported to cause delayed brain development and IQ decrease in children |
| Estrone and 17-β estradiol (steroidal estrogens) and 17-α ethynylestradiol (synthetic contraceptive), contained in contraceptive pills | Cause feminization which observed for fish in sewage treatment. The discharge causes mimicking estrogen/hormone effect to non-target |
| Antibiotics | Shown to cause resistance among bacterial pathogens, that lead to altered microbial community structure in nature and affect the higher food chain. |

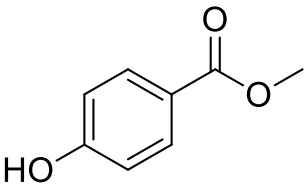
| | |
|---|---|
| Fragrances (musk) Musk xylol | Musk ambrette may damage the nervous system |
| Preservatives, used for anti-microbiological preservatives in cosmetics, toiletteries, and even foods | Shows weak estrogenic activity |
| Disinfectants/antiseptics, used in toothpaste, handsoaps, acne cream) | Found in the receiving waters, that cause toxic, biocide (kill microorganism) and also cause bacteria resistance development towards triclosan. |

### 2.1.1 Parabens

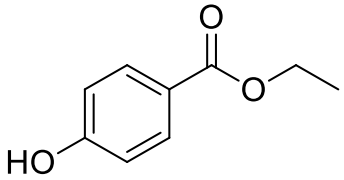
Parabens which constitute a family of p-hydroxybenzoic acid esters, esterified at the C-4 position (including methyl-, ethyl-, propyl- and butyl-), are a class of chemicals used in cosmetics and foodstuffs as preservatives and known to affect the endocrine system. Parabens have proved to be very effective antimicrobial agents and are widely used. Parabens meet several of the criteria of an ideal preservative, in that they possess a broad spectrum of antimicrobial activity, relatively non-irritating, non-sensitizing, and low toxicity, are stable over the pH range and are sufficiently soluble in water to produce the effective concentration in the aqueous phase. As the chain length of the ester group of paraben increases, antimicrobial activity increases, but water solubility decreases (Soni et al., 2005).

Parabens have been recognized as chemicals of concern for their potential risks to human health. Recent reports have indicated that exposure to parabens may modulate or disrupt the endocrine system and thus may have harmful consequences on human health. Several in vitro studies have shown that parabens exhibit specific activity toward the estrogen receptor, and may lead to a prolonged estrogenic effect in the skin (Prusakiewicz et al., 2007). In vivo studies have indicated that parabens, at a dose of approximately 10 mg/kg bw/day, induce oxidative stress via lipid peroxidation (Shah and Verma, 2011) and reduce testosterone secretion in male rodents (Oishi, 2004).

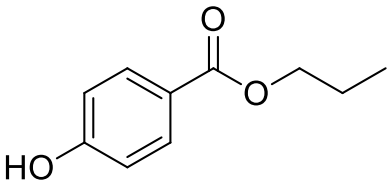
**Figure 2.1** and **Table 2.4** display the structures, names and the log octanol-water partition coefficients (LogKow) of four parabens and their properties.



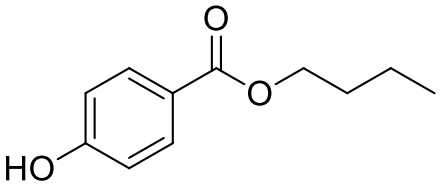

Methyl 4-hydroxybenzoate (MP)




Ethyl 4-hydroxybenzoate (EP)




Propyl 4-hydroxybenzoate (PP)




Butyl 4-hydroxybenzoate (BP)

**Figure 2.1 Structures, and names for four kinds of 4-hydroxybenzoates (parabens)**

**Table 2.4 Properties of parabens**

| **Analytes** | **Molecular weight (gram /mol)** | **Formula** | **logKow** |
|---|---|---|---|
| MP | 152.15 | $C_8H_8O_3$ | 1.96 |
| EP | 166.17 | $C_9H_{10}O_3$ | 2.49 |
| PP | 180.20 | $C_{10}H_{12}O_3$ | 2.98 |
| BP | 194.23 | $C_{11}H_{14}O_3$ | 3,47 |

## 2.2 Extraction Procedures of Parabens

In the interest for parabens determination in seafoods, the varied samples having a complex composition, need a pretreatment step prior in order to remove lipid as potential interference that can clog and foul the GC column. Additionally, the tendency to develop faster and environmental friendly analytical methodologies have led to the proposal of many extraction procedures.

Recent studies used solvent extraction method to extract paraben in seafoods. The new method was developed by Han in 2016, a more rapid pressurized solvent extraction (Pressurized-Liquid Extraction, PLE) plus gel permeation chromatography (GPC) and silica gel for clean-up. On 2013 and 2011, the QuEChERS extraction has developed and provided low LOD. However, these methods need more time-consuming and require a long-step extraction. **Table 2.5** compiled studies in recent years, the literature review of parabens detection in fish samples.

### 2.2.1 Matrix Solid Phase Dispersion

The biological specimen is a complex matrix samples, therefore, before analyzed it must be the extraction, purification, plus clean-up step in order to remove interferences. The conventional pretreatment methods, such as Soxhlet extraction, Ultrasonic Extraction, Solid-Liquid Extraction, Solid-Phase Extraction, have been successful to extract biological specimen, but these methods not only need multiple clean-up purification but also spend a lot of time and a large amount of organic solvent. Matrix solid-phase dispersion is extraction step that provides single step of extraction and clean-up, simple, low cost, convenient, and rapid procedure.

Matrix Solid Phase Extraction (MSPD) has been introduced by Professor Barker to obtain fast sample preparation techniques (Barker et al., 2007). MSPD is a microscale extraction method, typically using less than 1 gram of sample and low

volume solvents. It has been estimated to reduce solvent use by up to 98% and sample turnaround time by 90%. MSPD has found favor in its many applications because it eliminates most of the complications of performing classical liquid–liquid and/or solid phase extractions of solid and semisolid samples, particularly complex biological samples.

In the MSPD process, a biota sample is placed in a glass or agate mortar containing an appropriate bonded-phase or other solid support material, such as C18 or other suitable support. The solid support and sample are manually blended together using a glass or agate pestle. Internal standards or spikes may be added before this step. The blended material is then transferred and packed into a column suitable for conducting sequential elution with solvents. The eluates obtained in MSPD may be taken directly to instrumental analysis, being adequately "clean" for direct injection (Barker, 2007). The principle of MSPD is described in **Figure 2.2.** The advantage of MSPD are: this technique is applicable to solid and semi-solid extraction sample; the volume of extraction solvent used less than conventional Soxhlet extraction and have the rapid procedure.

**Table 2.5 Literatures review of parabens detection in fish sample**

| Compound | Sample matrix | Extraction and Preparation | Analytic method | LOD | Reference |
|---|---|---|---|---|---|
| MP, EP, PP, BP | Fish | High Speed Solvent Extraction (HSSE) | UFLC–MS/MS | 0.001–0.015 ng/g | (Kim et al., 2011) |
| MP, EP, PP, BP | Fish | Pressurized Liquid Extraction (PLE) coupled with SPE clean-up | LC-QqLIT-MS/MS | 3.3 µg/kg | (Han et al.,2016) |
| MP, EP, PP | Fish | QuEChERS extraction | LC-MS/MS | 0.04 ng/g | (Jakimska et al., 2013) |
| MP, EP, PP, BP | Fish | QuEChERS extraction | UHPLC–MS/MS | 0.015 ng/g | ( Ramaswamy et al., 2011) |

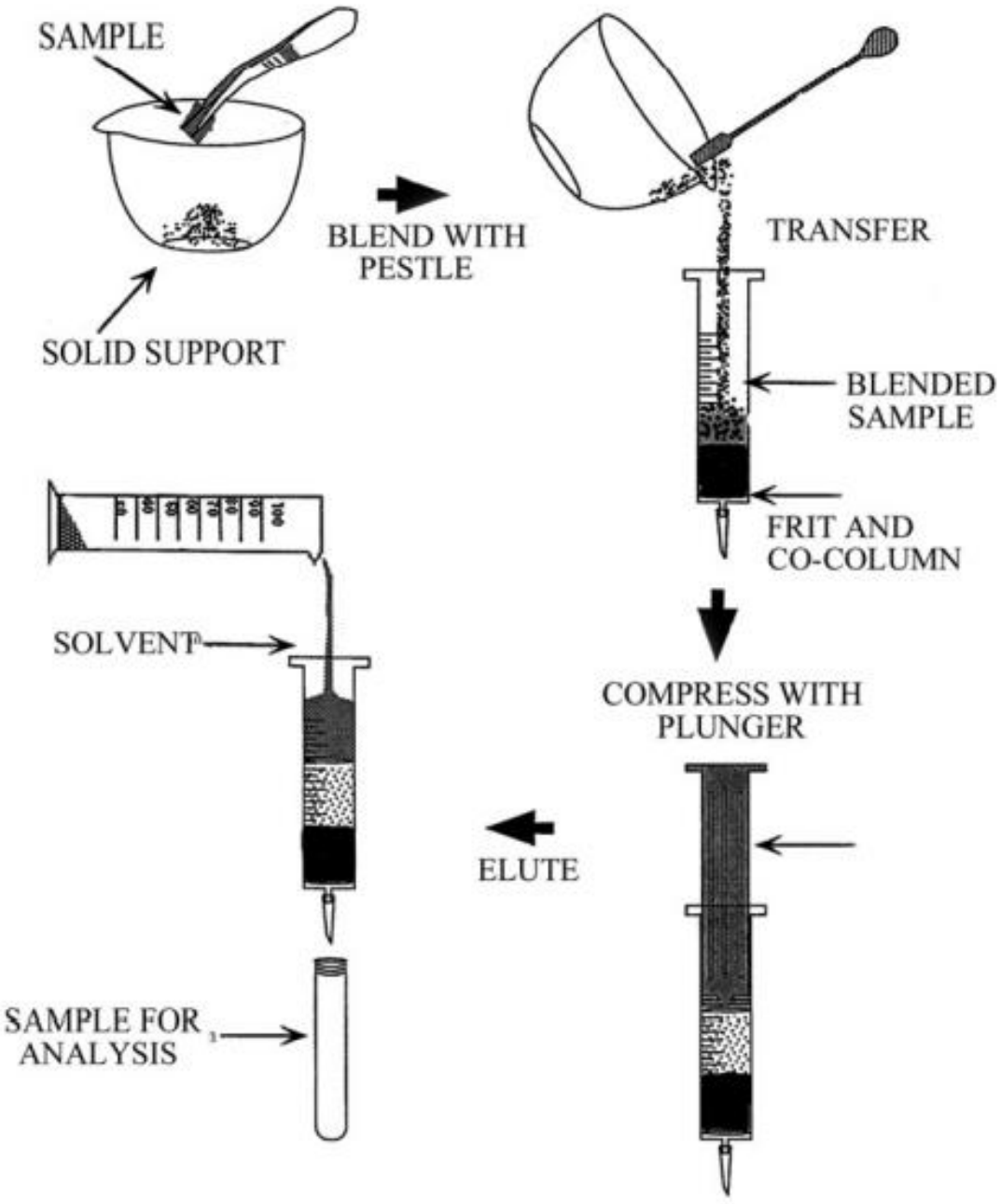



**Figure 2.2 Matrix Solid Phase Dispersion Principle (Barker, 2007)**

## 2.3 Gas Chromatography-Mass Spectrometer

Gas chromatography (GC) and mass spectrometry (MS) are two instrumental methods of analysis. They can be linked together as GC-MS to allow substances in a sample to be separated, then identified. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing some chemicals. This makes it ideal for the analysis of the hundreds of relatively low molecular weight compounds found in environmental materials. The application for GC-MS is numerous. They are used extensively in the medical, pharmacological, environmental, and law enforcement fields.

Analytical gas chromatography (GC) is a separation technique of components in mixtures (samples) with the purpose to obtain information about their molecular compositions and amounts. The information obtained from a chromatographic analysis can include a chromatograph (a graphical image of detector output), information regarding the height and the areas of the resolved (adequately separated) peaks in a chromatogram, their molecular identity, etc. (Poole, 2012).

The chromatographic separation relies on the interaction of the sample with a mobile phase and a stationary phase within the GC instrument column. The components of mixture, injected at column inlet and brought to travel through the column by the carrier gas (mobile phase). They migrate at a speed which is proportional to the carrier gas velocity depends on the strength of the interaction of each of these components with the stationary phase (Guillemin, 1988). Compound with different retention times in the column are phisically separated for presentation to a detector and analyzer. The schematic structure of the gas chromatograph is shown in **Figure 2.3.**

A mass spectrometer is an instrument that measures the mass-to-charge ratio (m/z) of gas phase ions and provides a measure of abundance of each ionic species. The measurement is calibrated against ions of known m/z. All mass spectrometers operate by separating gas phase ions in a low pressure environment by the interaction of magnetic or electrical fields on charged particles (McEwen, 1996). The mass spectrometer nowadays is a highly famous and computerized instrument. It consist of five part: sample introduction (inlet), gaseous ion source, mass analyzer, ion transducer and signal processor. The schematic structure of the mass spectrometer is shown in **Figure 2.4.**

As a sample constituent elutes from the GC column, it enters the ionization chamber of the mass spectrometer where the molecules are ionized, typically by electron impact. When an electron impact with a sample molecule results in the loss of an electron from the molecule, a positive ion is formed. The positive ions are separated according to their mass by a mass analyzer. The mass analyzer most commonly used in GC-MS is the quadrupole filter. The magnetic poles separate the ions by their mass/charge (m/z) ratio (McEwen, 1996).

In order for a compound to be analyzed by GC-MS it must be sufficiently volatile and thermally stable. In addition, functionalized compounds may require chemical modification (derivatization), prior to analysis and to eliminate undesireable adsorption affects.

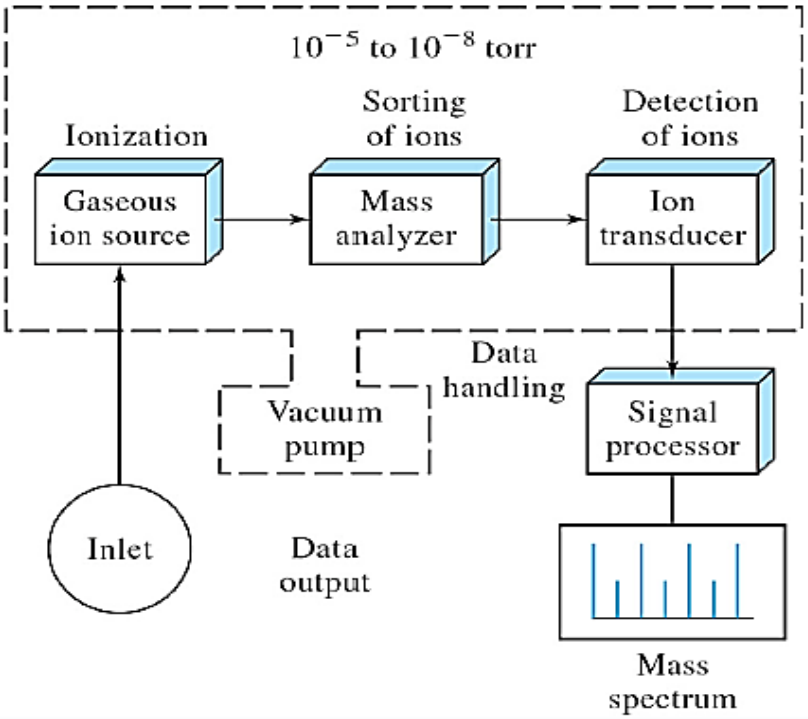



**Figure 2.3 Schematic structure of the gas spectrometry (Skoog, 2002)**

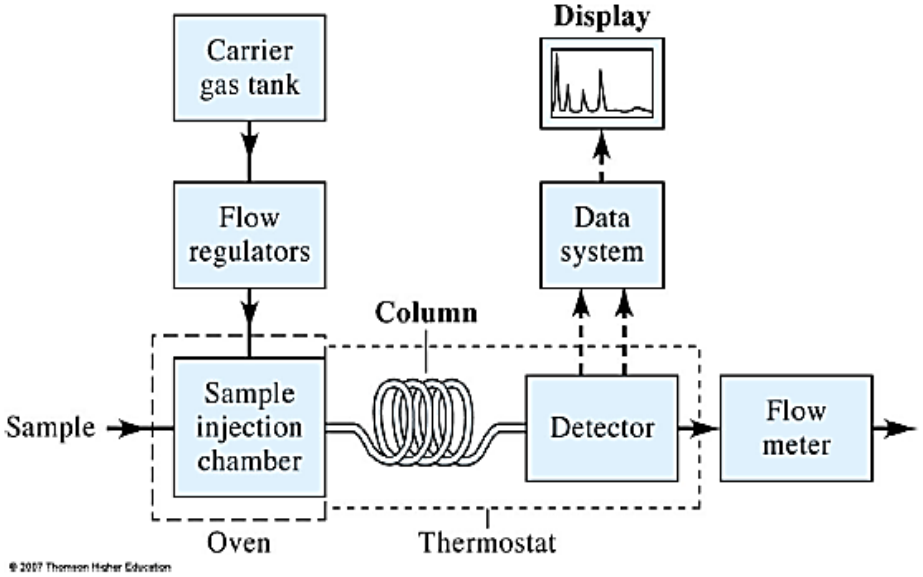



**Figure 2.4 Schematic structure of the mass spectrometry (Skoog, 2002)**

## 2.4 Derivatization

### 2.4.1 Injection-port derivatization (IPD)

In order for a compound to be analyzed by GC-MS it must be sufficiently volatile and thermally stable. However, not all the compounds can be directly analyzed by GC owing to the following reasons:

(i) polar compounds which contain polar functional groups, e.g. -OH, -COOH, -NH- and -SH, are of low volatility;

(ii) Some polar compounds have strong affinity toward the active sites of the GC column, resulting in asymmetric or ghost peaks, and even damaging the column;

(iii) some analytes are thermolabile and cannot survive a vaporization process at the high temperature;

(iv) No proper detectors fit well with certain compounds for detection.

These compounds may require chemical modification (derivatization) in order to increase their volatility and/or stability, in order to improve the GC separation and enhance the detectability.

Commonly derivatization reactions are performed off-line in reaction vessels which are separated from the GC instrument (Wang et al. 2013). However, off-line derivatization has many disadvantages. It needed multi-step reactions, consequently the procedure was laborious, tedious and time-consuming. In addition, offline derivatization used toxic and harmful reagents and the reaction efficiency is relatively low (Wang et al. 2013).

Injection-port derivatization (IPD) or on-line derivatization was a newly developed technique to simplify the derivatization procedure and enhance the analytical efficiency of organic compound, it also has short derivatization time (Wu et al. 2009).

IPD performed derivatization reaction occurred in the hot GC injection port by injecting the sample directly. Due to this simple procedure, the amount of derivatization reagent and the organic solvent is reduced (Wang et al. 2013).

### 2.4.2 Type of IPD reaction

Derivatization reagent is a compound containing carboxyl group, amino group or hydroxyl group and other functional groups. The active hydrogen in the polar groups (e.g. -OH, -SH, -NH-, -COOH, -CONH-) of the analytes is displaced by alkyl, silyl or acyl group during the derivatization reaction, which shields the polar groups for increasing volatility and stability of the analytes and weakening their affinity for the GC column (Wang et al., 2013). Depending on the functional group, there will be different types of derivatization:

(1) Alkylation

(2) Acylation

(3) Silylation

(4) Perfluoroacylation

(5) Esterification

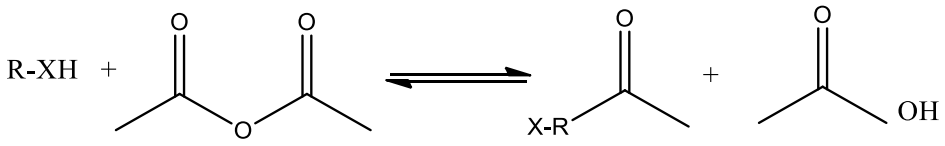
(6) Cyclization

#### 2.4.2.1 Acylation

Derivatization by acylation is a type of reaction in which an acyl group is introduced to an organic compound. In the case of a carboxylic acid, the reaction involves the introduction of the acyl group and the loss of the hydroxyl group. Compounds that contain active hydrogens (e.g.-OH, -SH and –NH-R) can be converted into esters, thioesters, and amides, respectively, through acylation. Acylation also improves the stability of those compounds that are thermally labile by inserting protective groups into the molecule. Acylation can render amenable separation by GC

to extremely polar materials such as sugars and, consequently, it is a useful alternative or complimentary to the silylation (Bizkarguenaga, et al., 2013).

Acetic anhydride is one of popular acylation reagent which commonly used in IPD. The equation below shows the general formula of acylation using acetic anhydride as reagent, and R-XH represents the compounds with –NH-, -OH, or -SH groups (Wang, et al., 2013).




## 2.5 Experimental Design

### 2.5.1 Introduction to Experimental Design

Statistical design of experiment is a statistical methods to analyze the data, and get the correct and objective conclusion in order to plan scientific experiments to get the most efficient way to carry out experiments. Planning an experiment provides a piece of information from the system or process under study. This information will guide the experimenter to make a decision or find the optimum point about the system or process. With the purpose to accommodate this plan, the multivariate statistical approach known as design of experiment (DoE) has been applied to different types of analytical chemistry problems. The experimental design is the most suitable method for identifying the effect of individual and interaction factors, detecting the optimum setting of every factor simultaneously, and reducing expended time and money (Heshmatollah et al., 2004). With the aim of performing an experimental design, the following five steps must be considered (Leardi, 2009):

(1) Define the goal of the experiments.

(2) Detect all the factors that can have an effect. Particular attention must be given to the words "all" and "can". It means that it is not correct to consider a predefined number of factors (e.g., let us take into account only three factors), and saying that a factor "can" have an effect which is totally different from saying that we think that a factor has an effect. One of the most common errors is indeed that of performing what has been defined a "sentimental screening", often based only on some personal feelings rather than on scientific facts.

(3) Plan the experiments. Once the factors have been selected, their ranges have been defined and the model to be applied has been postulated.

(4) Perform the experiments. While in the classical way of thinking this is the most important part of the process, in the philosophy of experimental design doing the experiments is just something that cannot be avoided, in order to get results that will be used to build the model.

(5) Analyze the data obtained by the experiments. This step transforms data into information and is the logical conclusion of the whole process.

### 2.5.2 Response Surface Methods (RSM) and Design

Response Surface Methods are designs and models for working with continuous treatments when finding the optimal or describing the response is the goal (Oehlert, 2000). The RSM is important in designing, formulating, developing, and analyzing new scientific studying and products. It is also efficient for the improvement of existing studies and products. This method has been widely used to optimize reacting parameters due to its benefit in minimizing the number of analysis to analyze the interaction between parameter (Montgomery, 2005), reducing chemical consumption and less analytical works (Ferreira et al., 2007). RSM examines the optimum conditions through experimental methods where the experiments can be designed according to many

options. Moreover, the RSM has been proven as an important statistical tool for examination of numerous treatment processes. The first goal for Response Surface Method is to find the optimum response. When there is more than one response, then it is important to find the compromise optimum that does not optimize only one response (Oehlert, 2000). When there are constraints on the design data, then the experimental design has to meet requirements of the constraints. The second goal is to understand how the response changes in a given direction by adjusting the design variables. In general, the response surface can be visualized graphically. The graph is helpful to see the shape of a response surface; hills, valleys, and ridge lines. Hence, the function f (x1, x2) can be plotted versus the levels of x1 and x2 as shown in **Figure 2.5**

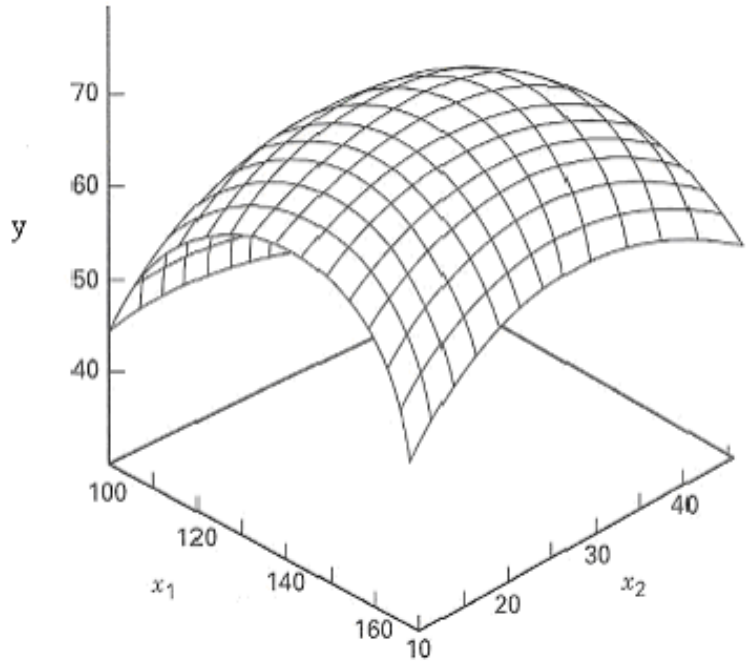



**Figure 2.5 Response surface plot (Montgomery, 2005).**

$y = f(x_1, x_2) + \varepsilon$

In this graph, each value of $x_1$ and $x_2$ generates a y-value. This three-dimensional graph shows the response surface from the side, and it is called a response surface plot. Sometimes, it is less complicated to view the response surface in two-dimensional graphs. The contour plots can show contour lines of x1 and x2 pairs that have the same response value y. An example of contour plot as shown in **Figure 2.6.**

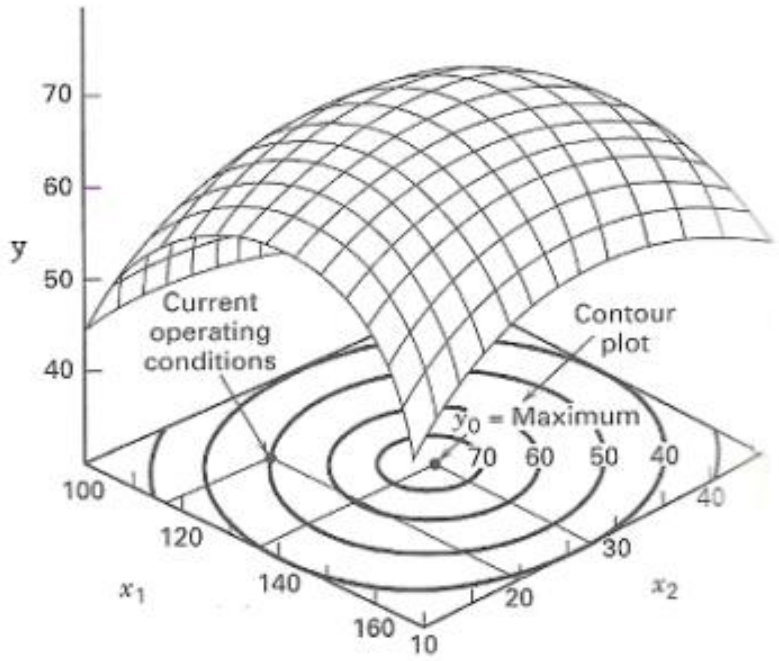



**Figure 2.6 Contour plot (Montgomery, 2005)**

In order to understand the surface of a response, graphs are helpful tools. But, when there are more than two independent variables, graphs are difficult or almost impossible to use to illustrate the response surface, since it is beyond 3-dimension. For this reason, response surface models are essential for analyzing the unknown function f (Montgomery, 2005).

### 2.5.3 Box-Behnken Design (BBD)

Box-Behnken design (BBD) is known as the one-step response surface design which requires only three levels to run an experiment. The use of Box-Behnken designs over central composite was promoted by Ferreira et al (2007) who suggested that it was a good design because it enables estimation of parameters for the quadratic model, detection of lack-of-fit of the model as well as building of a sequential design. However, the BBD might only include the responses in correlation with only a single factor. BBD is beneficial as the design is not presented in order where all the factors are at their highest or lowest levels, and this type of order inhibits errors in the resulting responses. BBD method is also considered a good choice in RSM study. **Table 2.6** shows the number of an experiment for different factors with three replications.

**Table 2.6 The number of experiments for different factors with three replications in the center of each design (Otto, 2007)**

| Number of Factors | Number of experiments |
|---|---|
| 3 | 15 |
| 4 | 27 |
| 5 | 46 |
| 6 | 54 |
| 7 | 62 |

A Box-Behnken design can be three levels, as shown in **Figure 2.7**, or more and can be applied to problems having three or more factors. There are no factorial or extreme points, and the design requires $2k(k - 1) + nc$ points (Anderson, 2004). Use of Box-Behnken should be contemplated for systems with greater than two factors where the optimum is known to lie in the middle of the factor ranges. Each point in Box-

Behnken design represents the factor values for one experiment (run) (Norton et al., 2009).

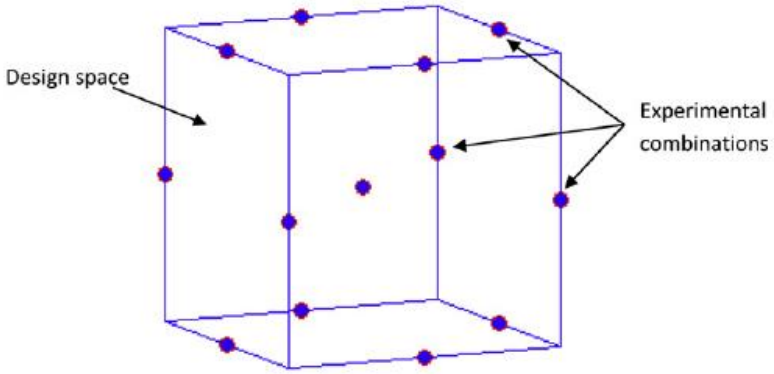



**Figure 2.7 A Box-Behnken design (BBD) for 3 factors (Norton et al., 2009)**

## 2.6 Linear Regression

### 2.6.1 Mandel's Fitting Test

Because a calibration curve model is not always appropriate to determine the suitability of linear equations to get accurate information, so statisticians develop a method called 'residual analysis". The difference between the observed value of the dependent variable ($y$) and the predicted value ($\hat{y}$) is called the residual ($e$). Each data point has one residual.

$$\text{Residual} = \text{Observed value} - \text{Predicted value}$$

$$e = y - \hat{y}$$

Both the sum and the mean of the residuals are equal to zero. That is, $\Sigma\, e = 0$ and $e = 0$.

The experimenters are able to assess the appropriateness of the residual model by defining residuals and examining residual plots. A residual plot is a graph that shows the residuals on the vertical axis and the independent variable on the horizontal axis. If the points in a residual plot are randomly dispersed around the horizontal axis, a linear regression model is appropriate for the data; otherwise, a non-linear model is more appropriate.

**Figure 2.8**, the residual plots, show three typical patterns. The first plot shows a random pattern, indicating a good fit for a linear model. The other plot patterns are non-random (U-shaped and inverted U), suggesting a better fit for a non-linear model.

a) b) c)

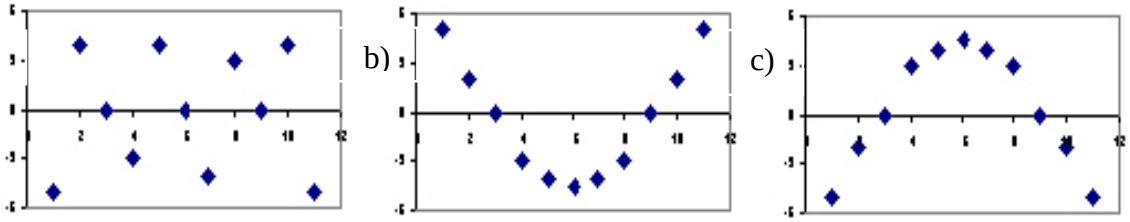


**Figure 2.8 Residual plot*:* a) Random pattern, b) Non random : U-shape, c) Non random : Inverted-U**

However, this method can not give us an objective answer, so Mandel proposes to define the mathematical and computing method. Mandel's fitting test is recommended for mathematical verification of linearity. The use of F-test in this method is for test compliance with a linear or quadratic equation linear equations to render.

It's F test equation such as the following equation:

$$\widehat{F} = \frac{S^2_{y.x} \cdot (n-2) - S^2_{y.x,2} \cdot (n-3)}{S^2_{y.x,2}}$$

**Linear Calibration model**

$$S_{y.x} = \sqrt{\frac{\sum_{o=1}^{n_c} (y_o - \hat{y}_l)^2}{df = n_c - 2}}$$

**Quadratic Calibration model**

$$S_{y.x,2} = \sqrt{\frac{\sum_{o=1}^{n_c} (y_o - \widehat{y_q})^2}{df = n_c - 3}}$$

### 2.6.2 The Lack-of-Fit by ANOVA

After a calibration curve, Mandel test determined, then do the appropriate test by Lack-of-Fit model. When we have repeated measurements for different values of the

variables, it is possible to test whether a linear model fits the data. In statistics, a lack-of-fit test is any of many tests that a proposed statistical model fits well. When we have repeated measurements for different values of the variables, it is possible to test whether a linear model fits the data. To calculate the selected calibration curve is correct, the experimental must be made to have repeated measures (replicate measurements), it is calculated before use ANOVA. **Table 2.7** lists the ANOVA simple representation.

**Table 2.7 ANOVA table of simple linear regression**

| Source of variation | SS | Df | MS (Mean Square) | $\hat{F}$ |
|---|---|---|---|---|
| Regression | $SS_{Reg}$ (sum of square due to regression) $SS_{Reg} = \sum_{i}^{k} n_i \cdot (\hat{y}_i - \bar{\bar{y}})^2$ | 1 | $MS_{Reg}$ | |
| Residual | $SS_R$ (sum of square due to residual) $SS_R = SS_{PE} + SS_{LOF}$ | n−2 | $MS_R$ | |
| Lack-of-fit | $SS_{LOF}$ (sum of squares due to lack-of-fit) $SS_{LOF} = \sum_{i}^{k} n_i \cdot (\bar{y}_i - \hat{y}_i)^2$ | k−2 | $MS_{LOF}$ | $\frac{MS_{LOF}}{MS_{PE}}$ |
| Pure error | $SS_{PE}$ (pure error sum of squares) $SS_{PE} = \sum_{i}^{k} \sum_{j}^{n_i} (y_{ij} - \bar{y}_i)^2$ | n−k | $MS_{PE}$ | |
| Total | $SS_{tot}$ (total sum of squares) $SS_{tot} = \sum_{i}^{k} \sum_{j}^{n_i} (y_{ij} - \bar{\bar{y}})^2$ $SS_{tot} = SS_{PE} + SS_{LOF} + SS_{Reg.}$ | n−1 | | |

*Symbol Definition:*

k: calibration curve of the concentration (xi)

$n_i$: each xi each repeated in ni times

$y_{ij}$: ni value of the repetitions in xi concentration

n: total concentrations of several experiments

$\bar{\bar{y}}$ : the value of all the experimental results

$\bar{\bar{y}}i$ : the average concentration ni of experimental values xi.

$\hat{y_i}$: the theoretical value of concentration xi

**2.7 Matrix Effect**

Gas chromatography (GC) analysis is often affected by the active sites in the GC system such as the inlet liner and column. A compound prone to thermal degradation and/or adsorption on the active sites has a decreased response. However, an analyte in a solution containing coextractives often gives a higher response than when analyzed in neat solvent. This phenomenon is known as the "matrix-induced chromatographic response enhancement" (matrix effect) (Erney, 1993). Matrix-induced response diminishment occurs when non-volatile coextracted matrix components, accumulated into the gas chromatographic system, help to the generation of new active sites. In both cases, the obtained signals from the calibration curve and in a real sample matrix (matrix standards) can be significantly different and may cause quantitative errors (Frenich et al., 2009 ).

There are various methods for addressing the matrix effect. It is desirable to use Isotopically labeled standards as internal standard (IS). This method compares an isotope labeled internal standard spiked into the actual sample compared to being spiked into the pure solvent. However, the disadvantage is within the isotope calibration standard goods are usually expensive and not every analyte isotope has its standards. Therefore, the solution mainly deal with before, we can do confirmed by the t-test (t-test).

A t-test between the two samples is typically used standard calibration curve of linear equation slope ($a_1$, c), the slope of the linear equation containing matrix effects ($a_1$, add). For comparison, the number is assumed that two slopes of variation:

$H_0$ : $a_{1,c}= a_{1,add}$ , $H_1$ : $a_{1,c}\neq a_{1,add}$, It may make use of t-test to prove:

$$\hat{t} = \frac{|a_{1,c}-a_{1,add}|}{s_p} \times \sqrt{\frac{n_c \times n_{add}}{n_c+n_{add}}}$$

$$S_p= \sqrt{\frac{(n_c-2)S^2_{a_{1,c}}+(n_{add}-2)S^2_{a_{1,add}}}{n_c+n_{add}-4}}$$

$n_c$ and $n_{add}$ are each several concentrations in the calibration curve, $S_{a_{1,c}} dan\, S_{a_{1,add}}$ It is the standard variance , df = $n_c+n_{add}-4$. When t-calculated is less than the theoretical test $\pm\hat{t}_{(95\%,\ 6)}$ value, suitable for $H_0$ : $a_{1,c}= a_{1,add}$, abtain of the matrix-free calibration curve or the calibration curve containing a small difference in the substrate, it indicates that matrix effects do not affect the concentration of the sample calibration curve for the quantification. F test also use to support t-test results. F value equation: $\hat{F} = \frac{S^2_{y,x,add}}{s^2_{y,x,c}}$

Theoretical F value is: $df_{add}=n_{add}-2$, the above t and F values can be calculated using JMP software, when $\widehat{F}$ less than the theoretical F-value indicates that no significant matrix effects. This conclusion can be obtained by the t-test results certified, accredited calibration curve was in line with experimental and matrix effects which are not significant.

# CHAPTER III

# Experimental

## 3.1 Chemical and equipment

### 3.1.1 Chemicals

All chemicals are analytical grade and used without further purification; all chemicals are used are as follows:

1. Methyl ester of p-hydroxybenzoic acid (Methylparaben, abbreviated MP), $C_8H_8O_3$, purity 99.9%, supplied by Alfa Aesar
2. Ethyl ester of p-hydroxybenzoic acid (Ethylparaben, abbreviated EP), $C_9H_{10}O_3$, purity 99.9%, supplied by Alfa Aesar
3. Propyl Ester of p-hydroxybenzoic acid (Propylparaben, abbreviated PP), $C_{10}H_{12}O_3$, purity 99.9%, supplied by Alfa Aesar
4. Butyl ester of p-hydroxybenzoic acid (Butylparaben, abbreviated BP), $C_{11}H_{14}O_3$, purity 99.9%, supplied by Alfa Aesar
5. P-Terphenyl-$d_{14}$ as an internal standard, $C_{18}D_{14}$, purity 98%, supplied by Sigma-Eldrich
6. Dichloromethane, $C_2H_2Cl_2$, purity 99.9 %，supplied by Macron
7. Methyl alcohol, $CH_3OH$, purity 99.9 %, supplied by Merck
8. Acetone, $CH_3COCH_3$, purity 99.9 %, supplied by Macron
9. Acetonitrile $CH_3CN$, purity 99.9%, supplied by Sigma-Aldrich
10. Sodium sulfate anhydrous, $Na_2SO_4$, purity > 99%, suplied by Fluka
11. Activated magnesium silicate (Florisil), $MgO_3Si$：< 200 mesh, supplied by Sigma-Aldrich
12. Silica-bound C18, supplied by Merck
13. Acetic Anhydride, supplied by Sigma- Aldrich

14. Deionized water, Milli-Q water produce by Millipore Elix 10 RO system and a Millipore Synergy UV system

### 3.1.2 Equipment

1. Four-digit microbalance: Mettler Toledo AG104 type, purchased from Mettler-Toledo company
2. Polypropylene centrifuge tube (volume 50 mL)
3. Solid phase extraction tube apparatus, purchased from Supelco company.
4. Multi-purpose tube shaker: Model Vortex-Genie 2, purchased from Scientific Industries companies.
5. Ultrasonic oscillator: Bransonic 5210 type, operating temperature 25-30 ℃, purchased from Branson company.
6. Gas Chromatography-Mass Spectrometry (GC-MS) Instrumentation. The GC-MS analysis was performed using a Varian 450 GC directly connected to a Varian 220 ion-trap mass spectrometer
7. Direct injection inductions (Direct sample introduction device, ChromatoProbe): purchased from Varian company.
8. Gas chromatography column: DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), temperature limits -60 ° C ~ 325 ° C, purchased from J & W Scientific Company.

## 3.2 Experimental procedures

### 3.2.1 Preparation of stock solutions

The methylparaben stock solution was prepared by dissolving 10 mg of methyl paraben solid standard with 10 mL methanol to make 1000 ppm of methylparaben (MP). The same procedure was applied to prepare ethylparaben (EP), propylparaben (PP), and butylparaben (PP) stock solutions. The internal standard stock solution was prepared by dissolving 10 mg of

internal standard with 10 mL dichloromethanes. These stock solutions were store at 4°C in the dark to prevent degradation by light.

**3.2.2 Preparation of working solutions.**

The working solution was prepared by diluting stock solution. In order to prepare MP, EP, PP, and BP working solution, the standard of stock solutions was diluted with methanol until reach concentration 1 ppm of MP, EP, PP, BP.

**3.2.3 Gas Chromatography Mass Spectrometer Settings**

**3.2.3.1 GC settings:**

(1) GC column : DB-5MS(30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W)

(2) Carrier gas: He

(3) Column flow rate : 1.0 mL/min

(4) Injection port temperature：

| Temp. (°C) | Rate (°C/min) | Hold (min) |
|---|---|---|
| 90 | - | 1.50 |
| 300 | 200 | 1.00 |
| 90 | 200 | 7.55 |

(5) Injector mode：

| Time(min) | Split state | Split ratio |
|---|---|---|
| Initial | On | 20 |
| 1.5 | Off | Off |
| 2.5 | On | 20 |

(6) Oven temperature program：

| Temp. (°C) | Rate (°C/min) | Hold (min) |
|---|---|---|
| 60 | - | 2.50 |
| 200 | 30 | 2.00 |
| 280 | 40 | 1.00 |

**3.2.3.2 MS Setting**

(1) Transfer line temperature: 280 °C

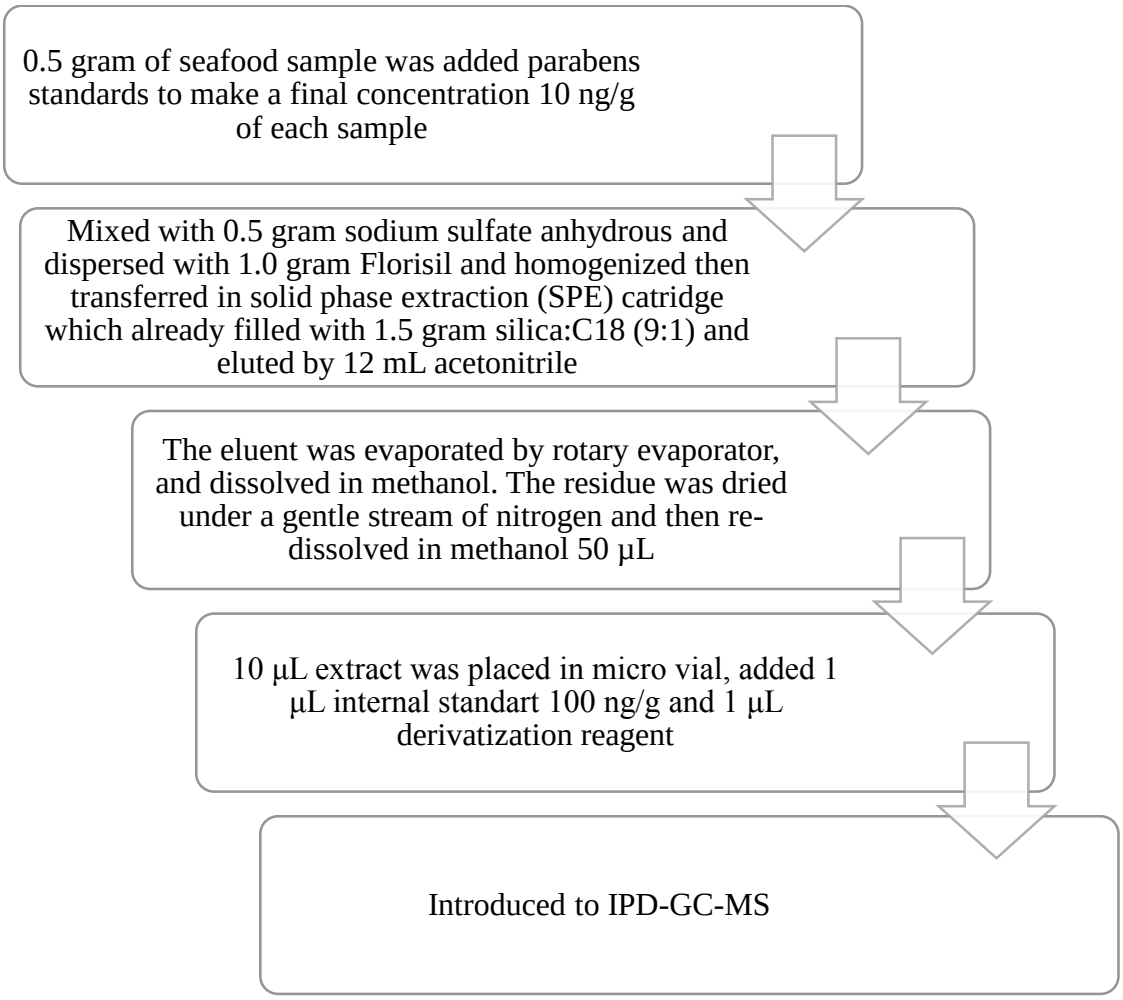
(2) Ion source temperature: 180 °C

(3) Scan mode: Full scan

**3.2.3 Sample Collection**

Two freshly killed fish (perch) were purchased from a local fish market in Chung-Li City, Taiwan. The fish samples were washed several times with deionized water. The fish skin was removed, and the muscle tissue was cut into small pieces, and then homogenized in a blender. The homogenate was then freeze-dried for three days and ground into powder.

### 3.2.4 Matrix Solid Phase Dispersion Procedures



# CHAPTER IV

## Result and Discussion

### 4.1 Determination of parabens using Gas Chromatography-Mass Spectrometry

#### 4.1.1 Parabens Derivatization and Gas Chromatography Analysis

Parabens derivatization was used to improve parabens volatility thus it could be amenable for GC-MS analysis. The derivatization reaction between parabens and acetic anhydride is described in **Figure 4.1.**

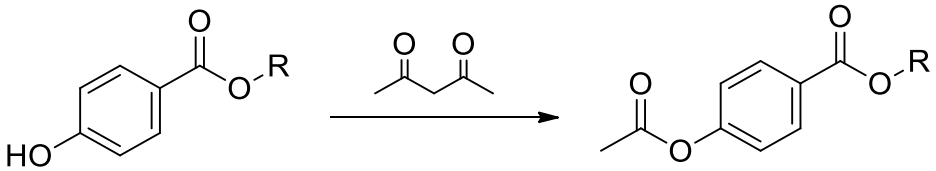



**Figure 4.1 The paraben derivatization reaction by acetic anhydride**

The paraben derivatization reaction by acetic anhydride involved the replacement of active hydrogen in hydroxyl group with the acyl group from acetic anhydride and converted into ester group. The ester group attached in derivative of parabens made them less polar than originally paraben which contain hydroxyl group. However, the attachment of ester group in parabens derivative could improve the volality of analytes. The ester group in parabens derivative has no hydrogen atom attached directly to an oxygen atom. Therefore, it is incapable of engaging in intermolecular hydrogen bonding thus has considerably lower boiling points than originally parabens.

After paraben derivatized by acetic anhydride, paraben was ready to analyze in GC for paraben determination procedure. The GC analysis was conducted by placing 10 μL of parabens (MP, EP, PP, BP) standard solution in gas chromatography microvial and then adding 1 μL acetic anhydride and 1 μL internal standard solution. The mixture was directly introduced to injector port of gas chromatography in order to obtain injection-port derivatization (IPD). The parabens derivatives was separated in gas chromatography based on its volatility and then

detected by mass spectrometry. The chromatogram of parabens derivatives is shown in **Figure 4.2** and their retention time are mentioned in **Table 4.1.**

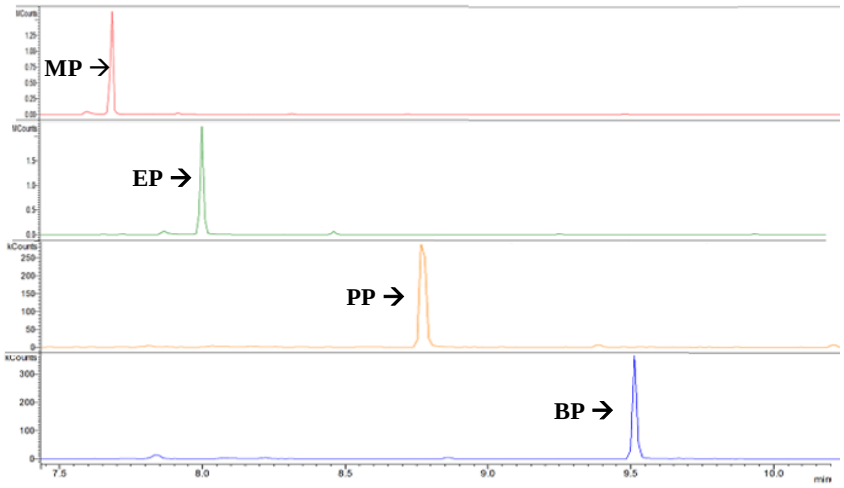



**Figure 4.2 Chromatogram of parabens derivatives***

***GC-MS condition:**

(7) GC column : DB-5MS(30 m × 0.25 mm i.d., 0.25 µm film thickness, J&W)

(8) Carrier gas : Helium

(9) Column flow rate : 1.0 mL/min

(10) Injector port temperature : 90°C

(11) Oven program : 60°C (2.5 min), 30°C/min to 200°C (2.0 min), 40°C/min to 280°C (1.0 min)

(12) Transfer line temperature : 280 °C

(13) Ion source temperature : 180 °C

(14) Sample volume : 10 µL

(15) Acetic anhydride volume : 1 µL

**Table 4.1 Retention time of the parabens derivatives***

| Compound | Derivate Compound | Molecular weight | Retention time (s) |
|---|---|---|---|
| MP | Methyl 4-acetoxybenzoate | 195 | 7.645 |
| EP | Ethyl 4-acetoxybenzoate | 208 | 8.040 |
| PP | Propyl 4-acetoxybenzoate | 222 | 8.713 |
| BP | Butyl 4-acetoxybenzoate | 236 | 9.515 |

***GC-MS condition:**

(1) GC column : DB-5MS(30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W)

(2) Carrier gas : Helium

(3) Column flow rate : 1.0 mL/min

(4) Injector port temperature : 90°C

(5) Oven program : 60°C (2.5 min), 30°C/min to 200°C (2.0 min), 40°C/min to 280°C (1.0 min)

(6) Transfer line temperature : 280 °C

(7) Ion source temperature : 180 °C

(8) Sample volume : 10 μL

(9) Acetic anhydride volume : 1 μL

**Figure 4.2** and **Table 4.1** bring out the success of online derivatization showing separation of parabens derivatives. The success of online derivatization characterized by the formation of derivatives MP, EP, PP, and BP. $[M]^+$ or molecular ions in mass spectra showed the molecular weight of the compound analyzed. In mass spectra showed in **Figure 4.2,** the derivatives MP, EP, PP, and BP exhibited the molecular ion at m/z 195, 208, 222, and 236, respectively, which indicated the molecular weight of derivatives MP, EP, PP, and BP.

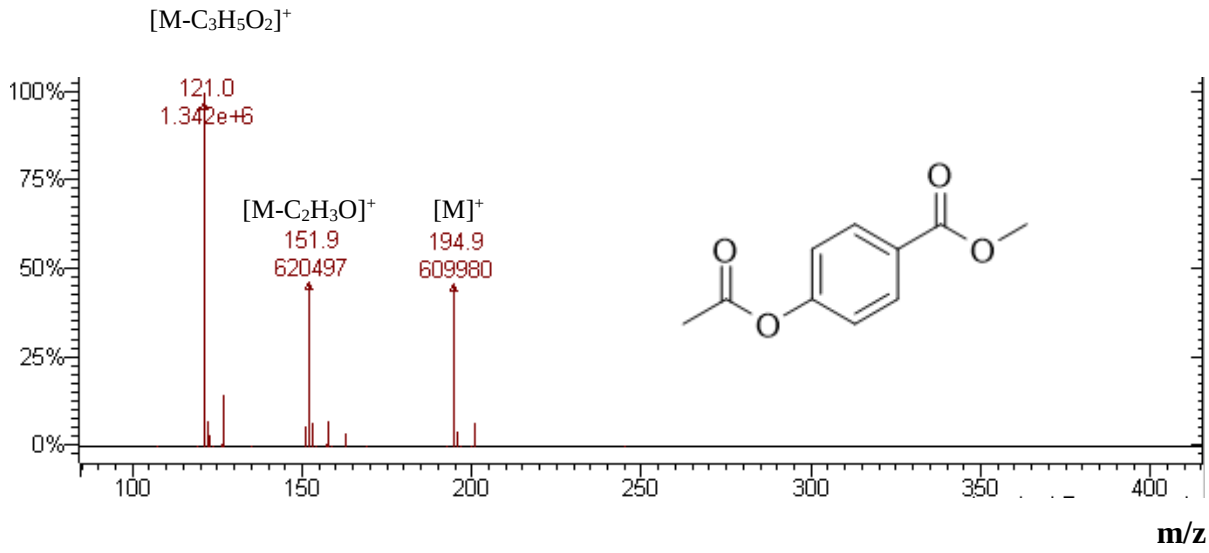
Therefore, it can be concluded that derivatives MP, EP, PP, and BP have been formed and an online derivatization has been successful applied.

The purpose of parabens derivatization was to increase parabens volatility of parabens. MP is more volatile than EP, PP, and BP because of its short chain. As a result, MP eluted first, folllowed by EP, PP, and BP.

### 4.1.2 Mass Spectrometry Analysis

In this study, ion trap mass spectrometer was used to detect the appearance of parabens derivatives. **Figures 4.3-4.6** showed mass spectra of parabens derivatives after analyzed by GC-MS. **Table 4.2** brings out the molecular weight and quantitation ions of the parabens derivatives.

The success of on-line derivatization was confirmed by appearance molecular ion peak at m/z 195, 208, 222, and 236 for derivates of MP, EP, PP, and BP, respectively, which represented the molecular weight of the parabens derivatives. **Figure 4.3** shows the fragmentation of methylparaben. The fragment at m/z 195 showed the molecular ion, fragment at m/z 151 is confirmed by the loss of – $C_2H_3O$ (acetyl group) and fragment at m/z 121 is confirmed by the loss of $-O(CH_3)$.



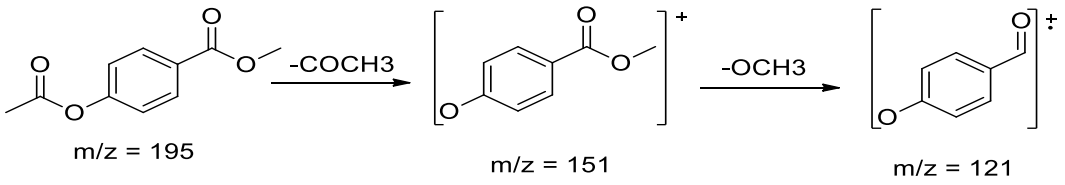



**Figure 4.3 Mass spectra of methylparaben derivatives (methyl 4-acetoxybenzoate) and the plausible interpretation of the MS fragmentation**

**Figure 4.4** brings out the fragmentation of ethylparaben. The molecular ion is showed by peak at m/z 208. Moreover, the fragment at m/z 165 is attributed to the loss of $-C_2H_3O$ (acetyl group), the fragment at m/z 138 is attributed to the loss of $-(CH_2)_2$, and the fragment at m/z 121 is confirm by the loss of $-O^-$.

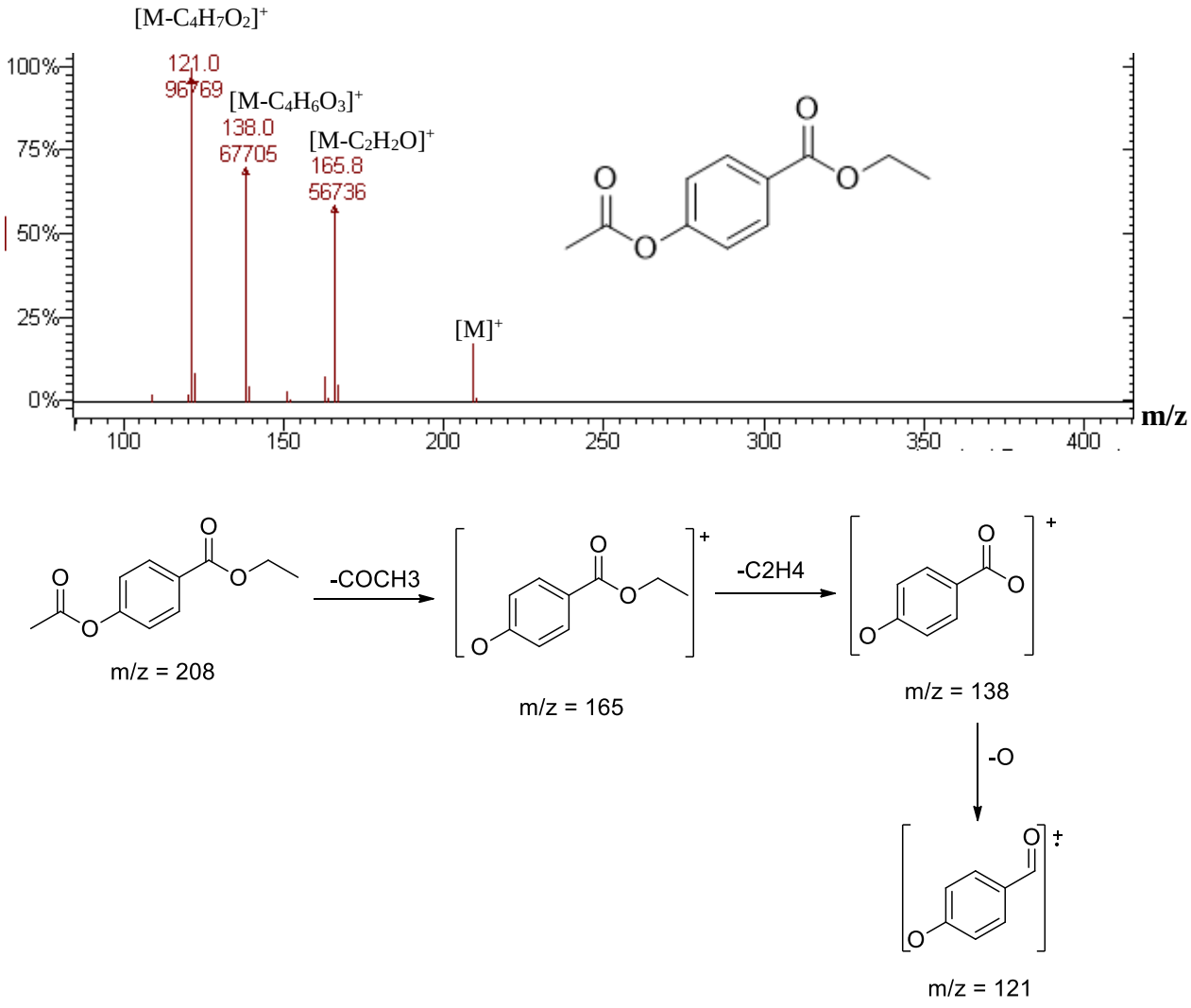



**Figure 4.4 Mass spectra of ethylparaben derivatives (ethyl 4-acetoxybenzoate) and the plausible interpretation of the MS fragmentation**

**Figure 4.5** and **Figure 4.6** exhibit the fragmentation of propylparaben and butylparaben. The fragment at m/z 222 for propylparaben and m/z 236 for butylparaben show molecular ion. Futhermore, fragment at m/z 138 for both propylparaben and butylparaben was attributed to the loss of $–COCH_3$ (acetyl group) and $–(CH_2)n$ (n = 3 to 4, for PP and BP, respectively). The fragment at m/z 121 is attributed by the loss of $–O^-$.

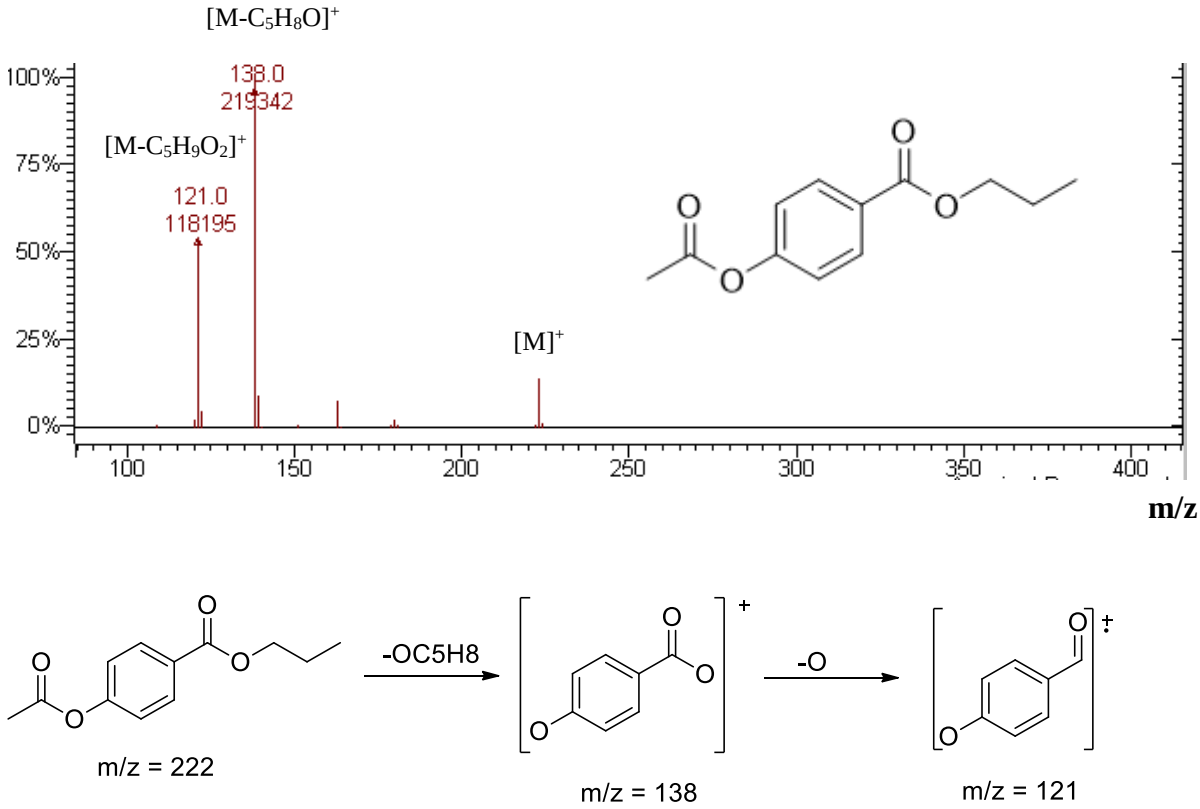



**Figure 4.5 Mass spectra of propylparaben derivatives (propyl 4-acetoxybenzoate) and the plausible interpretation of the MS fragmentation**

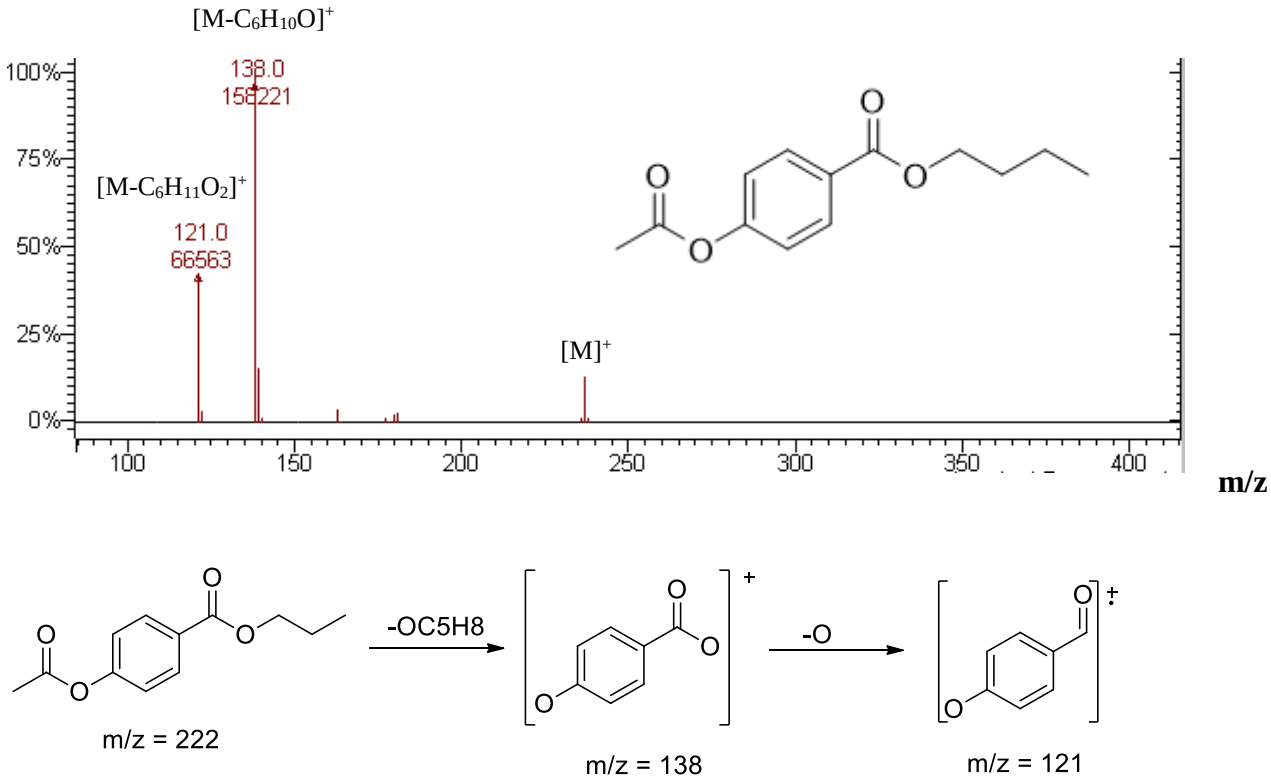



**Figure 4.6 Mass spectra of butylparaben derivatives (butyl 4-acetoxybenzoate) and the plausible interpretation of the MS fragmentation**

**Table 4.2 The quantitation ions of the parabens derivatives**

| Derivate Compound | Molecular weight | Quantitation ion (m/z) |
|---|---|---|
| Methyl 4-acetoxybenzoate | 195 | 121 + 151 |
| Ethyl 4-acetoxybenzoate | 208 | 121 +138 +165 |
| Propyl 4-acetoxybenzoate | 222 | 121 + 138 |
| Butyl 4-acetoxybenzoate | 236 | 121 +138 |

### 4.2 Optimization of Matrix Solid-Phase Dispersion (MSPD)

The main principle of Matrix Solid-Phase Dispersion (MSPD) is mixing the powdered sample with anhydrous sodium sulfate and dispersant reagent, then homogenizing this mixture. This blend was transferred to a polypropylene SPE cartridge containing clean-up co-sorbent packing at the bottom. The analytes were eluted with elution solvent by gravity flow. The extract was evaporated to dryness by a gentle stream of nitrogen at room temperature, then re-

dissolved with methanol. (Canosa, et.al., 2008; Tsai, et al., 2014). One of the most attractive advantage of MSPD procedure is that analytes can be extracted from the sample and separated from lipid and other interfering species in a single step. The challenge for the determination of trace levels of organic compounds in seafood samples is the removal of lipid from target analytes. In order to achieve this purpose, the parameters affecting MSPD were investigated to obtain the lipid-free extract and high recovery.

The parameter to compare of each condition are abundance and % recovery. Abundance is confirmed by the width of peak area, whereas, % recovery was calculated based on the following formula below:

$$\%Recovery = \frac{C\ spiked\ sample - C\ unspiked\ sample}{C\ spike\ added\ to\ sample}$$

### 4.2.1 Selecting type of dispersant and clean-up co-sorbent

The dispersant served to disrupt the lyphophilic sample, dispersed the analytes, and transfered it to the elution solvent. The clean-up co-sorbent is used to adsorb and clean the extract from the lipid fish and co-elution compuound that can interference in GC analysis. The nonpolar and polar adsorbent is needed to hold the nonpolar and polar lipid or other impurities. Finally, the elution solvent is served to bring the analytes come out from the cartridge. Therefore, the dispersant, clean-up co-sorbent and elution solvent type have main key in extraction efficiency, thus they are related to the success of the whole experiments..

The dispersant selected in this experiment were: Florisil, silica and alumina. Each dispersant gave different abundances depend on their polarity and hydrogen bond interaction. The result is shown in **Figure 4.8**, the silica and alumina ($Al_2O_3$) obtain the low abundance (less than 300.000) since the hydrogen bonding interaction between the oxygen atoms (O) on the silica and alumina and the hydrogen atoms (H) of the parabens was probably too strong, leading to difficulties in the elution process. Their high polarity caused the analytes could not well extracted. On the other hand, Florisil achieved the highest abundance. Based on “like

dissolve like" concept, Florisil which is semi-polar compound could extract the semi-polar parabens and transfered it to the elution solvent. Florisil and parabens interactions, including hydrogen bonding and electrostatic interaction, was significant, thus it could produce the best abundance. The hydrogen bonds between the Si-O groups in Florisil could bind the -OH groups of these parabens through the Florisil surface (Xu, 2016). The interaction between Florisil and parabens was presented by **Figure 4.9**.

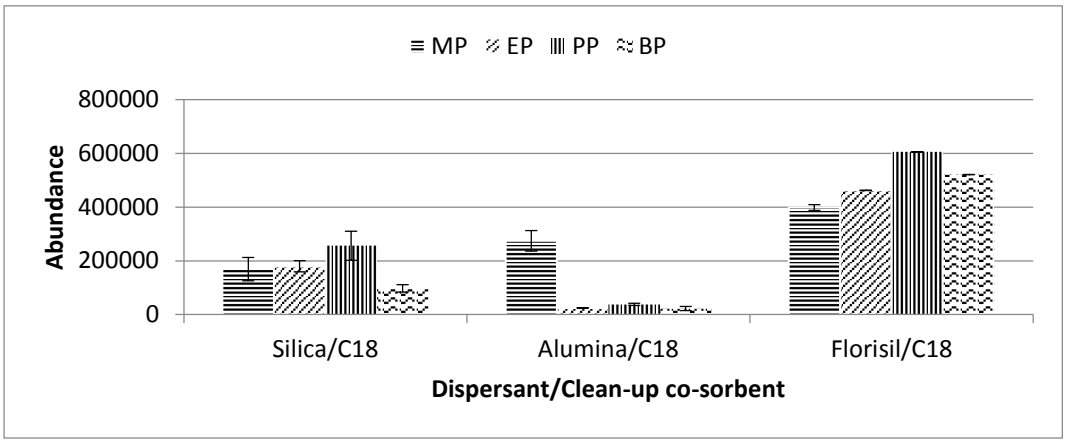



**Figure 4.8 Selecting type of dispersant***

***MSPD condition**

a) Dispersant amount : 2.0 gram
b) Clean-up co-sorbent amount : 1.0 gram
c) Elution solvent volume : 10 mL

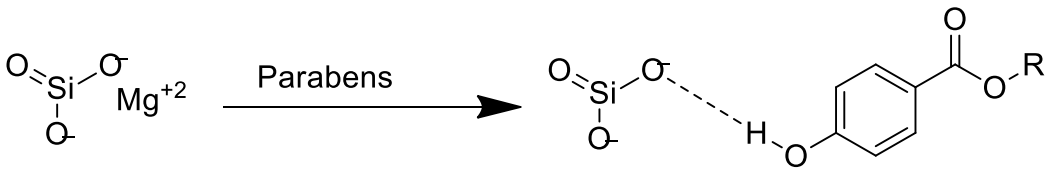



**Figure 4.9 The interaction between Florisil and parabens**

The abundance of parabens showed at Figure 4.8 was satisfied, otherwise there is yellow colored of impurities in the extract, so that the various clean-up co-sorbent has applied to get the clean extract. This study was done in order to obtain good abundance with the clean extract. The nonpolar C18 polar clean-up co-sorbent was applied in order to retain the non

polar lipid fish. Otherwise, the yellow colored compound was observed in the extract which supposed it was polar lipid or polar co-elution compound that did not retain to non polar clean-up co sorbent. Therefore, various combination of type clean-up co-sorbent was applied including the adding of silica to C18 in various comparison for retaining the polar impurities. The effect of clean-up co-sorbent type is shown in **Figure 4.10** presenting silica+C18 (9:1) producing the greatest abundance based on this study (more than 300.000). This result showed that the silica+C18 (9:1) could retain polar and non-polar interferences and obtained the clear extracts.

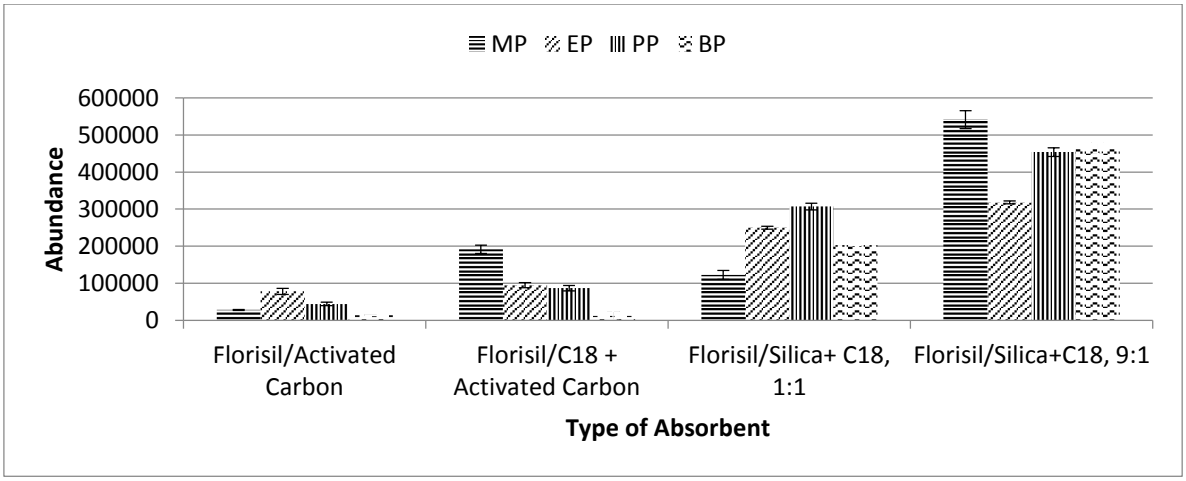



**Figure 4.10 Selecting type of clean-up co-sorbent***

***MSPD condition**

a) Dispersant amount : 2.0 gram
b) Clean-up co-sorbent amount : 1.0 gram
c) Elution solvent volume : 10 mL

### 4.2.2 Dispersant and clean-up co-sorbent amount and elution solvent volume

The type of elution solvent gives the fundamental effect in MSPD because it bring the analytes come out from the cartridge. In this experiment, dichloromethane, ethyl acetate and acetonitrile was used to was used to elute the parabens from the Florisil surface. The high lipid content was observed when the less polar solvents, dichloromethane and ethyl acetate, were employed. Dichloromethane and ethyl acetate were less polar so they can elute the non-polar

lipid out of the cartridge. This result is in agreement with previous research by Canosa et.al (2008) that dichloromethane and ethyl acetate failed to recover parabens. Otherwise, the good abundance and clear extract was achieved when semi-polar elution solvent, acetonitrile, was used. When dispersant and sample were eluted with semi-polar elution solvent, hydrogen bond between dispersant and parabens was broken. The impurities could be adsorbed onto the clean-up co-sorbent and parabens passed through the column.

The amount of dispersant and clean-up co-sorbent, as well as the volume of elution solvent have important effects on analytes recovery. These effects are summerized in **Figures 4.11-4.13. Figure 4.11** presents that 1.0-gram Florisil as dispersant obtain the best recovery (more than 50% of each analyte). When 0.5-gram Florisil was used, the recovery was low because of the lack of dispersant which disrupted the sample and dispersed the analytes to elution solvent. As a result, some analytes remained in the sample. Moreover, when 1.5-gram and 2.0-gram Florisil was used, too much dispersant cause analytes settling in the dispersant and can not transfer well to elution solvent.

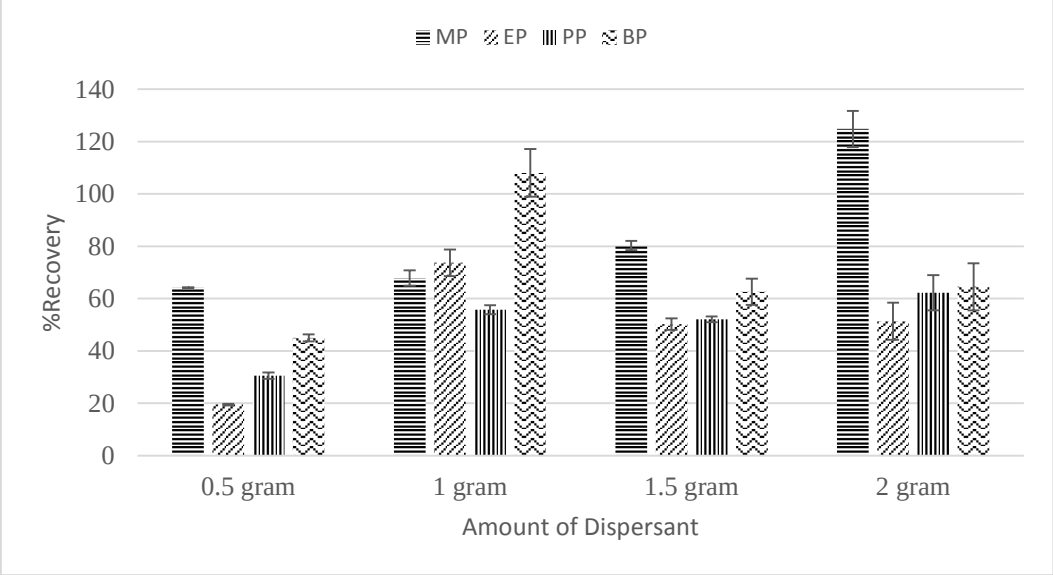



**Figure 4.11 Selecting dispersant amount***

***MSPD condition**

a) Dispersant type : Florisil
b) Dispersant amount : varied

c) Clean-up co-sorbent type : silica+C18 (9:1)
d) Clean-up co-sorbent amount : 1.0 gram
e) Elution solvent type : Acetonitrile
f) Elution solvent volume : 10 mL

As shown in **Figure 4.12**, the highest extraction recovery was reached by 12 mL acetonitrile. The less recovery achieved when 10 mL acetonitrile was used because deficiency of elution solvent volume to elute the analytes. As a result, not all analytes eluted through the cartridge. The less recovery also accomplished when 14 mL of elution solvent was applied. It because of the excess elution solvent volume used thus it not only elutes the analytes but also other interference which would disturb chromatographic analysis and influence on recovery alleviation.

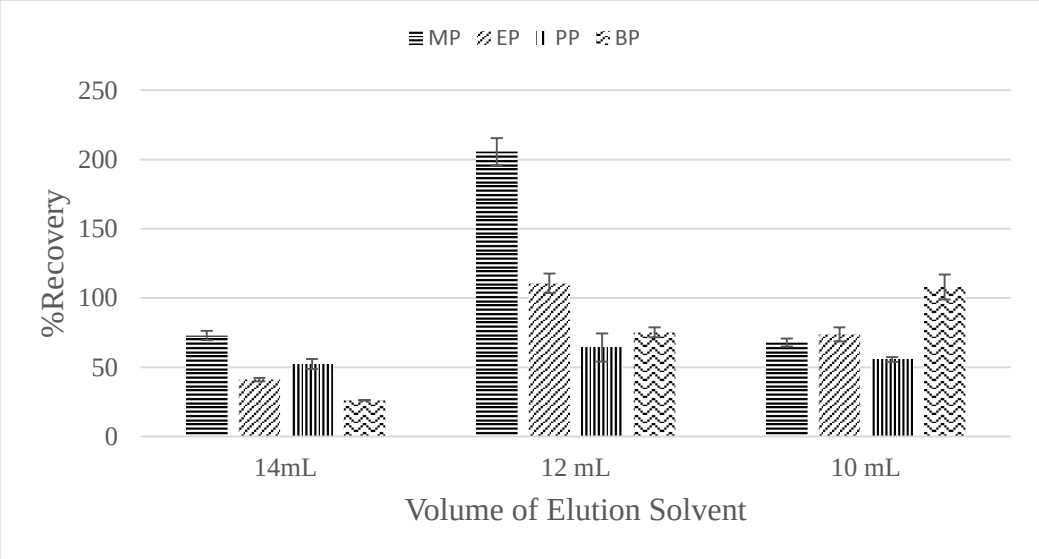



**Figure 4.12 Selecting volume of elution solvent***

***MSPD condition**

a) Dispersant type : Florisil
b) Dispersant amount : 1.0 gram
c) Clean-up co-sorbent type : silica+C18 (9:1)
d) Clean-up co-sorbent amount : 1.0 gram
e) Elution solvent type : Acetonitrile
f) Elution solvent volume : varied

**Figure 4.13** displays the effect of the amount of clean-up co-sorbent. The highest recovery was achieved by 1.5-gram clean-up co sorbent. The less amount of clean-up co-sorbent give less recovery too. It is caused by the lack of clean-up co-sorbent, thus lipid and other interference did not adsorb well to clean-up co-sorbent. The extracts would contain interference that disturbed chromatographic analysis and effected in decreasing of recovery. Otherwise, 2.0-gram clean-up co-sorbent did not give much different result as 1.5-gram clean-up co-sorbent was used. Therefore, with the reason of environmental friendly, 1.5-gram silica+C18 (9:1) as clean-up co-sorbent was used.

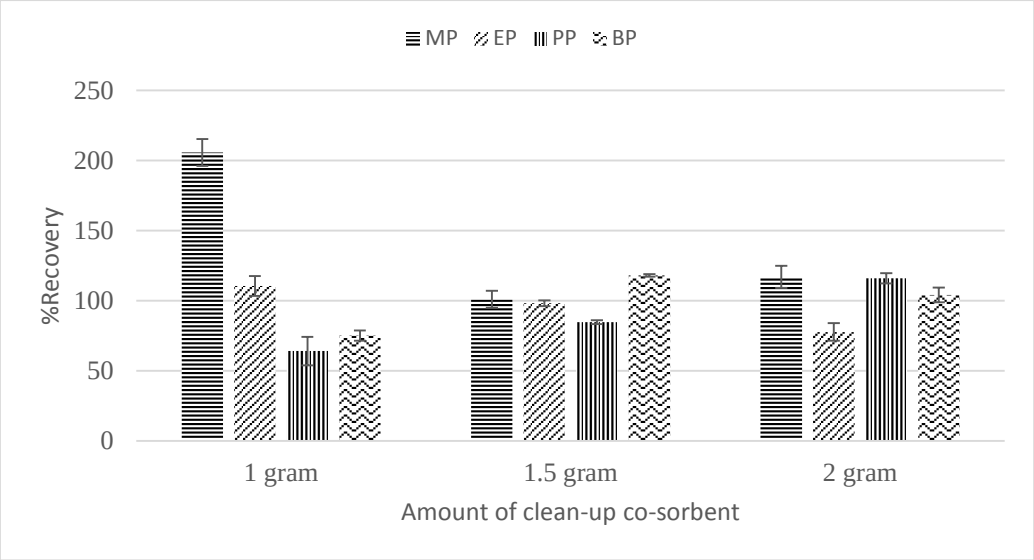



**Figure 4.13 Selecting clean-up co-sorbent amount**

***MSPD condition**

a) Dispersant type : Florisil
b) Dispersant amount : 1.0 gram
c) Clean-up co-sorbent type : silica+C18 (9:1)
d) Clean-up co-sorbent amount : varied
e) Elution solvent type : Acetonitrile
f) Elution solvent volume : 12 mL

According to the experiments results, the MSPD condition with 1 gram of Florisil, 1.5 gram of silica+C18 and 12 mL of acetonitrile was chosen as the optimal recovery since it gave satisfactory MSPD performance with recoveries from 84-118 %..

### 4.2.3 MSPD Optimization by Statistical Experimental Design

The present study was proposed to investigate the parameters affecting MSPD efficiency (i.e: type and amount of dispersant and clean-up co-sorbent, as well as the volume of elution solvent), in order to simplify the experiment and improve MSPD efficiency, as well as, to reduce the number of experiments, experimental design method was applied. The experiment designed to investigate the effects of the interaction between the parameters themselves resulting the total recovery. The experimental design was utilized the Box-Behnken Design (BBD) which accomplished by Stat-Ease Design-Expert 8.0.6 software (Stat-Ease, Inc., Minneapolis, MN, USA). The BBD was designed 3 factor and the range of studied variables were: the amount of clean-up co-sorbent (1.0, 1.5 and 2.0 gram), the amount of dispersant (0.5, 1.0 and 1.5 gram) and volume of elution solvent (10, 12 and 14 mL) as shown in **Table 4.3**. **Table 4.4** shows the BBD matrix design comprised 15 randomized experiments (as evidenced by total recovery). According to **Table 4.4**, the highest total recovery achieved when 1.0 gram of Florisil, 1.5 gram of silica+C18 and 12 mL of acetonitrile was applied which showed by total recovery is ± 400% for all the analyte. The results of these 15 experiments design were represented and visualized by the response surface plot as shown in **Figure 4.14**. Response surface plot was used to find the optimal condition of some variabled. The best results were on the highest point of the response surface plot.

**Table 4.3 Design of BBD**

| Factor | Low(-1) | Center(0) | High(+1) |
|---|---|---|---|
| Florisil (g) | 0.5 | 1 | 1.5 |
| Silica+C18 (g) | 1.0 | 1.5 | 2.0 |
| ACN (mL) | 10 | 12 | 14 |

**Table 4.4 Fifteen randomized experiments results based on Box-Behnken designed 3 factor for parabens determination in marketed fish**

| Run Sample | Florisil (gram) | Acetonitrile (mL) | Silica-C18 (gram) | Total Recovery |
|---|---|---|---|---|
| 1 | 1 | 10 | 1 | 235.82 |
| 2 | 1 | 14 | 2 | 319.64 |
| 3 | 0.5 | 10 | 1.5 | 184.89 |
| 4 | 1.5 | 12 | 2 | 291.89 |
| 5 | **1** | **12** | **1.5** | **391.76** |
| 6 | 1.5 | 10 | 1.5 | 203.742 |
| 7 | 1.5 | 12 | 1 | 232.63 |
| 8 | 1 | 14 | 1 | 221.13 |
| 9 | **1** | **12** | **1.5** | **415.20** |
| 10 | 0.5 | 12 | 1 | 315.526 |
| 11 | 0.5 | 14 | 1.5 | 209.28 |
| 12 | 1.5 | 14 | 1.5 | 198.05 |
| 13 | 0.5 | 12 | 2 | 240.22 |
| 14 | **1** | **12** | **1.5** | **413.09** |
| 15 | 1 | 10 | 2 | 235.43 |

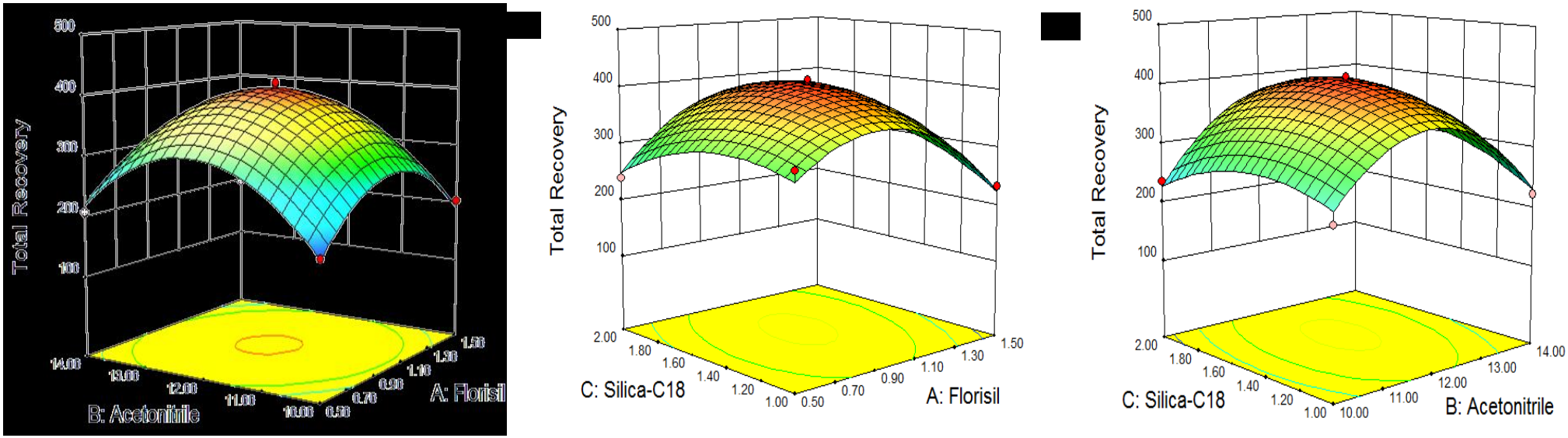



**Figure 4.14 Response surface plot for the total recovery of all target analytes estimated from BBD on each pair of independent variables: (a) amount of Florisil against volume of acetonitrile, (b) amount of silica: C18 against amount of Florisil, (c) amount of silica: C18 against volume of acetonitrile. Fixed optimal condition based on BBD and response surface method were: 1.0 gram of Florisil, 1.5 gram of silica: C18 and 12 mL of acetonitrile.**

The highest point of the response surface plot on **Figure 4.14** shows the best result from since it gave highest total recovery for all the analytes. The optimal parameters could be found in the middle of the response surface plot : 1.0 gram of Florisil, 1.5 gram of silica+C18 and 12 mL of acetonitrile. These results was suitable with the previous experiments that 12 mL acetonitrile, 1.5 gram Silica+C18 and 1.0 gram Florisil obtained the optimal condition that produced the best recovery for MP, EP, PP and BP, thus it was chosen as the optimal condition of MSPD for parabens determination.

Under the optimized condition of BBD and response surface method, 0.5 gram of powdered fish sample was homogenized with 0.5-gram anhydrous sodium sulfate and 1.0 gram of Florisil as dispersant. Sample was added parabens standart solution for sample spiking to make a final concentration 10 ng/g of each sample. This blend was transferred to SPE column containing 1.5-gram clean-up co-sorbent, silica: C18 (9:1). Analytes were then eluted with 12 mL of acetonitrile and concentrated to dryness under a gentle stream of nitrogen. The extract was redissolved with 50 µL methanol. Then, 10 µL of extract fraction was mixed with 1.0 µL acetic anhydride reagent and directly injected to IPD GC system. The extraction recovery under optimum condition are presented in **Table 4.5**.

**Table 4.5 The extraction recovery under optimum condition**

| | MP | EP | PP | BP |
|---|---|---|---|---|
| Extraction | 98[a] | 98 | 84 | 118 |
| Recovery | (6)[b] | (2) | (1) | (1) |

[a] Recovery (%)
[b] Relative Standard Deviation (%), n=3

### 4.2.4 Analysis of variance (ANOVA)

Analysis of variance (ANOVA) was accomplished with aim to evaluate the significance of the of the regression responses considering the experimental variance. ANOVA is the most powerful numerical method for model validation and experimental interpretation (Stalikas,

Fiamegos, Sakkas, & Albanis, 2009). The combination of 15 randomized experiment data made up by ANOVA using Design-Expert software which set α= 0.05 and confidence level at 95%. The results are shown in **Table 4.6**. P-value less than 0.05 showed that the variables have a significant influence on the experiment.

**Table 4.6 Analysis of variance (ANOVA)**

| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob > F |
|---|---|---|---|---|---|
| A-Florisil | 69.6436 | 1 | 69.6436 | 0.142088 | 0.7217 |
| B-Acetonitrile | 958.7383 | 1 | 958.7383 | 1.956027 | 0.2208 |
| C-Silica-C18 | 828.937 | 1 | 828.937 | 1.691205 | 0.2502 |
| AB | 226.2317 | 1 | 226.2317 | 0.46156 | 0.5271 |
| AC | 4527.002 | 1 | 4527.002 | 9.236032 | 0.0288 |
| BC | 2413.757 | 1 | 2413.757 | 4.924569 | 0.0772 |
| A^2 | 33488.61 | 1 | 33488.61 | 68.32378 | 0.0004 |
| B^2 | 46695.19 | 1 | 46695.19 | 95.26796 | 0.0002 |
| C^2 | 6322.711 | 1 | 6322.711 | 12.89965 | 0.0157 |

The JMP software also applied to calculate the variables effect of the whole experiment on the analytes recovery. **Figure 4.15** shows the JMP calculation, Plato factor effect, which displays the graph of the variables effect on the total recovery. When the graph more than the reference line, indicates that it has significant effect.

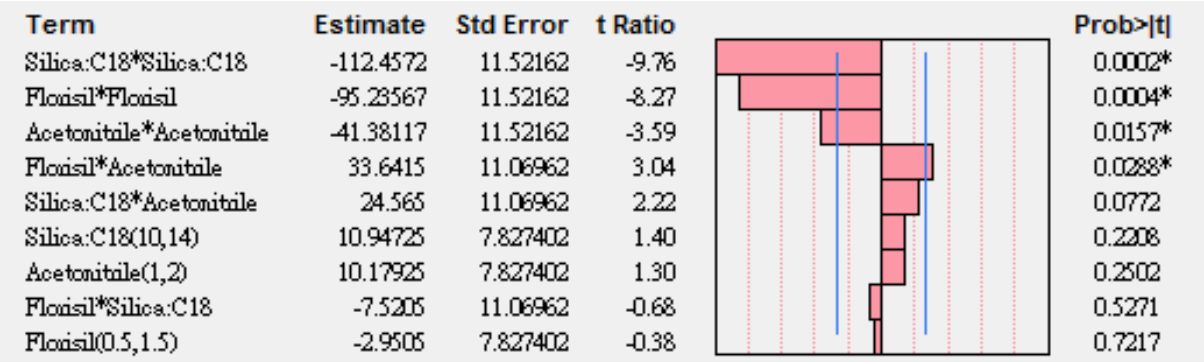



**Figure 4.15 Plato factor effect of JMP calculation**

**Figures 4.16-4.18** show the residual plots. These residual plots was used to assess and validate the regression model, whether it was appropriate the data or not. **Figure 4.16** displays the normal plot of residuals, **Figure 4.17** shows the residuals versus run number and **Figure 4.18** shows the residual versus predicted. If the resulting plot in the **Figure 4.16** is approximately linear, assumed that the error terms are normally distributed. The random plot pattern shown in **Figure 4.17** is indicated a good fit for a linear model. The residuals plotted against the predicted values in the **Figure 4.18** were randomly scattered, showed that the data was fit with the regression model.

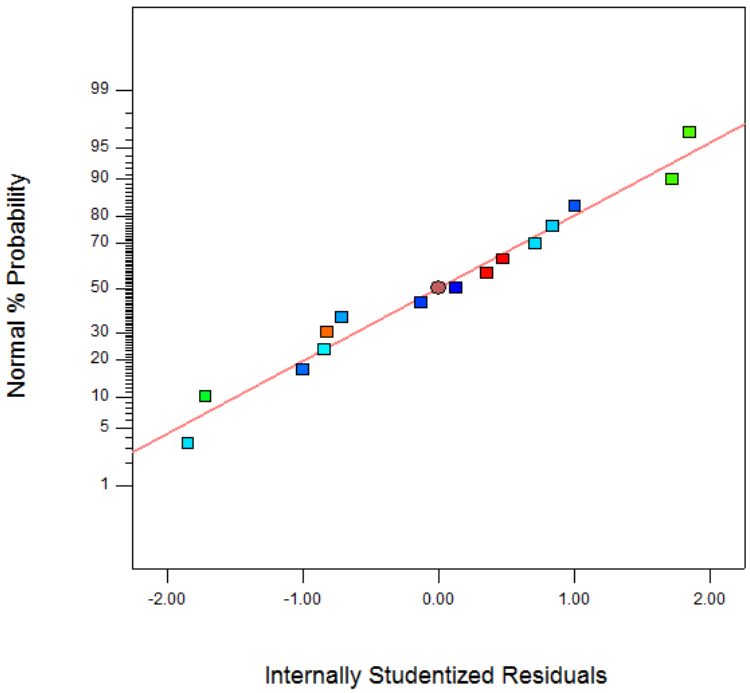



**Figure 4.16 Normal plot of residuals**

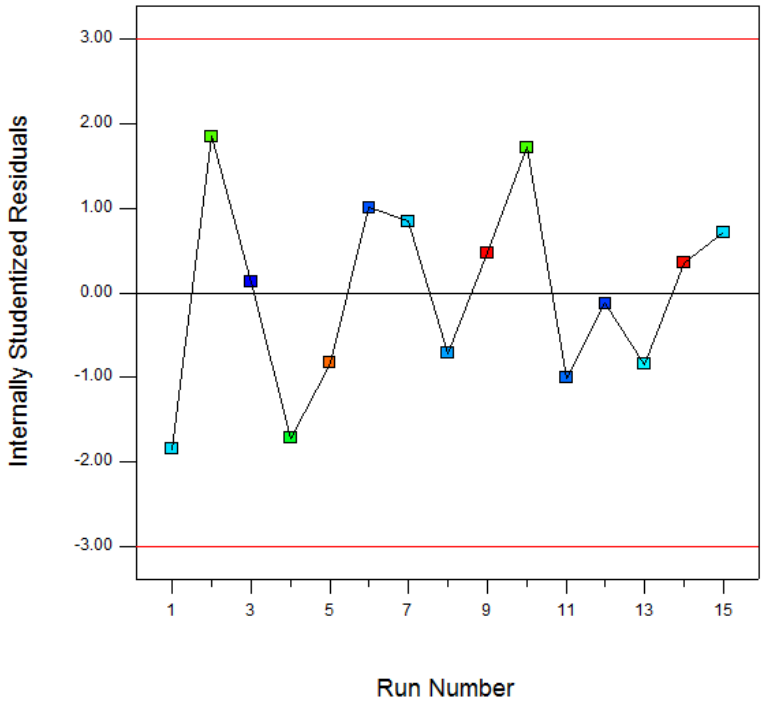



**Figure 4.17 The residuals versus run number**

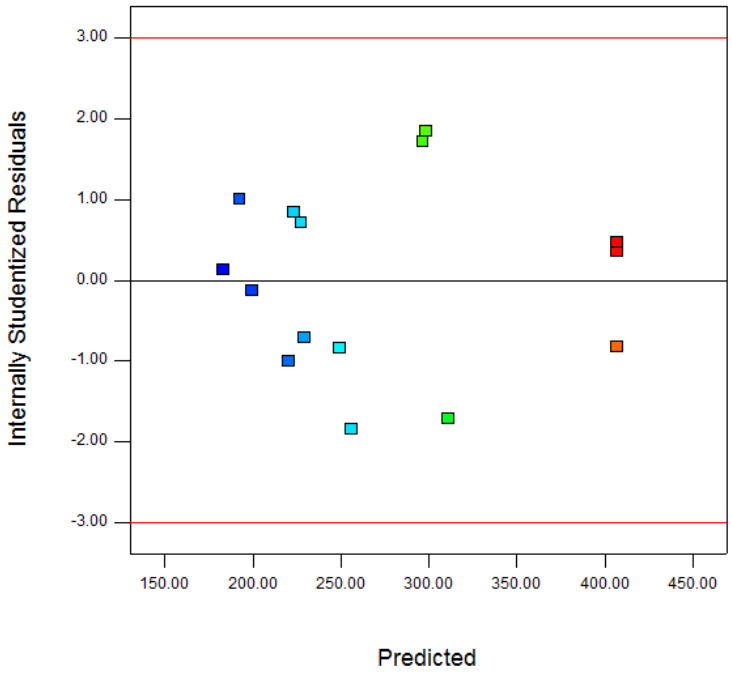



**Figure 4.18 The residuals versus predicted**

### 4.3 Method Performance and Validation

In order to determine the feasibility and efficiency of the optimized MSPD method coupled with on-line acylation GC-MS, the analytical characteristic in term of linearity in the

response of the GC-MS system, repeatability, reproducibility, LODs, and LOQs were investigated.

### 4.3.1 Linearity

#### 4.3.1.1 Calibration Curve

A large number of analytical methods require the calibration of an instrument. This typically involves the preparation of a set of standards containing a known amount of the analyte of interest, measuring the instrument response for each standard and establishing the relationship between the instrument response and analyte concentration. A calibration curve demonstrates the relationship between instrument response and concentration. This relationship is usually linear and used to transform measurements made on test samples into estimates of the amount of analyte present. Calibration is the key to accurate data.

Most calibration curves are based on a linear relationship that can be expressed using the equation for a straight line, $y = mx + b$. In this equation, y is the instrument response, x is the concentration, m represents the slope of the line, and b is the y-intercept. To make certain that a curve is linear, correlation coefficient ($R^2$) is used.

The linearity of the response of the GC-MS system in this study was evaluated at six concentration level in the range 4 to 500 ng/g (i.e., 4, 10, 50, 100, 250, and 500 ng/g), each divided by 10 ng/g of internal standard. **Figures 4.16 – 4.19** display the calibration curve of MP, EP, PP, BP and also their correlation coefficient, respectively. **Table 4.7** summarizes the results of the calibration curves. A good linearity was confirmed by the $R^2$ value of MP, EP,

PP, BP which are more than 0.998. It means that they are good correlation between the instrument response and analytes concentration.

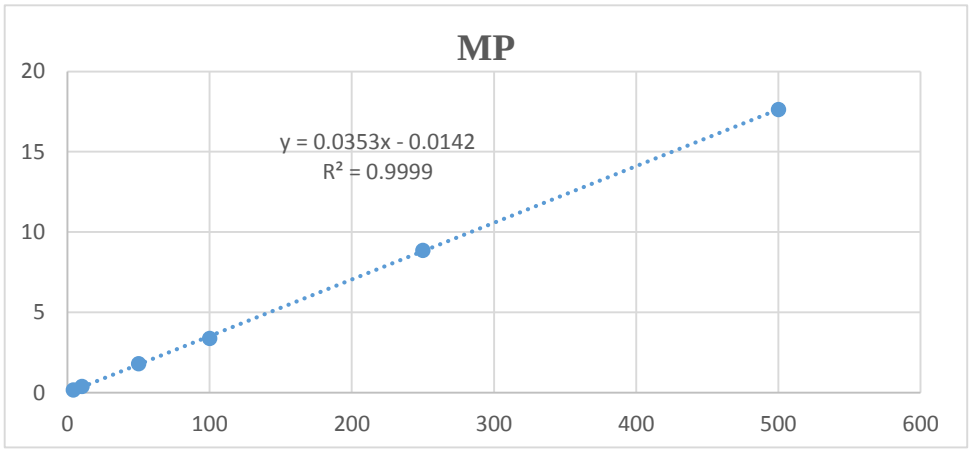



**Figure 4.19 Calibration curve of Methyl Paraben**

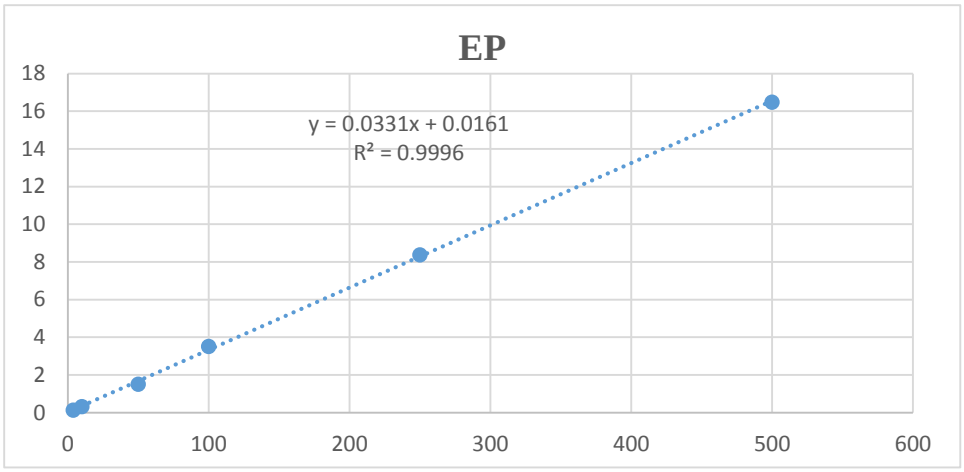



**Figure 4.20 Calibration curve of Ethyl Paraben**

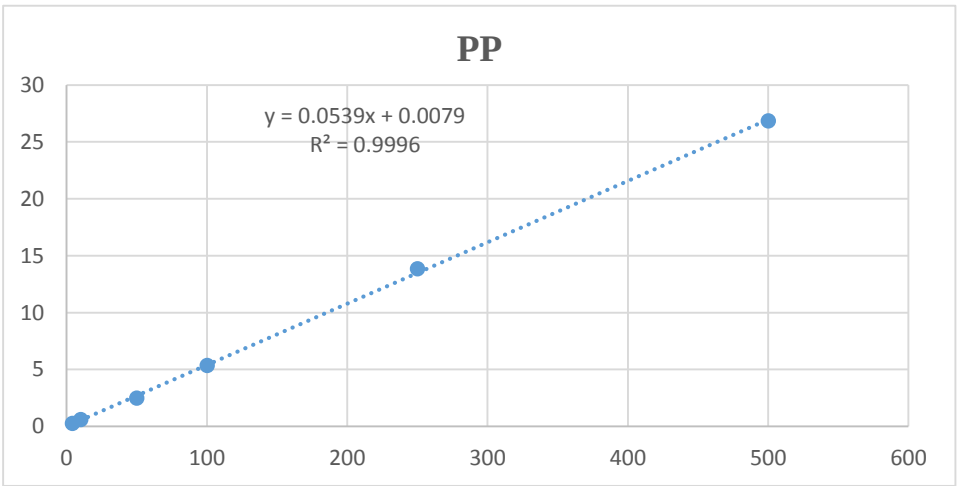



**Figure 4.21 Calibration curve of Propyl Paraben**

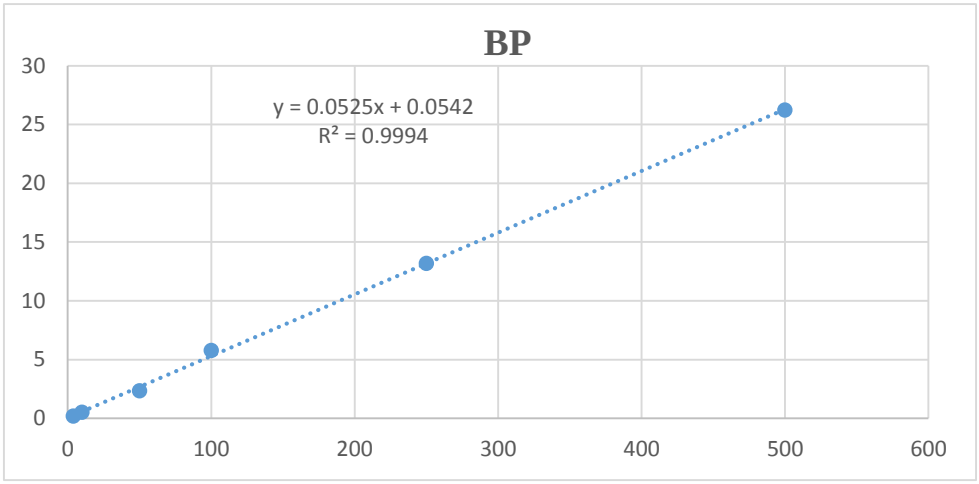



**Figure 4.22 Calibration curve of Butyl Paraben**

**Table 4.7 Linear range, equation, and correlation coefficient of the analytes**

| Analytes | Linear Range (ng/g) | Equation | $R^2$ |
|---|---|---|---|
| MP | 4-500 | y = 0.0353x - 0.0142 | 0,9999 |
| EP | 4-500 | y = 0.0331x + 0.0161 | 0,9996 |
| PP | 4-500 | y = 0.0539x + 0.0079 | 0,9996 |
| BP | 4-500 | y = 0.0525x + 0.0542 | 0,9994 |

### 4.3.1.2 Mandel Fitting Test

The linearity was insured by Mandel's fitting test. Mandel's fitting test was confirmed by F-value which calculate using this following formula:

$$F = \frac{S_{y.x}^2 \cdot (n-2) - S_{y.x,2}^2 \cdot (n-3)}{S_{y.x,2}^2}$$

Mandel Fitting test experiment applied six concentration points of calibration curve (n), and 3 degrees of freedom. The theoretical F-value $_{(95\%,\ 1,3)}$ is 10.128. The results show in **Table 4.8.** The experimental F-value of four analytes were less than the theoretical F value. It suggest that the experimental data was fit with the linear regression model.

**Table 4.8 Mandel Test**

| Compound | $\hat{F}_{experimental}$ | $F_{critical}$ |
|---|---|---|
| MP | 0,183 | |
| EP | 4,614 | $F_{(95\%,1,3)}$= **10.128** |
| PP | 2,744 | |
| BP | 1,431 | |

### 4.3.1.3 The Lack-of-fit (LOF) test

Clarification of the calibration curve also performed by Lack-of-fit (LOF) test model. The LOF test is carry out by ANOVA based on the equation that has been mentioned in the session 2.6.2. The LOF test was establish by six concentration point of calibration curve (k) which each point was repeated three times (ni), and total of experiments is 18 (n). The results are show in **Table 4.9**. The F-value is less than the theoretical F-value $_{(95\%,\ 4,12)}$ = 3.259 indicating that the experimental data was fit with the linear regression model.

**Table 4.9 The Lack of Fit (LOF) test**

| Compound | $\hat{F}_{experimental}$ | $F_{critical}$ |
|---|---|---|
| MP | 0,00033 | |
| EP | 0,00096 | $F_{(95\%,4,12)}$= **3.259** |
| PP | 0,00093 | |
| BP | 0,00168 | |

### 4.3.2 Matrix effect

The matrix effect was also evaluated in this study. The step of this experiment was dried the standard mixture solution with the gentle stream of nitrogen and then analyzed by GC-MS.

The standard mixture concentration for matrix effect test are 10, 50, 100, 250, 500 ng /g. The GC-MS analysis data then compare with the calibration curve. The ± t critical $_{(95\%, 6)}$ is ± 2.44 and F critical $_{(95\%,3,3)}$ is 9.27. **Table 4.10** presented the t experimental value and **Table 4.11** presented experimental values of F-test $_{(95\%, 6)}$ which performed by the JMP software. These data show that t-test and F-test were less than t and F critical values. It represent that matrix effect is not significant. It means that the interference did not affect quantitative calibration curve and the calculation value for sample concentration was on the exact value. It was confirmed by the two tests below.

**Table 4.10 Matrix Effect t-test**

| Compound | $\hat{t}_{experimental}$ | $t_{critical}$ |
|---|---|---|
| MP | 0,121 | |
| EP | 1,000 | $\pm t_{(95\%,6)} = \pm 2.44$ |
| PP | 0,306 | |
| BP | 0,128 | |

**Table 4.11 Matrix Effect F-test**

| Compound | $\hat{F}_{experimental}$ | $F_{critical}$ |
|---|---|---|
| MP | -0,8955 | |
| EP | 0,5524 | $F_{(95\%,3,3)}=9.27$ |
| PP | -0,9996 | |
| BP | -0,9827 | |

### 4.3.3 LOQ (Limit of Quantification), LOD (Limit of Detection), precision and accuracy

Precision of the method was evaluated with relative standard deviation (RSD) under repeatability (intra-day) condition performed 5 extraction in same day and reproducibility

(inter-day) condition performed 20 extraction in four different days. Accuracy was defined by average of recovery for each analyte in spiked sample. Instrumental quantification limit and detection limit were determined by means of MSPD coupled with on-line acylation GC-MS which defined for signal-to-noise (S/N) ratio of 10 and 3, respectively. **Table 4.12** summarizes the LOQ, LOD, repeatability, reproducibility, and recovery of the analytes.

**Table 4.12 LOQ, LOD, repeatability and reproducibility of the analytes**

| Analytes | LOQ (ng/g) | LOD (ng/g) | Intra-day (n=5) | Inter-day (n=20) |
|---|---|---|---|---|
| MP | 0.2 | 0.06 | 107 (6) [a] | 111 (5) [a] |
| EP | 0.4 | 0.12 | 93 (6) | 102 (8) |
| PP | 0.4 | 0.12 | 112 (3) | 106 (6) |
| BP | 1.0 | 0.30 | 101 (7) | 102 (3) |

[a] Relative standard deviation (%RSD) are given in parentheses (n=3)

The relative standard deviations of intra-day and inter-day analysis are less than 8% that indicate that the method has a good precision. The method accuracy was ensured by recovery of the analytes which in ranged 93 - 112%. This result confirm that MSPD optimized method obtain good accuracy. The low LOQ and LOD of the analytes show that this method can be used in low analytes concentration in real sample.

**4.4 Method application for real samples analysis**

The optimized MSPD method was applied to assess the marketed seafoods in order to evaluate method feasibility and applicability. Commercially seafoods samples used in this experiment were purchased in Chungli market. They are tilapia, perch, cod, and shrimp. Four kinds of seafoods samples were pretreated as described in section 3.2.4 and 3.2.5 **Figures 4.20-4.23** exhibit on-line acylation GC-MS chromatograms for (a) a non-spiked, (b) a spiked shrimp, tilapia, cod and perch sample. The peak intensity of spiked sample were higher than peak

intensity of unsipked sample. **Table 4.13** lists the recovery of spiked sample under 10 ng/g of final concentrations and the concentrations of parabens detected in 4 seafoods samples.

**Table 4.13 Unspike and spike of real samples**

| Samples | Analytes | | | |
|---|---|---|---|---|
| | **MP** | **EP** | **PP** | **BP** |
| *Shrimp* | | | | |
| Unspike conc. (ng/g) [a] | 10.75 (2) [c] | 8.01 (1) | 5.45(4) | 7.44 (1) |
| Spike recovery (%) | 109.67 [b] (1) [c] | 98.59 (0.3) | 83.80 (10) | 98.07 (3) |
| *Cod* | | | | |
| Unspike conc. (ng/g) | 11.47 (0.7) | 5.63 (7) | 6.81 (0.1) | 5.60 (9) |
| Spike recovery (%) | 102.56 (4) | 95,60 (1) | 81.28 (2) | 94.99 (0.5) |
| *Tilapia* | | | | |
| Unspike conc. (ng/g) | 6.23 (4) | 5.47 (2) | n.d. | 5.02 (5) |
| Spike recovery (%) | 77.59 (8) | 95.98 (3) | 74.94 (8) | 88.58 (4) |
| *Perch* | | | | |
| Unspike conc. (ng/g) | 18.53 (7) | 15.12 (3) | 4.89 (9) | 6.24 (3) |
| Spike recovery (%) | 107.47 (7) | 87.98 (1) | 116.09 (2) | 104.97 (1) |

n.d., not detected at LOQ as listed at Table 4.11
[a] Original concentration (ng/g) of analytes found in fish samples (n=3)
[b] Spiked mean recovery (%, n=3) at final concentration 10 ng/g for each analyte.
[c] Relative standard deviation (%RSD)

**Table 4.14** shows the method comparison between MSPD and other method for parabens determination. Compared to others experiment for parabens determination mentioned in **Table 4.14**, optimized MSPD method had advantages since the procedure was organized in single step for extraction and clean-up, thus made MSPD method was simple and more save-time. MSPD also offered the less used of organic solvent as it used only 12 mL of acetonitrile

for extraction procedure and about 50 µL methol for the other next step. However, optimized MSPD method produced higher LOD than QuEChERS method explained by Jakimska (2013).

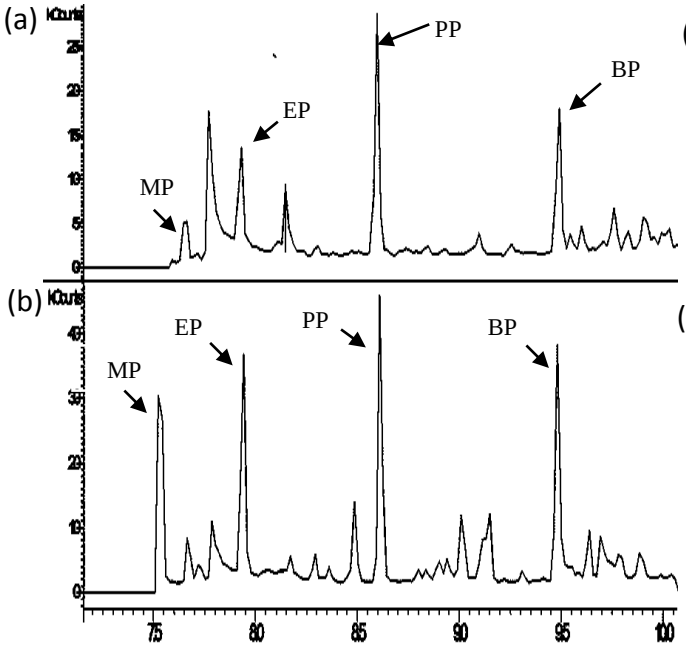



**Figure 4.23 GC-MS chromatogramps for (a) a non spiked and (b) a spiked shrimp sample**

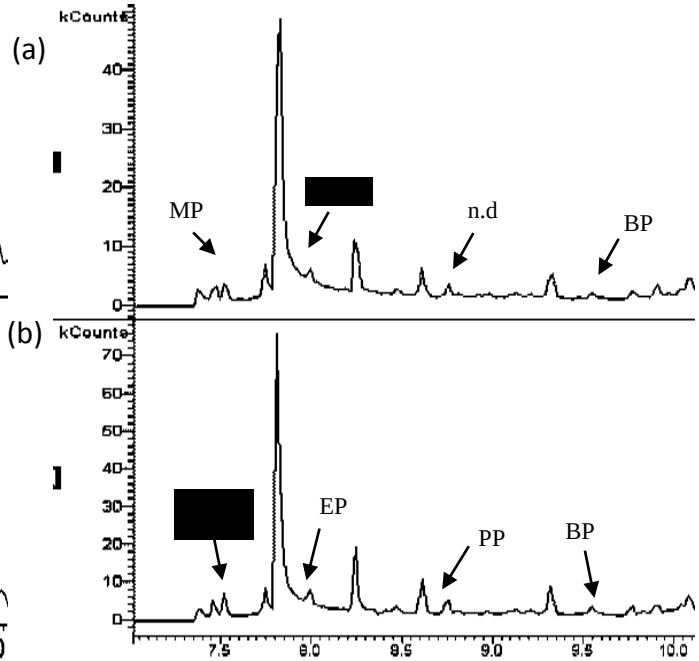



**Figure 4.24 GC-MS chromatogramps for (a) a non spiked and (b) a spiked tilapia**

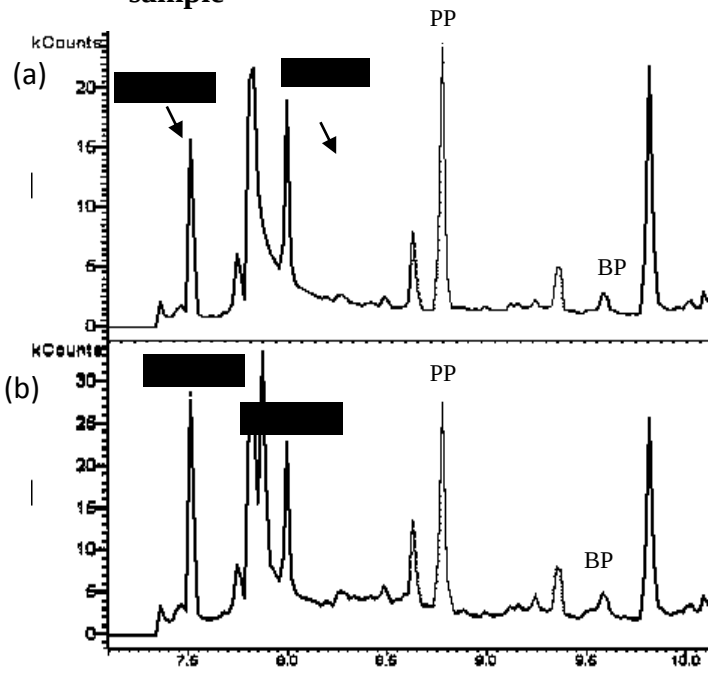



**Figure 4.25 GC-MS chromatogramps for (a) a non spiked and (b) a spiked cod sample**

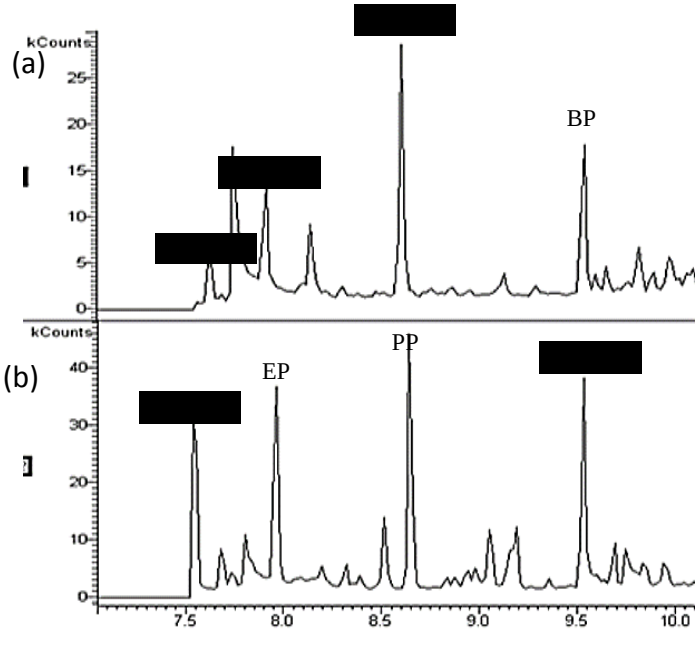



**Figure 4.26 GC-MS chromatogramps for (a) a non spiked and (b) a spiked perch sample**

**Table 4.14 Comparison Table of MSPD and other pretreatment methods for parabens determination**

| Compound | Extraction and Preparation | Sample amount (gram) | Analytes volume (μL) | LOD | Reference |
|---|---|---|---|---|---|
| MP, EP, PP, BP | High Speed Solvent Extraction (HSSE) | 5.0 | 10 μL | 1.0-15 pg/g | (Kim et al., 2011) |
| MP, EP, PP, BP | Pressurized Liquid Extraction (PLE) coupled with SPE clean-up | 1.0 | 10 μL | 3.3 μg/kg | (Han et al.,2016) |
| MP, EP, PP | QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction | 1.0 | 5 μL | 0.04 ng/g | (Jakimska et al., 2013) |
| MP, EP, PP, BP | MSPD | 0.5 | 10 μL | 0.06-0.30 ng/g | This study |

# CHAPTER V

# Conclusions

## 5.1 Conclusions

Matrix Solid-Phase Dispersion (MSPD) method coupled with on-line acylation for parabens determination in seafoods has been successfully optimized. Based on this study, it can be concluded that :

- Compared to off-line derivatization, on-line acylation derivatization provides time-saving and environmentally-friendly that can be introduced to hot injection port without requiring further treatment process.
- MSPD appears to be a rapid, simple and effective method for sample pre-treatment to determine parabens in seafood samples.
- MSPD method presents a good precision, low detection limit and high recovery for parabens in real seafood samples.

## Reference


- Anderson, M.J. & Whitcomb P.J. 2004. RSM Simplified: Optimizing Processes Using Response Surface Methods for Design of Experiments. Productivity Press, USA., 292
- Azzouz, A., Rascón, A. J. & Ballesteros, E. 2016. Simultaneous determination of parabens, alkylphenols, phenylphenols, bisphenol A and triclosan in human urine, blood and breast milk by continuous solid-phase extraction and gas chromatography–mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis,* 119**,** 16-26.
- Baker, S.A. 2007. Matrix solid phase dispersion (MSPD). *Journal of Biochemical and Biophysical Methods,* 70, 151-162
- Barcelo, D., Grathwohl, P., Jones, K., Totsche. 2003. EUGRIS: Futher Description Emerging Pollutants.
- Bizkarguenaga, E., Iparragirre, A., Navarro, P., Olivares, M., Prieto, A., Vallejo, A. & Zuloaga, O. 2013. In-port derivatization after sorptive extractions. *Journal of Chromatography A,* 1296**,** 36-46.
- Bowden, J. A., Colosi, D. M., Mora-Montero, D. C., Garrett, T. J. & Yost, R. A. 2009. Enhancement of chemical derivatization of steroids by gas chromatography/mass spectrometry (GC/MS). *Journal of Chromatography B,* 877**,** 3237-3242.
- Canosa, P., Rodríguez, I., Rubí, E., Ramil, M. & Cela, R. 2008. Simplified sample preparation method for triclosan and methyltriclosan determination in biota and foodstuff samples. *Journal of Chromatography A,* 1188**,** 132-139.
- DOD. 2011. *Chemical & Material Risk Management Program* [Online]. Available: www.denix.osd.mil/cmrmd/ECMR/ECProgramBasics.cfm. Accessed at 16th March 2016

- Ferreira, A. M. C., Möder, M. & Laespada, M. E. F. 2011. Stir bar sorptive extraction of parabens, triclosan and methyl triclosan from soil, sediment and sludge with in situ derivatization and determination by gas chromatography–mass spectrometry. *Journal of Chromatography A,* 1218**,** 3837-3844.
- Ferreira, S. L. C., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandão, G. C., Da Silva, E. G. P., Portugal, L. A., Dos Reis, P. S., Souza, A. S. & Dos Santos, W. N. L. 2007. Box-Behnken design: An alternative for the optimization of analytical methods. *Analytica Chimica Acta,* 597**,** 179-186.
- Frenich, A. G., Vidal, J. L. M., Moreno, J. L. F. & Romero-González, R. 2009 Compensation for matrix effects in gas chromatography–tandem mass spectrometry using a single point standard addition. *Journal of Chromatography A,* 1216**,** 4798–4808.
- Gao, Y., Ji, Y., Li, G. & An, T. 2016. Theoretical investigation on the kinetics and mechanisms of hydroxyl radical-induced transformation of parabens and its consequences for toxicity: Influence of alkyl-chain length. *Water Research,* 91**,** 77-85.
- Gomes R.L., Lester J.N. 2003. Endocrine Disrupters in Receiving Waters, Chapter. 6, CRC Press, Boca Raton, 203-206
- Guillemin, G. G. C. 1988. Quantitative gas chromatography for laboratory analyses and on-line process control. *Journal of Chromatography Library.* New York, USA: Elsevier.
- Han, Chao., Xia, Biqi., Chen, Xiangzhun., Shen, Jincan., Miao,Qian., & Shen,Yan. 2016. Determination of four paraben-type preservatives and three benzophenone-type ultraviolet light filters in seafoods by LC-QqLIT-MS/MS. *Journal of Chromatography A.* 194. 1199-1207
- Heshmatollah, Leardi, R. & Jalali-Heravi, M. 2004. Experimental Design in Analytical Chemistry–Part I: Theory. *Journal of AOAC International,* 97**,** 1-10.

- Hines, E. P., Mendola, P., Von Ehrenstein, O. S., Ye, X., Calafat, A. M. & Fenton, S. E. 2015. Concentrations of environmental phenols and parabens in milk, urine and serum of lactating North Carolina women. *Reproductive Toxicology,* 54**,** 120-128.
- Jakimska, A., Huerta, B., Barganska, Z., 2013, Development of a liquid chromatography–tandem mass spectrometry procedure for determination of endocrine disrupting compounds in fish from Mediterranean rivers. *Journal of Chromatography A*, 1306, 44-58
- Kajornkavinkul, S., Punrat, E., Siangproh, W., Rodthongkum, N., Praphairaksit, N. & Chailapakul, O. 2016. Graphene/polyvinylpyrrolidone/polyaniline nanocomposite-modified electrode for simultaneous determination of parabens by high performance liquid chromatography. *Talanta,* 148**,** 655-660.
- Kang, H.-S., Kyung, M.-S., Ko, A., Park, J.-H., Hwang, M.-S., Kwon, J.-E., Suh, J.-H., Lee, H.-S., Moon, G. I., Hong, J.-H. & Hwang, I. G. 2016. Urinary concentrations of parabens and their association with demographic factors: A population-based cross-sectional study. *Environmental Research,* 146**,** 245-251.
- Kim, J.-W., Ramaswamy, B. R., Chang, K.-H. & Tanabe, S. 2011. Multiresidue analytical method for the determination of antimicrobials, preservatives, benzotriazole UV stabilizers, flame retardants and plasticizers in fish using ultra high performance liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography A,* 22**,** 11-20.
- Leardi, R. 2009. Experimental design in chemistry: A tutorial. *Analytica Chimica Acta,* 652**,** 161-172.
- McEwen, F. K. B. L. C. 1996. *Gas Chromatography and Mass Spectrometry, a practical guide*, California, Academic Press.

- Montgomery, D. C. 2005. Design and Analysis of Experiments : Response surface method and designs. New Jersey: John Wiley and Sons, Inc.
- Moos, R. K., Koch, H. M., Angerer, J., Apel, P., Schröter-Kermani, C., Brüning, T. & Kolossa-Gehring, M. 2015. Parabens in 24 h urine samples of the German Environmental Specimen Bank from 1995 to 2012. *International Journal of Hygiene and Environmental Health,* 218**,** 666-674.
- Norton, T. J., Fallon, R., Grant, J. & Sun, D.-W. 2009. *Building and Environment,* 45**,** 983-995.
- Ocana-Gonzales, J.A., Villar-Navarro, M., Ramos-Payan, M., Torres, F., Bello-Lopez, M.A. 2015. New developments in the extraction and determination of parabens in cosmetics and environmental samples. *Analytica Chimica Acta,* 858, 1–15
- Oehlert, Gary W. 2000. Design and analysis of experiments: response surface design. New York: W.H. Freeman and Company
- Oishi, S. 2002. Effects of propyl paraben on the male reproductive system. *Food and Chemical Toxicology,* 40**,** 1807-1813.
- Oishi, S. 2004. Lack of spermatotoxic effects of methyl and ethyl esters of p-hydroxybenzoic acid in rats. *Food and Chemical Toxicology,* 42**,** 1845-1849.
- Otto, M., 2007. Chemometrics-Statistics and Computer Application in Analytical Chemistry. 2nd Edn., Wiley-VCH, Weinheim, Germany, 328
- Poole, C. F. 2012. *Gas Chromatography*, USA, Elsevier, 124-135
- Prusakiewicz et al. 2006.Comparison of skin esterase activities from different species. *Pharmaceutical Research*., 23, 1517–1524
- Ramaswamy, Rajendran, B., Kim, J., Isobe, T., Chang, Kwang. 2011, Determination of preservative and antimicrobial compounds in fish from Manila Bay, Philippines using

ultra high performance liquid chromatography tandem mass spectrometry and assessment of human dietary exposure, *Journal of Chromatography A*, 192, 1739-1745

- Rocío-Bautista, P., Martínez-Benito, C., Pino, V., Pasán, J., Ayala, J. H., Ruiz-Pérez, C. & Afonso, A. M. 2015. The metal–organic framework HKUST-1 as efficient sorbent in a vortex-assisted dispersive micro solid-phase extraction of parabens from environmental waters, cosmetic creams, and human urine. *Talanta,* 139**,** 13-20
- Shah, R.J. Verma, R.J. 2011. Butyl p-hydroxybenzoic acid induces oxidative stress in mice liver, *Acta poloniae pharmaceutica.*, 68, 875–879
- Shanmugam, G., Ramaswamy, B. R., Radhakrishnan, V. & Tao, H. 2010. GC–MS method for the determination of paraben preservatives in the human breast cancerous tissue. *Microchemical Journal,* 96**,** 391-396.
- Skoog, D. A., Holler, F. J., Nieman, T. A. 2002. Principles of instrumental analysis. 5th ed., Thomoson Learning
- Soni, M. G., Carabin, I. G. & Burdock, G. A. 2005. Safety assessment of esters of p-hydroxybenzoic acid (parabens). *Food and Chemical Toxicology,* 43**,** 985-1015.
- Tsai, D.-Y., Chen, C.-L. & Ding, W.-H. 2014. Optimization of matrix solid-phase dispersion for the rapid determination of salicylate and benzophenone-type UV absorbing substances in marketed fish. *Food Chemistry,* 154**,** 211-216.
- Xu, J.J., Yang, R., 2016, Application of ionic liquids for elution of bioactive flavonoid glycosides from lime fruit by miniaturized matrix solid-phase dispersion, *Food Chemistry,204,* 167–175
- Wang, Q., Ma, L., Yin, C.-R. & Xu, L. 2013. Developments in injection port derivatization. *Journal of Chromatography A,* 1296**,** 25-35.

- Wu, J., Hu, R., Yue, J., Yang, Z. & Zhang, L. 2009. Determination of fecal sterols by gas chromatography–mass spectrometry with solid-phase extraction and injection-port derivatization. *Journal of Chromatography A,* 1216**,** 1053-1058.
- Zhu, L. 2015. Rejection of Organic Micropollutants by Clean and Fouled Nanofiltration Membranes. *Journal of Chemistry,* 2015**,** 9.