Quantitative Analysis of 3-Monochloropropane-1, 2-diol (3-MCPD) in Soy Sauce by Chromatographic Techniques

Nittaya Sudsiri

Master of Science Thesis in Analytical Chemistry

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Thesis Title	Quantitative Analysis of 3-Monochloropropane-1, 2-diol (3-MCPD) in Soy Sauce by Chromatographic Techniques
Author	Miss Nittaya Sudsiri
Major Program	Analytical Chemistry

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The Graduate School, Prince of Songkla University, has approved this thesis as a partial fulfillment for the Master of Science degree in Analytical Chemistry.

.....
(Surapon Arrykul, Ph.D)
Associate Professor and Dean
Graduate School

ชื่อวิทยานิพนธ์	ปริมาณวิเคราะห์สาร 3-โมโนคลอโรโพรเพน-1, 2-ไดออล (3-เอ็มซีพีดี) ในซอสปรุงรสถั่วเหลือง โดยเทคนิคโครมาโทกราฟี
ผู้เขียน	นางสาวนิตยา สุดศิริ
สาขาวิชา	เคมีวิเคราะห์
ปีการศึกษา	2546

บทคัดย่อ

การวิเคราะห์เชิงคุณภาพและเชิงปริมาณของสาร 3-โมโนคลอโรโพรเพน-1, 2-ไดออล หรือ 3-เอ็มซีพีดี (3-Monochloropropane-1,2-diol, 3-MCPD) ในตัวอย่างซอสปรุงรสใช้วิธีทำให้ 3-เอ็มซีพีดี เป็นอนุพันธ์ โดยกรดฟีนิลบอรอนิก (Phenylboronic acid) เป็นสารก่ออนุพันธ์ ในการเตรียมอนุพันธ์ของ 3-เอ็มซีพีดี อุณหภูมิ และเวลาที่เหมาะสม คือ 90 องศาเซลเซียส และ 30 นาที ตามลำดับ หลังจากที่ปฏิกิริยาเกิดขึ้นสมบูรณ์แล้ว ผลิตภัณฑ์ที่เกิดขึ้นจะถูกสกัดขึ้นไปอยู่ในชั้นของสารละลาย ซึ่งใช้เวลาในการสกัด 20 วินาทีและยืนยันโครงสร้างของอนุพันธ์ 3-เอ็มซีพีดี โดยใช้ไอ อาร์ สเปกโตรเมตรี (IR spectrometry) และแก๊สโครมาโทกราฟีร่วมกับตัวตรวจวัดแมสสเปกโทรเมตรี การวิเคราะห์อนุพันธ์ของ 3-เอ็มซีพีดี ด้วยเทคนิคแก๊สโครมาโทกราฟี ใช้คาปิลลารีคอลัมน์ชนิดเอชพี 5 (HP 5) ยาว 30 เมตร ขนาดเส้นผ่านศูนย์กลาง 0.32 มิลลิเมตร และความหนาของฟิล์ม 0.25 ไมโครเมตรร่วมกับตัวตรวจวัดชนิดเฟลมไอออไนเซชัน

สภาวะที่เหมาะสมของแก๊สโครมาโทกราฟี ร่วมกับตัวตรวจวัดเฟลมไอออไนเซชัน ได้แก่ อัตราการไหลของแก๊สพา (ฮีเลียม) 3.0 มิลลิลิตรต่อนาที โปรแกรมอุณหภูมิคอลัมน์เริ่มต้นจาก 90 องศาเซลเซียส จากนั้นเพิ่มอุณหภูมิด้วยอัตรา 15 องศาเซลเซียสต่อนาที จนถึง 270 องศาเซลเซียส อุณหภูมิหัวฉีด 250 องศาเซลเซียส และอุณหภูมิของตัวตรวจวัด 280 องศาเซลเซียส ที่สภาวะที่เหมาะสมนี้ ขีดกำจัดของการตรวจวัดของสาร 3-เอ็มซีพีดี คือ 0.5 ไมโครกรัมต่อมิลลิลิตร โดยมีช่วงความเป็นเส้นตรง 0.5 ไมโครกรัมต่อลิตร ถึง 1000 ไมโครกรัมต่อลิตร ด้วยค่าสหสัมพันธ์เชิงเส้นตรง (R^2) มากกว่า 0.99 และให้ความแม่นยำสูง โดยมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์น้อยกว่า 4%

ในการศึกษาเทคนิคการเตรียมตัวอย่าง ใช้เทคนิคการสกัดด้วยคอลัมน์ (column chromatographic extraction) สาร 3-เอ็มซีพีดีจะถูกสกัดออกจากตัวอย่าง โดยใช้อัลตร้าโซนิกเป็นเวลา 15 นาที และถูกดูดซับด้วยซิลิกาเจล 60 ขนาด 230-400 เมช ในคอลัมน์ที่มีเส้นผ่านศูนย์กลาง 20 มิลลิเมตร ความยาว 400 มิลลิเมตร สารตัวอย่างจะถูกชะด้วยเอทิลอะซิเตต 150 มิลลิลิตร ด้วย

อัตราการไหล 8.0 มิลลิลิตรต่อนาที ในการเตรียมตัวอย่างครั้งนี้ไม่จำเป็นต้องมีการทำให้สะอาด ซึ่งเปอร์เซ็นต์การได้กลับคืนของ 3-เอ็มซีพีดี 95±9 เปอร์เซ็นต์

การวิเคราะห์ 3-เอ็มซีพีดีในตัวอย่างซอสปรุงรสจำนวน 5 ตัวอย่างที่ได้จากการสุ่มจากห้างสรรพสินค้าใน อำเภอหาดใหญ่ จังหวัดสงขลา โดยการวิเคราะห์เชิงปริมาณที่ใช้นอร์มอล-เฮกซะเดคเคน (n-hexadecane) เป็นอินเทอร์นอล สแตนดาร์ด (Internal standard) พบ 3-เอ็มซีพีดีปนเปื้อนอยู่ในช่วงที่ไม่สามารถตรวจวัดได้ ถึง 57.07±8.03 ไมโครกรัมต่อกรัม

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Abstract

Qualitative and Quantitative Analysis of 3-Monochloropropane-1, 2-diol (3-MCPD) in soy sauce were performed by derivatized 3-MCPD using phenylboronic acid as derivatizing reagent. The optimum temperature and time of derivative reaction were 90 °C and 30 minutes respectively. After the reaction was completed, the analyte was extracted for 20 seconds to a hexane layer. The derivative of 3-MCPD was analysed by gas chromatography equipped with a 30 m × 0.25 mm i.d. × 0.32 μm film thickness HP 5 capillary column and flame ionization detector and confirmed by IR spectrometry and gas chromatography with mass spectrometry.

Optimum conditions for gas chromatograph-flame ionization were, carrier gas (He) flow rate 3.0 ml min^{-1}, column temperature programming –initial temperature 90 °C for 1 minute, ramped to 270 °C at 15 °C min^{-1} , injector temperature 250 °C and detector temperature 280 °C. At optimum conditions the system provided a limit of detection of 0.5 μg ml^{-1} and a linear dynamic range of 0.5 -1000 μg ml^{-1} with linear regression (R^2) of greater than 0.99 and the relative standard deviation (%RSD) of less than 4%

Column chromatographic extraction was used for sample preparation. 3-MCPD was extracted in an ultrasonic bath for 15 minutes and adsorbed on silica gel 60, 230-400 mesh in a column of 20 mm i.d. × 400 mm length. The analyte was eluted by ethyl acetate at 150 ml with a flow rate of 8.0 ml min^{-1}.

In this study, the sample preparation did not required any clean up and gave a high percentage recovery of 95±9 %

Five brands of soy sauce were sampled from the local department store in Hat Yai, Songkhla. Internal standard using n-hexadecane was applied for quantitative analysis of 3-MCPD in soy sauce samples. Contaminations of 3-MCPD in these samples were found in the range of non-detectable to 57.07±8.03 $\mu g\ g^{-1}$.

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The completion of this thesis would be quite impossible without the help of many people, whom I would like to thank.

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Nittaya Sudsiri

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Chapter 1

Introduction

1.1 Introduction

Food is a biological necessity for human survival. At present, there are varieties of foods and each type of food has different ingredients. Soy sauce is one of the savories in several types food. It is made from soy bean and fermented by microbes and acid hydrolysis. Soy sauce has been traditionally produced in Asia by the fermentation process for thousands of years. The used of acid-hydrolyzed vegetable protein (acid-HVP) reaction to produce a seasoning sauce is more recent and has been used in Thailand for the last forty years.

Thailand is one of the world largest exporters of soy and seasoning sauces. There are 62 producers with a production of approximately 44,000 metric tons, some of which the following amount of seasoning sauce were exported to the European Union (EU) (Table 1).

Generally, traditional soy sauce is fermented by a two-stage fermentation process for up to 6 months then, processed further, by filtering and heat treatment prior to bottling for distribution. Any further ingredients, such as flavors, caramels, sugar and salt are added just

Table 1 Amount of seasoning sauce were exported to EU (Commission of the European communities, 2002)

Years	Weight (metric tons)
1999	75.17
2000	130
2001	1.46

before the sauce is bottled. Compound soy sauce is prepared by the addition of acid-HVP to sauce that has not completed the full fermentation to accelerate the process, or by the direct adding of hydrochloric acid to soy-bean protein and water. The latter is the most common process used to produce the soy and seasoning sauces in Thailand. The time and the cost involved in the production of such compound sauce is significantly less than the traditional fermentation method. However, the used of the acid resulted in the formation of 3-Monochloropropane-1, 2-diol (3-MCPD). The level of 3-MCPD formation in such a process can be lowered by reducing the fat content of the soy bean protein, and by altering variables in the acid hydrolysis reaction, *i.e.* temperature, pressure, time and the neutralization of the acid by strong alkali following the reaction (Commission of the European communities, 2002). 3-MCPD has recently become a big issue and was reported in the news media as follows.

On June 22, 2001, soy sauce products from Thailand were banned by the European Union (EU) because they were contaminated by 3-monochloro-1, 2-propanediol or 3-chloro-1, 2-propanediol or 3-MCPD. For 3-MCPD a high concentration of 2.7-85 mg/kg was found (www.fda.moph.go.th/fda-net/html/chemical/News_ipcs6-3/mcpd.htm).

On the same day under "Soy sauce makers call for calm" in Japan Today it was written that soy sauce products imported from Thailand, China, Hong Kong, and Taiwan contained levels of 3-MCPD above the safety guidelines (www.japantoday.com/gidx/news37324.html.).

On June 23, 2001, under "UK soy sauce scare leaves bad taste" Japan Today also reported that Australia, New Zealand and Great Britain warned consumers that certain soy sauce products were contaminated by trace substances that could cause cancer if taken daily and some supermarkets in New Zealand pulled supplies from their shelves. But a producer in Thailand said that it would take at least until early 2002 for the companies to reduce levels of 3-MCPD. The Great Britain's Food Standards Agency (FSA) also singled out brands and products imported from Thailand, China, Hong Kong and Taiwan (www.japantoday .comgidx/news37499.html.).

On July 19, 2001, The Gulf news reported under "The United Arab Emirates (UAE) banned the soya products from East Asia" – The United Arab Emirates banned the import and sale of soya products and ordered the withdrawal of all stocks nationwide. The General Secretariat of Municipalities said they had imposed the ban after the products were found a carcinogenic chemical in UK when tested at excessive levels. The Britain Food Standard Agency had warned consumers to avoid certain brands of soy sauce and soya-based products from Thailand, China, Hong Kong, Taiwan and Singapore after finding unacceptable levels of 3-MCPD. Municipalities requested the shops and supermarkets to remove all Asian soya-based products, including the popular soy sauce, from shelves and also for restaurants not to serve (www.gulf-news.com/Articles/news.asp.ArticleID=22557). In "Soy sauce

contaminant is widespread in UK" on July 20, 2001 the Food Commission reported that the cancer-causing chemical found in soy sauce, 3-MCPD was also found in wide range of UK-made foods. The finding of high levels of 3-MCPD in several brands of soy sauce hit the United Kingdom national headlines in June (www.foodcomm .org.uk/soya_press.html). On August 15, 2001, under the headline "Malaysia banned cancer-risk soy sauce" the BBC reported that Malaysia's Food Standard Agency (FSA) had blocked the sale of more than 20 types of soy sauce and other popular cooking sauces since they contained 3-MCPD at unsafe levels. The cooking sauce contained 3-MCPD more than 20 parts per billion and the FSA issued a warning the consumers on cancer-risk from soy sauce in June 2001 (www.news.bbc.co.uk/1/hi/business/1492704.stm.)

Reuters reported on May 28, 2002 that the Australia New Zealand Food Authority (ANZFA) pointed out that a King brand "New soy sauce" imported from Vietnam had an unacceptably high levels of 3-MCPD, 200 times higher than the safe level. (www.planetark.com/ dailynewsstory.cfm/newsid/16167/newsDate/28-May-2002/story.htm.)

3-MCPD is the most common form of chloropropanols. Which consist of

- 3-Chloro-1, 2-propandiol (3-MCPD)
- 3-Chloro-1, 2-dihydroxypropane (a-chlorohydrin)
- 2-Chloro-1, 3-propanediol (2-MCPD)
- 1, 3-dichloro-2-propanol (DCP a-dichlorohydrine)
- 2, 3-dichloro-1-propanol (www.dmsc.moph.go,th/ webroot/Ubon/food/3-MCPD.htm.))

3-MCPD could cause cancer in animals when fed with large amounts over their lifetime. Although human usually get chloropropanols only at low levels, but it was still a concern health risk. In addition, 3-MCPD has probably been presented in foods for a long time, but scientists were only recently able to detect it reliably. 3-MCPD in soy sauce products is formed at high temperature (www3.jaring.my/kongguan/mcpd.html) due to chlorination of glycerol. This glycerol is present in fat and oil of vegetable materials (Fromberg, 2001.), or chemical hydrolysates of proteins, or hydrolysed vegetable proteins (HVP) which are produced by the hydrolysis of various proteinaceous vegetable material, for example, oilseed meals and wheat gluten, by hydrochoric acid (HCl). It has been established that HCl reacted with lipid presented in raw material used for production of HVP yielding free fatty acid, partial acrylglycerols, glycerol and other compounds. Glycerol chlorohydrins have been identified as minor products resulting from the reaction of HCl with acrylglycerols (Figure 1), phospholipids (Figure 2) and glycerol (Figure 3). Investigations focus on these glycerol monohydrins have shown that the relative proportion of 3-MCPD and 2-chloropropane-1, 3-diol (2-MCPD), is approximately 10:1.

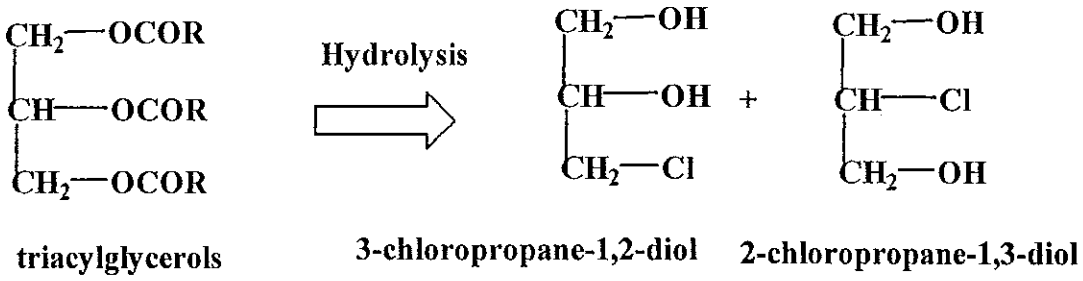


Figure 1 Formations of chloropropanediols from acylglycerols

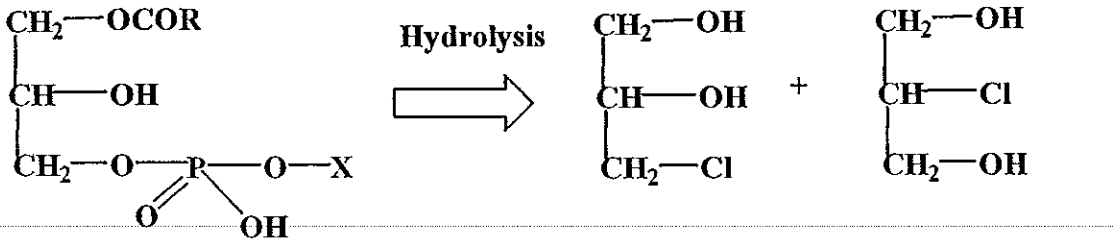


Figure 2 Formations of chloropropanediols from phospholipids

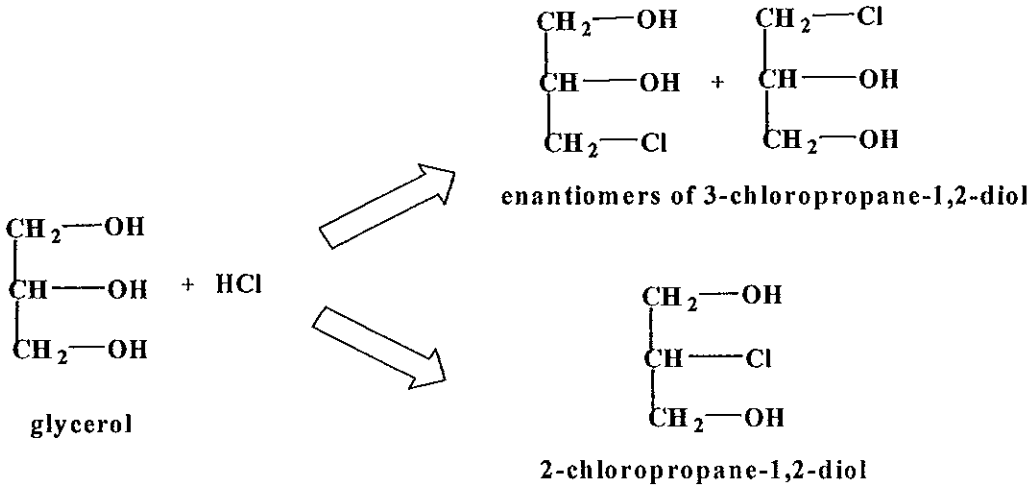


Figure 3 Reaction of glycerol with HCl (Velisek, 2002).

1.1.1 Possible sources of chloropropanols in diet

a) Acid –HVP

Since the 1980s, the procedure used to manufacture the savoury food ingredient can result in the formation of 3-MCPD. Most acid-HVP is produced by using strong hydrochloric acid to cause a high temperature chlorination of liquid present in protein starting materials. The surveys carried out by the Ministry of Agriculture, Fisheries and Food (MAFF) of United Kingdom Government in 1990 and 1992 showed that 3-MCPD level up to 100 mg/kg was quite common in acid-HVP (Joint MAFF/DH Food Safety and Standards Group (JFSSG), 2002). However the levels of

3-MCPD in acid-HVP used in the UK has declined markedly and in 1999 showed undetectable amount of 3-MCPD in acid-HVP (JFSSG, 1999).

b) Roasted cereals, dark malts and dark malt extracts

The report from the UK brewing and malting industries indicated that 3-MCPD levels of up to 0.3-0.4 mg/kg can occur in roasted cereals (JFSSG, 2002). To colour and flavour most dark beers and some lager dark speciality malts are used. Extracts derived from these ingredients, which are used for certain foods and drinks flavour, may also contain 3-MCPD levels of over 0.1 mg/kg (JFSSG, 2002).

c) Fermented sausages

Certain types of fermented sausages such as salami have also been shown to be contaminated by 3-MCPD. This may be due to the formation of 3-MCPD within the meat (due to the formation between fat and salt in the product, couple with its long shelf life) and/or due to the presence of 3-MCPD in the resins used in sausage casing (JFSSG, 2002). The casing industry is carrying out work at European level to determine the contribution of casing to the 3-MCPD content in salami. Like other users of epichlorohydrin-based wet strength resins, the industry has also started to use higher grade resins that contain much lower levels of 3-MCPD (Bodén *et al,* 1997)

d) Soy sauces

Several grades of soy sauce are manufacturing in the Far East. Those include the traditional fermented product as well as some lower grades which may involve the use of an acid treatment or include acid-

HVP as an ingredient (JFSSG, 2002). It is known that such acid treatments can generate very large amounts of 3-MCPD. They can also occur during the manufacture of acid-HVP. While many European manufacturers have made changes to their HVP manufacturing processes over the last 3-4 years which have reduced 3-MCPD levels, some manufacturers in other parts of the world may not have changed their process (JFSSG, 2002). Furthermore, the Joint MAFF/DH Food Safety and Standards Group (JFSSG) and the UK food industry have indicated that the contaminant can also occur in several other foods and ingredients as a result of processing, storage condition or migration from certain food contact materials (JFSSG, 2002).

Not all soy sauce contains 3-MCPD. Only about 22 percent of tested soy sauces were found to have detectable levels. The traditionally brewed soy sauce did not contain the contaminant. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has concluded that 3-MCPD is an undesirable contaminant in food and recommended that its concentration in hydrolyzed proteins should be reduced to the lowest level technically feasible (www2.ctahr.hawaii.edu/oc/freepubs/pdf/fst-1.pdf).

e) Domestic cooking and formation of 3-MCPD in food

3-MCPD has also been found in low levels in food that prepared without hydrolysis such as cheese and during the domestic cooking of prepared cereal products such as bread (Crew *et al.*, 2002). 3-MCPD can occur in toast (0.02-0.32 mg/kg), with slightly lower concentration in some grilled cheeses (0.08-012 mg/kg) and fried batters (0.07-0.09

mg/kg). In contrast, 3-MCPD is undetectable or present only at very low levels in cooked meat, and gravy (JFSSG, 2002).

f) Food contact materials

Information from the packaging industry and others indicated that very low levels of 3-MCPD may migrate into food and beverages from packaging materials. 3-MCPD is present in certain types of epichlorohydrin-based wet strength resins used in paper (e.g. tea bag paper, coffee filters, adsorbent meat padding) and cellulose casings (JFSSG, 2002).

1.1.2 Limits and analysis of 3-MCPD

Different limits have been established for 3-MCPD based on the same general scientific studies. For examples, the European Scientific committee for Food (SCF) established a legal limit of 10 parts per billion for 3-MCPD in soy sauce-type product. In contrast, Canada has set a limit on 3-MCPD of 1 part per million, which is more in line with JECFA's finding. (www2.ctrhr.hawaii.edu). European Union has set a limit of 3-MCPD of 20 parts per billion and for England -10 parts per billion (www.fda.moph.go.th/fda-net/HTML/PRODUCT/3-MCPD.htm).

For the analysis, High Performance Liquid Chromatography (HPLC) method for 3-MCPD has not been reported. Gas chromatography is the most widely used technique. The direct determination 3-MCPD by gas chromatography is rather difficult because 3-MCPD can react during the analysis with other compounds in the sample, *i.e.*, active site on the column and non-volatile residues at the inlet. As a result, peak shape deteriorates with repeated injections and precision is poor (Kissa, 1992).

These problems can be prevented by derivatization of the hydroxyl groups with a suitable reagent to produce a more volatile derivative and provide a less polar and more thermally stable form (Poole, 1978). Suitable derivatizing agents used for derivatization were shown in Table 2. However, some of the derivatives still need a rather time-consuming gas chromatographic procedure (Meierhans *et al.*, 1998).

Table 2 Chemical derivatization with various GC detector

Chemical Deivatization	Detector	References
1.Heptafluorobutyric acid anhydride (HFBA)	GC-MS, GC-ECD	(Matthew,2000), (Chung, 2002)
2. Phenylboronic acid	GC-FTIR GC-FID, GC-MS	(Rodman, 1986), (Plantinga, 1991)
3. Butaneboronic acid	GC-ECD	(Pesselman, 1988)
4.heptafluorobutyrylimidazole (HFBI)	GC-MS, GC-ECD	(Bergen van, 1992), (Hamlet, 2002)
5.N,O-bis-(trimethylsilyl) trifluoroacetamide(BSTFA)	GC-MS, GC-FID	(Kissa, 1992) (Bodén, 1997)
6. Acetone	GC-MS	(Meierhans, 1998)

1.1.3 Derivatization techniques

Gas chromatography is the technique of choice for the separation of thermally stable volatile organic and organometallic compounds. Unfortunately, many compounds of biomedical and environmental interest, particularly those of high molecular weight and/or contain polar functional groups, are thermally labile at the temperature required for their separation. Derivatization, in effect a microchemical organic

synthesis, is used to improve the thermal stability of such compounds which would otherwise not be suitable for gas chromatographic analysis. In most instances, derivatization reactions are performed to convert protonic functional groups into thermally stable non-polar groups. Thus, as well as improving the thermal stability of compound, the derivatized compound often exhibits improved peak shape with a minimization of undesirable column interactions which could lead to irreversible adsorption and skew peak formation (Poole, 1984). Derivatization is useful in many instances where it may:

a) increase the volatility and decrease the polarity of polar components (Baugh, 1993).

b) improves both selectivity and chromatographic efficiency.

c) enhances detectabity and sensitivity (Lawrence, 1981).

Detvatization reactions are usually simple chemical reactions such as acylation, alkylation, or silylation (Baugh, 1993).

In this work, phenylboronic acid was chosen as derivatizing reagent because it has been utilized as the basis for chromatographic separations (Sienkiewiczt and Robert, 1980). The quantitative analysis of 3-MCPD in soy sauce was focused an Chromatographic techniques, that is, High Performance Liquid Chromatographic technique with UV-Visible and Gas Chromatographic technique with flame ionization detector. The sample preparation was also investigated using the chromatographic column.

1.2 Background

1.2.1 Chemical formula and chemical structure

The chemical formula of 3-MCPD is $C_3H_7O_2Cl$ and chemical structure is shown in Figure 4

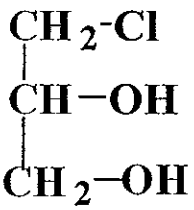


Figure 4 3-MCPD structure

1.2.2 Common name and synonym

The 3-MCPD has large number of common name and synonym (www. cdc.gov/niosh/rtec/ty3d6aa8.html). These are

- Monochlorohydrin
- Epibloc
- 1-chloropropane-2,3-diol
- Glycerin alpha-monochlorohydrin
- glycerol chlorohydrin
- Glycerol alpha-chlorohydrin
- Alpha- monochlorohydrin
- U-5897
- 2, 3-Dihydroxypropyl chloride
- Glyceryl-alpha-chlorohydrin
- 3-chloropropylene glycol

- 3-chloro-1, 2-propanediol
- 1-chloro-2, 3-propanediol
- alpha-Chlorohydrin
- 3-Chloro-1, 2-dihydroxypropane
- 1-Chloro-2, 3-dihydroxypropane
- beta,beta'-Dihydroxyisopropyl chloride
- 3-Chloropropane-1,2-diol
- 3-Monochloropropane-1,2-diol

1.2.3 Physical properties

- Hazard symbol: very toxic
- Appearance: very faintly yellow clear viscous liquid
- Boiling point: 216-219 ^{0}C
- Density $D_{20/4}$ (D_4^{20}): 1.321 g/ml
- Refractive index $N_{20/D}$ (N_D^{20}): 1.481 (www.sigmaaldrich.com/cgi-bin/hsrun/Suite7/Suit/HAHT page/Suite.HSview Hier).

1.2.4 Toxicity

Many studies have shown that 3-MCPD caused a variety of toxicological changes in blood, kidneys, testes and sperm in experimental animals. Furthermore, the European Union Scientific Committee for Food concluded that 3-MCPD should be classified as non-genotoxic carcinogen. On the other hand, other toxicological studies have demonstrated that 3-MCPD produced neurotoxic changes inastrocytes and induced severe fore- and hind-limbs paralysis in mice at single doses of 90 mg/kg of body weight. It has also been shown that ingested 3-

MCPD can be widely distributed in bodily fluid and can also cross the blood-brain barrier (Kim, 2004). Committee on the Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) concluded that 3-MCPD can be regarded as having no significant genotoxic potential *in vivo*. However, Committee on Carcinogenicity of Chemicals in Food (COC) confirmed its carcinogenicity (Crew, 2002).

1.2.5 Phenylboronic acid

The chemical formula of phenylboronic acid is $C_6H_7BO_2$ and its chemical structure is shown in Figure 5. The synonyms are acid phenylboronique (French), boronic acid, phenyl-(9CI), borophenylic acid, dihydroxyphenylborane, kyselina fenylborita (CZECH), phenylboric acid, and phenydihydroxyborane. It has the following physical properties;

Appearance: white to off-white power,

Melting point: 217 – 220 ^{0}C,

Solubility (color): faintly brown

Solubility (method): 1g in 10 ml MeOH

Harzard symbol: harmful (www.camd.lsu.edu/msds/p/phenyl_ boronicacid .htm)

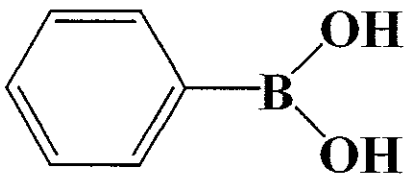


Figure 5 Phenylboronic acid structure

Phenylboronic acid spontaneously converts to phenyl boric anhydrous or phenylboroxide, therefore, it must be stored in a cool dry place. In addition, it is a strong oxidizing agent and it should be protected from moisture (www.easternct.edu/depts/env_saf/Phenylboronic_acid.html).

1.3 Literature Review

The safety of 3-MCPD and 1,3-DCP has been evaluated by Joint FAO/WHO Export Committee on Food Additive (JECFA), which concluded that 3-MCPD and 1, 3-DCP were undesirable contaminants in food and their levels should be reduced to the lowest with technologically achievable (www.legco.gov.hkl/yr00-01/english/panels /fseh/papers/e2097-07.pdf). Therefore, the method of analysis is very important to determine the concentration of 3-MCPD and 1, 3-DCP in order to obtain the real concentration of 3-MCPD in food and food ingredients.

Several methods have been developed for the analysis of 3-MCPD. It was first determined in 1986 by Rodman and Ross (1986) using gas-liquid chromatography. They improved the technique by using 3-MCPD derivative. Because of a well-characterized reaction between phenylboronic acid and dihydroxy compound, it was used to prepare 3-MCPD derivative for gas chromatographic determination. The structure of the derivative was confirmed by gas chromatography - matrix isolation-fourier transforms infrared spectroscopy (GC-MI-FT-IR) and gas chromatography mass spectrometry that used a 10 m methylsilicone wide-bore capillary column (0.53 mm. I.D. film thickness, 1.2 μm).

In 1988, 3-MCPD and epichlorohydrin in water were investigated by gas chromatography-electron capture detector. 3-MCPD had been successfully chromatographed as n-butylboronic acid derivative and as 3-chloropropanediol n-butylboronate. The column was 20 ft ×1/8 in I.D. stainless-steel packed with 10 % SP1000 (on supelcoport). Limits of detection were 0.05 μg/ml for epichlorohydrin extracts and 0.1 μg/ml for 3-MCPD solutions. These methods were rapid, sensitive, and precise for routine residual determinations of either epichlorohydrin or 3-MCPD in aqueous solution (Pesselman and Feit, 1988).

In addition, 3-MCPD has been derivatized with N,O-bis (trimethylsilyl) trifluoroacetamide and determined by capillary gas chromatography in extracts of resins and in solvent containing ketones and the corresponding ketals (dioxalanes), n-tatradecane was used as the internal standard. The underivatized 3-MCPD was separated by either a 30 m × 0.75 mm I.D., SPB-5 megabore borosilicate glass capillary column, or a SPB-5 0.25 mm I.D. capillary fuse-silica column. The SPB-5 column has a bonded 1.0 μm thick film of 94% dimethyl-5% diphenyl-1% vinyl polysiloxane. The limit of detection in this method is 5 μg ml^{-1}. (Kissa, 1992).

In 1997, Bodén *et al.* applied GC-MS for determination of 1, 3-dichloro-2-propanol and 3-chloro-1,2propanediol in papers treated with polyamidoamine-epichlorohydrin wet-strength resins. An analytical method has been developed for the simultaneous determination of 1, 3-dichloro-2-propanol and 3-chloro-1,2propanediol in 0.05-2 mg/kg range. The compounds were simultaneously extracted and silylated with a solution of N,O-bis-(trimethylsilyl) trifluoroacetamide in acetonitrile and finally determined by capillary GC-MS in selective ion monitoring mode

(GC-MS-SIM). A 25 m × 0.32 mm I.D., CP SIL 5 CB (100% methyl silicone) fused silica capillary column with a phase thickness of 1.2 μm was used. The detection limit was 0.04 mg/kg for both 1, 3-dichloro-2-propanol and 3-chloro-1,2propanediol.

The determination of 3-MCPD in liquid hydrolysed vegetable proteins (such as soy sauce, salami, cheese, etc.) have been reported by Plantiga *et al.*(1991) using capillary gas chromatography with flame ionization detector. A phenylboronic acid derivative was prepared and extracted with hexane. The identification of its derivative was confirmed by GC-MS. For both GC-FID and GC-MS analyses the chromatographic separation was carried out by a 50 m ×0.32 mm I.D., CP-SIL 5 CB fuse-silica column with 0.12 μm film (Chrompack) with internal standard, n-haptadecane. The limit of detection of this method was 0.2 ppm.

Bergen van *et al.*(1992) developed a method for identification and determination of full range of chloropropanols in protein hydrolysates and composite savoury food product based on capillary gas chromatography of heptafluorobutyrate derivatives with electron capture and mass spectrometric detection. Heptafluorobutylrate esters of chloropropanols were separated and determination by 25 m ×0.2 mm. I.D. fuse-silica column of immobilized OV-1 (0.33 μm). *p*-dichlorobenzene (PDCB) was used as an internal standard. The limit of detection were 50 - 100 μg/kg for diols and 10 μg/kg for dichloroproanols. For GC-MS the detection limit was 10-20 μg/kg for chloropropane monols and 20-80 μg/kg for diols.

In 1993, 3-MCPD in hydrolyzed vegetable protein was analyzed by using capillary gas chromatography coupled with an electrolytic conductivity detector and a packed-column inlet was fitted with a SGE

on-column adapter to allow direct injection onto a 60 m × 0.75 mm I.D. borosilicate glass supelcowax 10 column with 1 μm film. The column was fitted with 50-cm retention gap of 0.53 I.D. deactivated fused silica. The 1-chlorotetradecane was used as an internal standard. Confirmation of peaks observed for 3-MCPD and 2-MCPD were obtained with GC-MSD. The limit of detection for this method was 0.25 mg/kg sample (Spyre, 1993). Later, in 1998, the method was improved for determination of 3-MCPD and 2-MCPD in different savoury food using GC-MS. Monochloropropanediols was derivatized with acetone and the derivative of 3-MCPD and 2-MCPD were separated on a DB-5, capillary column (30 m ×0.25 mm., 0.25 μm.). Detection was performed by two different mass spectrometers using ion monitoring, to reach ultimate sensitivity in low μg/kg range (Meierhans *et al.*, 1998).

In year 1999 and 2001 3-MCPD was derivatized with heptafluorobutyrylimidazole prior to injection onto the GC-MS (JFSSG, 1999 and Food Standard Agency UK, 2001). Using the same technique Hamlet *et al.* (2002) surveyed 3-MCPD in a range of food ingredients in the UK. They found that 49 samples were not quantified of 3-MCPD. The remaining 14 samples contain levels of 3-MCPD between 0.014 and 0.488 mg/kg. In the same year, Chung *et al.* (2002) used heptafluorobytyric acid anhydride as derivatizing reagent to derivatize 3-MCPD. The detector response was obtained over a concentration range of 10-1000 μg/kg. In another research, 3-MCPD contaminated levels more than 0.1 mg/kg for three samples (crackers) and the highest level being 0.134 mg/kg (Crew *et al.*, 2002).

Sample preparation

After sampling, it is necessary to prepare the sample for determination of analytes through its dissolution, trace enrichment and interferences removal. For the preparation of an appropriate number of sample, the methods have to be selected not only on the basis of the expected concentration of the analytes in the solution that results from the decomposition process, but also according to general requirement such as multielement analysis and dynamic range of the determination, number and mass of sample, laboratory equipment and the experience of the analytical staff (Buldini *et al.*, 2002). The techniques commonly used for extraction from liquid are liquid-liquid extraction (LLE), solid-phase extraction (SPE), and solid-phase microextraction (SPME). Other techniques may be useful in selected circumstances (Mitra, 2003). Basically, the methods depend on differential solubilities (or absorpivities) of substances to be separated relative to the two phases between with they are to be partitioned (www.ares.unimet.edu.ve).

Breden and Anastasio (2000) extracted 3-MCPD and other halogenated mono-alcohol in water by ethyl acetate. The recovery of 3-MCPD was 79.5% and other halogenated diol was in the range 83-120 %. 3-MCPD in papers sample was extracted with acetonitrile and simultaneously derivatized with BSTFA. The liquid phase was analyzed by GC-MS in SIM mode. The recoveries of 3-MCPD and DCP were 80-100% (Bodén *et al.*, 1997). For the solid-liquid extraction, Extrelut column and column chromatography were used for extraction in food. Spyre (1993) reported the application of Extrelut column to extract 3-MCPD in hydrolyzed vegetable protein (HVP). The column was eluted with ethyl acetate and obtained the recoveries more than 95 %.

According to the expected 3-MCPD levels or the desired limit of detection, two very similar procedures were applied: for routine analysis, Extrelut 3 columns were used, whereas for ultimate sensitivity, Extrelut 20 columns were used. The method proved to be fast and reliable and showed good recoveries and accuracy (Meierhans *et al.*, 1998). Usually, the sorbent fall into three general classes; non-polar, polar, and ion-exchange and their activity is dependent on the properties of bonded phase and of any active site not end capped on the sorbent. The choice of the sorbent is dependent on the food matrix, analytes of interest and their interferents (Buldini *et al.*, 2002). The appropriate adsorbents for 3-MCPD extraction were Extrelut^{TM} refill pack (JFSSG, 1999), diatomaceous earth (JFSSG, 1999) and silica gel (Chung *et al.*, 2002). The size of column for extraction were 2 cm. I.D.× 60cm. with PTFE stopcock (Hamlet, 2001), 2 cm. I.D.× 40 cm length with sintered glass disc and trap (www.aoac.org/vmeth/C_methodEX 2000.01.pdf) and 3 cm. I.D. × 100 cm length (Chung *et al.*, 2002).

1.4 Objectives

1.4.1 To study the appropriate sample preparation technique and the qualitative and quantitative analysis of 3-Monochloropropane1, 2-diol (3-MCPD) in soy sauce using gas chromatography with flame ionization detector technique.

1.4.2 Quantitative analysis 3-MCPD concentration in soy sauce.

Chapter 2

Experimental

2.1 Chemicals and materials

2.1.1 Standard chemicals

- 3-Chloro-1, 2-propanediol purity 98.0 % (Fluka, Switzerland)
- Phenylboronic acid purity 97.0 % (Sigma, USA)

2.1.2 Genearal chemicals and solvents

- Sodium Chloride (NaCl), AR grade (Carlo Erba, USA)
- Acetone, AR grade (Carlo Erba, USA)
- n-Hexane, AR grade (Lab-scan, Thailand)
- n-Hexadecane, AR grade (Merck, Germany)
- Sodium sulphate anhydrous, AR grade (Merck, Germany)
- Siliga gel 60 (230-400 mesh) (Merck, Germany)
- Ethyl acetate, AR grade (Lab-scan, Thailand)
- Dichloromethane, AR grade (Lab-scan, Thailand)
- Diethyl ether, AR grade (Fisher ChemAlert Guid, USA)
- Acetonitrile, AR grade (Lab-scan, Thailand)
- Glass wool

2.1.3 Samples

Soy sauce samples were purchased from department stores in Songkhla province.

2.2 Instruments and apparatus

2.2.1 IR spectra were obtained on FT-IR spectrometer model FTS165 (Perkin Elmer, USA)

2.2.2 Gas Chromatograph-mass spectrometer (GC-MS)

- Gas chromatograph model 6891 Series (Agilent, USA)
- An auto-sampler injection model 7860 Series (Agilent, USA)
- Mass Selective Detector (MSD) model 5973 (Agilent, USA)
- Computer system model KAYAK (Hewlette Packard, USA)

2.2.3 High Performance Liquid Chromatography (HPLC) system consisted of;

- High pressure liquid Chromatographic pump model 515
- HPLC pump (Waters, USA)
- Ultraviolet-Visible (UV-Vis) model 2487 Dual λ Absorbance
- Detector (Waters, USA)
- Auto-sampler injection model 717 plus autosampler (Waters, USA)
- Millennium^{32} software (Waters, USA)
- Hypersil BDS C18 column: 250 mm × 4.6 mm.i.d., 5μm. (Supelco, USA)

2.2.4 Gas chromatography- Flame Ionization Detector (GC- FID)

- Gas chromatograph model GC 8960 series equipped with flame ionization detector (Agilent, USA.)
- Capillary Column: 30 m. × 0.32 mm I.D., 0.25 μm film thickness of 5% phenyl 95 % dimethylpolysiloxane, HP-5 (Agilent, USA).
- Computer system model VL Vectra (Hewlette Packard, USA)
- Chemstation sorfwares (Agilent, USA).
- Helium (He) Carrier Gas high purity 99.99% (TIG, Thailand)
- Oxidant gas, Air, zero grade 99.995% (TIG, Thailand)
- Nitrogen gas, high purity grade 99.99% (TIG, Thailand)
- Hydrogen gas, high purity 99.99% (TIG, Thailand)

2.2.5 Apparatus

- Heating block (Laboratory built), 4 cm × 20cm × 20 cm
- Aluminium block
- Glass column, 20 mm I.D. × 400 mm
- Clear vial 1.0 ml and 5.0 ml with caps (waters, USA)
- Amber vial 2.0 ml with silver aluminum cap (Agilent, USA
- 11-mm crimper and 11-mm decrimper (Agilent, USA)
- Filter paper-No.3 (Whatman, Maidstone, England)
- Microlitre pipette: model P1000, 100-1000 μl; model P200, 50-200 μl; and model P20, 5-20 μl (Gilson,

France)

- General glasswares such as volumetric flask 10, 100, 250 ml; glass round bottom 250 ml and pear-shaped flask.
- Evaporating rotator (EYELA, Japan)
- Ultra sonic bath transonic Digitals (Elma, Germany)
- Syringe Filter, Filter device Nylon filter Media with Polypropylene Housing, 13 mm. i.d., 0.45µm. pore size (Orange Scientific, Belgium)
- Membrane filter, 47 mm, supor®-450 membrane (Waters, USA)
- Syringe for sample filter, Micro-Mate® interchangeable (Popper & Son, Inc, ITALY)
- Thermocouple Module 80TK with sensor (Fluke, USA)
- Multimeter (Fluke, USA)
- Thermometer

2.3 Methodology

2.3.1 Standard solution of 3-Monochloropropane-1,2-diol (3-MCPD)

Standard solution was prepared by accurately pipetting 75.7 µl of 3-MCPD and placed into a 100 ml volumetric flask and diluted with 20% sodium chloride solution. The final concentration was 1000 µg ml^{-1}.

2.3.2 Phenylboronic acid as derivatization reagent

The derivatization reagent was prepared by weighing 0.200 g phenylboronic acid into a 100 ml volumetric flask and diluted with 95% acetone + 5% distilled water. The final concentration was 2.00 g L^{-1}

Sodium chloride solution contained 200 g L^{-1} sodium chloride was prepared by dissolving 20.0 g sodium chloride in 100 ml. distilled water.

Sodium chloride solution, 5M, was prepared by dissolving 73.125 g sodium chloride in distilled water in a 250 volumetric flask.

Internal standard was prepared by pipetting 1.29 µl of n-Hexadecane into a 100 ml volumetric flask and diluted with n-hexane. The final concentration was 10 µg ml^{-1} (D = 0.777 g/ cm^3).

2.3.3 Derivatization of 3-MCP-phenylboronate ester derivative

The derivatization technique was adapted from Plantinga *et al.*, (1991); 3-MCP-phenylboronate ester derivative was prepared by pipetting 600 µl of 1000 µg ml^{-1} 3-MCPD standard solution into a 2.0 ml vial. Then added 400 µl of 2.00 g L^{-1} of derivatization solution, phenylboronic acid, and capped. The mixture was placed in a heating block at 90 °C for 20 min for the derivatization reaction to take place. Then cooled the derivatized solution to room temperature and removed the vial cap. 3-MCP-phenylboronate ester was extracted by adding 1.0 ml n-hexane and shaking the mixture by hand for 30 seconds. The hexane extractant was placed into a 2.0 ml vial with a new cap. The concentration of 3-MCP-phenylboronate ester was 1066 µg ml^{-1}, calculated from the reaction. The structure of 3-MCP-phenylboronate

ester was confirmed by IR spectrometry and gas chromatography- mass spectrometry to ensure the ester derivative.

After 3-MCP-phenylboronate ester derivative was extracted into n-hexane layer the extractant was evaporated to dryness at 60 °C. A 1.0-ml of acetonitrile was then added to the residue of 3-MCP-Phenylboronate ester derivative. A 10-µl 3-MCP-Phenylboronate ester derivative was injected to an HPLC system operated in reverse-phase. A flow rate of 0.8 ml min^{-1}, injection volume of 10 µl and isocratic elution of 75% v/v acetonitrile in water were employed. Detection of 3-MCP-Phenylboronate ester derivative was monitored at 268 nm. The chromatogram of 3-MCP-Phenylboronate ester derivative was compared with reagent blank.

2.3.4 Lab built heating block for derivatization reaction

A lab built heating block was designed for the derivatization reaction of 3-MCPD and phenylboronic acid. The heating block (Figure 6) consisted of an aluminium block, a hot plate, and a thermostat to control the temperature. The square aluminium block (20 cm × 20 cm × 4 cm) had 45 holes each with an inner diameter of 1.0 cm and 2.0 cm deep to fit the 2.0 ml vials. When used, the aluminium block was placed on the hot plate. The temperature of this lab built heating block was calibrated by a digital thermocouple.

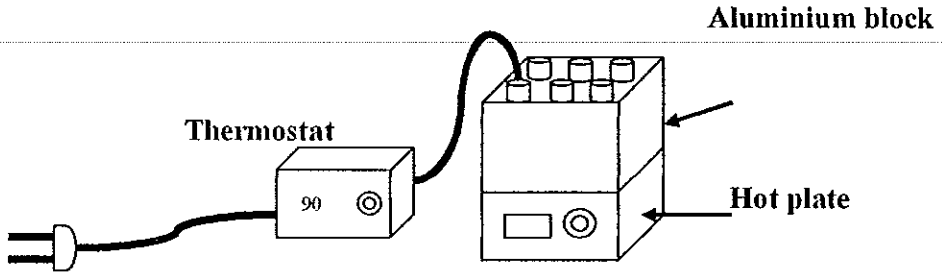


Figure 6. Lab built heating block

2.3.5 Optimization of 3-MCP-Phenylboronate ester derivative reaction

Parameters for derivatization reaction were optimized, *i.e.*, reaction temperature, time of reaction, and extraction time. For gas chromatographic analysis the concentration and volume of 3-MCP-phenylboronate ester derivative used in all optimization experiments were 100 µg ml^{-1} and 1.0 µl respectively.

2.3.5.1 Temperature for derivatization reaction

Temperature of the derivatization reaction was optimized by varying the temperature of the heating block at 30, 40, 50, 60, 70, 80, 90, 100 and 110 °C, 1.0-µl of 100 µg ml^{-1} 3-MCP-phenylboronate ester was injected to the gas chromatograph at optimum conditions (Rodman and Ross, 1986), *i.e.*, injector temperature 250 °C, detector 300 °C and column temperature programming were set as 100 °C (4 min), 10 °C/min

to 280 °C. Five replications were done for each temperature for derivatization reaction.

2.3.5.2 Time for derivatization reaction

The temperature of the reaction was set at optimum from 2.3.5.1. Time of the derivatization reaction was varied at 10, 20, 30 and 40 min. Each experiment was done in five replications. The derivatization product was analyzed by GC at optimum conditions.

2.3.5.3 Extraction time

The reaction conditions were set following the results from 2.3.5.1-2.3.5.2. Extraction time was varied at 10, 20, 30 and 40 seconds. Each experiment was done in five replications. The products were analyzed by GC at optimum conditions.

2.3.6 Optimization of gas chromatograph-flame ionization detector conditions

The chromatographic optimum conditions of GC-FID system were studied for carrier gas flow rate, fuel gas (H_2) flow rate, oxidant gas flow rate (Air), programming temperature, injector temperature and detector temperature.

2.3.6.1 Carrier gas flow rate

A 1.0-µl 100 µg ml^{-1} aliquot of 3-MCP-phenylboronate ester derivative was injected into GC -FID system. Nitrogen, hydrogen and air were used as make up, fuel and oxidant gases and were initially

maintained at flow rates of 30, 30, and 300 ml/min, respectively. These values were suggested in the Operating Manual of GC-FID model HP 6890 series. Optimum carrier gas flow rate was investigated by varying the flow rate of helium carrier gas at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 ml/min. The retention time and peak height of ester derivative were obtained from the chromatogram. The van Deemter graph was plotted and the optimum flow rate was obtained from the lowest HETP.

The injector temperature, detector temperature and the column temperature programming were set followed the application note of Gas chromatography (Rodman and Ross, 1986). These values were 250 °C, 300 °C and 100 °C (4min), 10 °C/min to 280 °C respectively.

2.3.6.2 Column temperature programming

Column temperature programming consists of the following steps: initial temperature, hold time of the initial temperature, final temperature and ramp rate of temperature. The chosen condition was the one that provide the highest response, best resolution of analyte and minimum time. These steps of column temperature programming were studies as the following.

Step I Initial temperature

The initial temperature was varied from 60 to 100 °C with an increment of 10 °C; and hold for 4 min. The ramp rate was 10 °C/min and the final temperature was 280 °C. Carrier gas flow rate was set at optimum condition obtained from 2.3.6.1

Step II Hold time at initial temperature

The carrier gas flow rate and initial temperature (step I) were set at optimum conditions. The hold time was varied at 0, 1, 2, 3, and 4 min. Other parameters were set as, injector temperature 250 °C, detector temperature 300 °C and the column temperature programming was 100 °C (4min), 10 °C/min to 280 °C.

Step III The final temperature

Optimum final temperature was investigated by varying the temperature at 250, 260, 270, 280, 290, and 300 °C. The carrier gas flow rate, initial temperature and hold time were set at optimum conditions. Other parameters were set as 2.3.6.1.

Step VI The ramp rate of temperature

All parameters were set at optimum conditions and the ramp rate was varied at 5, 10, 15, and 20 °C/min.

2.3.6.3 Injector temperature

The optimum carrier gas flow rate and the column temperature programming were set at optimum conditions obtained from previous experiments. Other parameters were the same as 2.3.6.1-2.3.6.2. The injector temperature was investigated by varying the temperature at 200, 225, 250, 275, and 300 °C, respectively.

2.3.6.4 Detector temperature

The optimum carrier gas flow rate, the column temperature programming and injector temperature were set at optimum conditions. Other parameters were the same as 2.3.6.1-2.3.6.2. The detector temperature was varied at 200, 220, 240, 260, 280, and 300 °C.

2.3.6.5 Optimization of carrier, fuel and oxidant gases

The carrier gas flow rate, the column temperature programming, injector and detector temperatures were set at optimum conditions. The flow rate of air (oxidant) was set at 100 ml min^{-1} and then varied the flow rate of hydrogen (fuel) at 20, 30, 40, and 50 ml min^{-1}.The flow rate of hydrogen at optimum condition and varied the flow rate of air at 100, 200, 300, and 400 ml min^{-1}.

The responses of all parameters obtained from chromatogram were then compared. The optimum of each parameter was selected to be the one that provided the highest response.

2.3.7 Limit of detection (LOD)

3-MCPD was prepared at 1000 μg ml^{-1} and diluted with 20% sodium chloride to several concentrations in the range of 0.4–1.0 μg ml^{-1}. Each concentration was derivatized as in 2.3.5. A 1.0-μl aliquot of each 3- MCP-phenylboronate ester derivative was injected into the GC-FID system at the optimum conditions obtained from 2.3.6.1 – 2.3.6.5

Limit of detection was the lowest concentration that the detector could detect and had a signal on the chromatogram with the signal to noise ratio (S/N) of more than three (S/N > 3).

2.3.8 Linear dynamic range (LDR, Linearity)

The standard stock solution of 3-MCPD was diluted with 20% sodium chloride to various concentration in the range of 0.5-1000 μg ml^{-1}. Each concentration was derivatized as in 2.3.5. A 1-μl aliquot of each 3-MCP-phenylboronate ester derivative was injected into the GC-FID system at the optimum conditions from 2.3.6.1 – 2.3.6.5.

Linearity of 3-MCP-phenylboronate ester derivative was determined by plotting the peak area versus the concentration of 3-MCP-phenylboronate ester derivative. The linearity was justified by considering the linear regression coefficient.

2.4 Sample preparation

The column chromatographic technique was used to extract 3-MCPD in soy sauce sample. The column was packed with the mixture of sample and adsorbent

Soy sauce sample, 8.00 g, was placed into a 250 ml-beaker then 80 μl of 1000 μg ml^{-1} standard stock solution was added. The 5M sodium chloride solution was then added into the mixed sample to make up a total weight (soy sauce + salt solution) of 20.0 g. The mixture was then stirred using a spatula until it became homogeneous. It was then extracted in an ultrasonic bath at room temperature (~30 °C).

A 16.0 g of silica gel 60 (230-400 mesh) was added to the soy sauce mixed sample and mixed thoroughly by stirring and crushing with a spatula. The reconstituted mixture was transferred to a chromatographic glass column (Figure 7). The column was shaken or vibrated to compact the content, 1.0-cm layer of sodium sulphate anhydrous was placed on the top of the column and left for 15 minutes. Ethyl acetate was used for eluting at a flow rate 8 ml min^{-1}.The eluate was collected in a pear-shaped flask.

The eluate was concentrated to about 5.0 ml by the rotary evaporation at 70 °C. The concentrated extract was then transferred to a 10 ml volumetric flask and adjusted the volume to 10 ml with ethyl acetate. A small quantity of sodium sulphate anhydrous was added to the flask, which was then shaken and left to stand for 5 minutes. A 1-ml aliquot of the sample extractant was then transferred into a 2.0 ml vial. The solution was evaporated just to dryness under stream of nitrogen, and derivatized with phenylboronic acid in 2.3.5. After the separation of the organic and aqueous layers, 1.0-µl of the hexane layer was injected to the GC-FID system at optimum conditions.

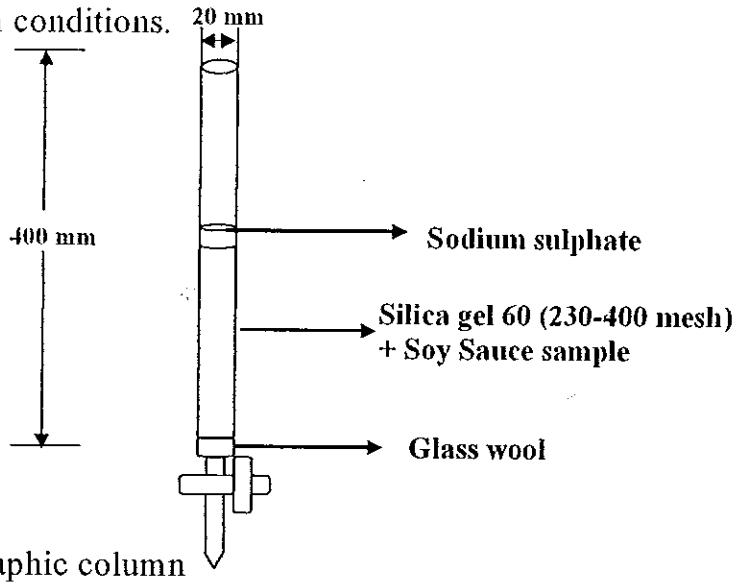


Figure 7.Glass chromatographic column

The recovery of 3-MCPD was obtained by optimizing the extraction conditions.

2.4.1 Flow rate

The flow of solvent through the column was to elute analyte and was optimized for high selectivity and maximum efficiency. Four flow rates for eluting 3-MCPD were investigated, *i.e.* 4, 6, 8, and 10 ml min^{-1}, and each flow rate was done in five replications. The resulting extraction was analyzed by GC-FID. The optimum flow was the one that gave the highest response.

2.4.2 Extraction time

Good extraction method should provide high extraction efficiency in a short period of time. The extraction time was varied at 5, 10, 15, and 20 minutes. Five replications was done for each extraction time. The extractant was analyzed by GC-FID. The highest response was selected.

2.4.3 Eluting solvent

The eluting solvent should be strong with respected to the analyte for high selectivity. Four elution solvents for 3-MCPD extraction were investigated, ethyl acetate, diethyl ether and dichloromethane. Each experiment was done in five replications. The resulting extract was analyzed by GC-FID and the solvent that provided the best and highest response extraction efficiency was selected.

2.4.4 Volume of eluting solvent

In the procedure for sample preparation by chromatographic column, the volume of eluting solvent should be enough to elute the analyte in order to obtain the highest yield. The volume of eluting solvent was varied at 50, 100, 150, and 200 ml and each experiment was done in five replications. The extractant was analyzed by GC-FID at optimum conditions and the best response was selected.

2.4.5 Clean up & Recovery

Clean up was done by using 80 ml of n-hexane-ethyl acetate (90:10 v/v) mixture to remove all non polar compounds in the sample. Each experiment was done in five replications.

2.5 Qualitative and quantitative analysis of 3-MCPD in soy sauce samples

2.5.1 Sampling

Five brands of soy sauce samples were sampling from department stores in Hat Yai district as in Table 3. All soy sauce samples were stored at room temperature.

Table 3.The information of samples

Samples	Net. Volume	Date of Expire	Date of analysis
Soy sauce1	100	10-12-04	05-03-04
	740	10-10-06	23-02-04
Soy sauce 2	700	12-01-07	23-02-04
Soy sauce 3	700	01-06-05	23-02-04
Soy sauce 4	600	09-03-05	24-02-04
Soy sauce 5	700	01-12-06	24-02-04

2.5.2. Qualitative Analysis

Qualitative analysis was carried out by comparing the retention time of the chromatogram of 3-MCP-phenylboronate ester derivative standard to unknown samples. The confirmation was done by GC-MS.

2.5.3 Quantitative Analysis

Quantitative analysis depended on the response from GC-FID, *i.e.*, area of chromatographic peak that was proportional to the amount of analyte. The analytical standardization techniques applied to this work were 'External standardization' and 'Internal standard method'.

External standard solution was 3-MCP-phenylboronate ester derivative working standard solution prepared at the same levels of concentration close to the unknown samples. These standards were then run chromatographically under identical condition as the samples. A direct relationship between peak size and composition of one or more components can then be established, and the unknowns compared graphically or mathematically to the standards for analysis (Grob, 1985).

Internal standard method was performed by adding other compound which was similar in functional group type to the analyte, with known concentration, into the sample in order to compare the signal intensity from the instrument. Four concentrations of 3-MCPD were added to soy sauce samples and extracted by chromatographic glass column. The resulting solution was derivatized and injected to GC-FID at optimum conditions. The results were plotted between ratio of peak area of analyte to peak area of internal standard (A_a/A_{is}) and concentration of 3-MCP-phenylboronate ester derivative. The concentration of 3-MCPD in the sample can then be determined.

Chapter 3

Results and Discussion

In gas chromatography it is preferable to separate compounds in their native state but some compounds are unstable at the temperatures used in GC or do not possess a detectable chromophore for ultraviolet detection in HPLC. In that case, derivatization is required to form a derivative that increases their volatility, suitable for GC analysis. Derivatization involves a chemical reaction between an analyte and reagent to change its chemical and physical properties. The main uses of derivatization in chromatography are to change the molecular structure or polarity of anayte for better chromatography, stabilize an analyte, improve detectability, and change the matrix for better separation

The derivatization reaction should be rapid and quantitative, with minimal production of by product. Any excess reagent should not interfere with the analysis or should be easily removed from the reaction mixture (Settle, 1997).

In this thesis, phenylboronic acid was chosen as a derivatizing reagent, *i.e.*, protecting 1, 2-diol in 3-MCPD and produces cyclic boronates. The stability of the boronates depends on the nature of the diol. Many boronates are moisture sensitive and unstable under protic conditions. More hindered diols form is more hydrolytic stable boronates (Piŝ and Harmatha, 1992). The proposed structure of the 3-MCP-phenylboronate ester derivative is shown in Figure 8.

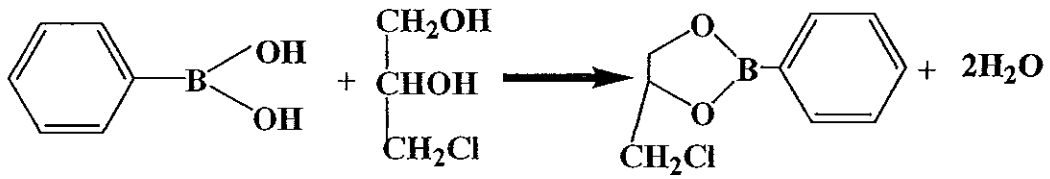


Figure 8 The proposed structure of 3-MCP-phenylboronate ester derivative

3.1 Confirmation the structure of 3-MCP-phenylboronate ester derivative by IR spectrometry

The confirmation of 3-MCP-phenylboronate ester was done by IR spectrometry and the result IR-spectrum is shown in Figure 9. It showed the aromatic CH stretch above 3000 cm^{-1}. The methyl CH bending absorbance showed the wavenumber closed to 1370 cm^{-1} and 1440 cm^{-1} and methylene C-H stretch absorbance was closed to 2940 cm^{-1}.The aromatic disubstitued ring bending is obscured by the strong C-Cl absorbance at 763 cm^{-1}. The IR spectrum of 3-MCP-phenylboronate agreed with Rodman and Ross (1986).

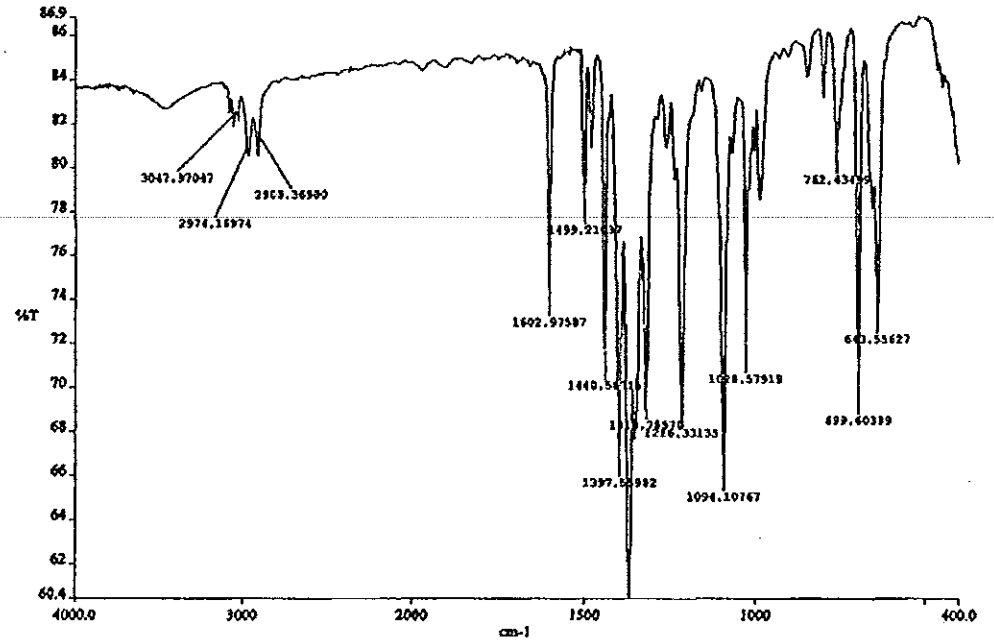


Figure 9 The spectra of 3-MCP-phenylboronate ester derivative

3.2 Confirmation the structure of 3-MCP-phenylboronate ester derivative by gas chromatography-mass spectrometry

The other confirmation of the 3-MCP-phenylboronate ester derivative was done by GC-MS in 2.2.4. Figures 10 and 11 showed the chromatogram and mass spectra of the phenylboronic acid derivative of 3-MCPD. In Figure 11 the base peak is the $C_6H_5BO^+$ peak corresponded to the loss of CH_2Cl from 3-MCP-phenylboronate ester.

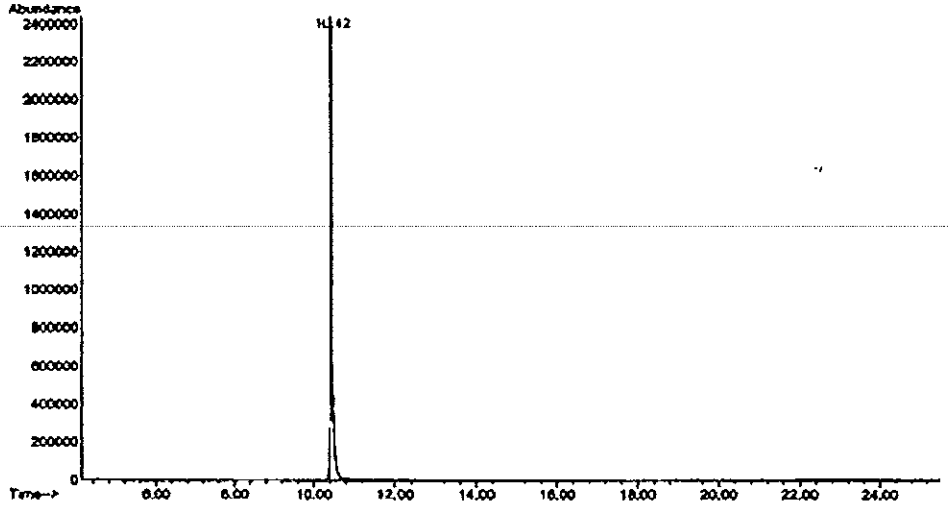


Figure 10 The chromatogram of 3-MCP-phenylboronate ester derivative

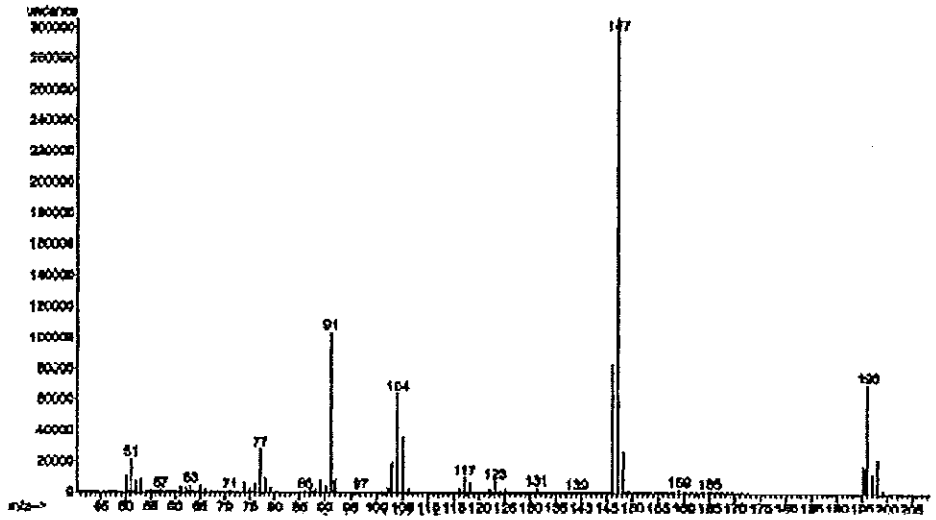


Figure 11 The mass spectra of 3-MCP-phenylboronate ester derivative

3.3 High performance liquid chromatography-UV/Vis (HPLC-UV/Vis)

The HPLC system was used in the reverse phase liquid chromatographic mode. The mobile phase was 75%v/v acetonitrile in water. 3-MCP-phenylboronate ester derivative was separated by Hypersil BDS-C18. The result is shown in Figure 12.

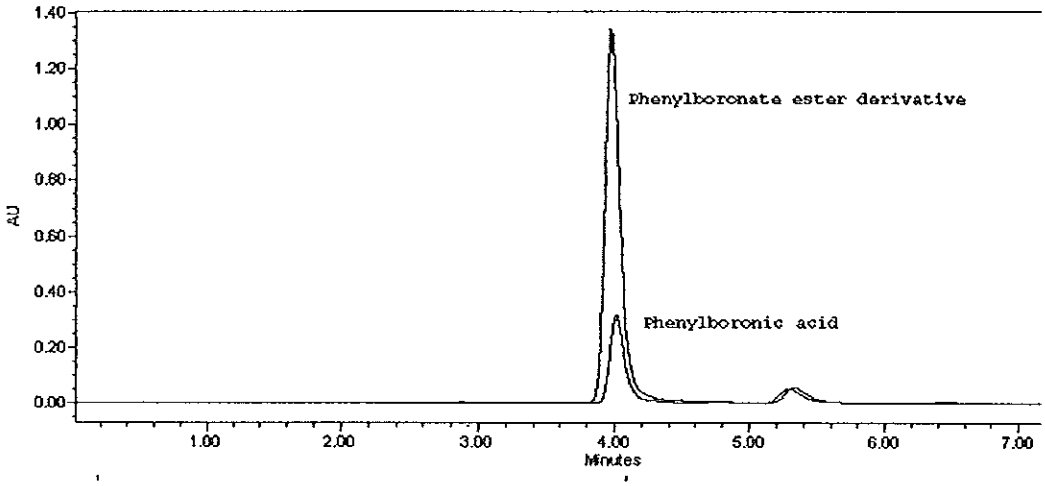


Figure 12 Chromatogram of 3-MCP-phenylboronate ester derivative and phenylboronic acid

The excess phenylboronic acid, the derivatizing reagent, and 3-MCP-phenylboronate ester derivative were eluted at the same retention time. It was found that the reverse phase was not suitable for the analysis of 3-MCP-phenylboronate ester due to the limited stability of the prepared boronates under its operating, *i.e.*, flow rate 0.8 ml min^{-1}, 75% v/v acetonitrile, wavelength 268 nm and 10 µl of standard injected to HPLC system. This could be explained by the properties of the compounds, the limitation of instrument and the process of separation.

Properties of compound

Boronic acid (phenylboronic acid is one of derivatives of boronic acid) is weak acid, (pKa = 8.8 for phenylboronic acid (Springsteen and Wang, 2002)) which produced boronates ions ($RB(OH)_3^-$ in alkaline solution with the change in coordination number and structure (trigonal to tetrahedral(Figure 13)). It can form covalent bonds with compounds having a cis-diol moiety (Soh *et al.*, 2002) or react with bidentate ligand, such as dicaboxylic acid, α-hydroxy carboxylic acids, 1,2-benzenediol, polyols, *etc.*, produces four coordinate complexes (Figure 14). Most of these complexation reactions have no spectral changes in the UV-vis region, so the reaction rate has been measured by pH indicator which follows the proton released during reaction (Ito *et al.*, 2003). However, phenylboronic acid has been used for the protection of 1, 2 and 1, 3-diol. The stability of the boronates depends on the nature of the diol. Many boronates are moisture sensitive and unstable under protic conditions (Piŝ and Harmatha, 1992). This ester was generally stable in non-polar solvent but readily hydrolyzed in water (Shafizadeh, 1971). The stability of the boronate ester is pH and solvent dependent but the factors that govern these processes are not-well understood. Phenylboronic acid structures are neutral and anionic forms. The neutral form linked to the phenyl moiety has a planar triangular conformation with a sp^2-hybridized boron atom. On the other hand, the anionic form has a tetrahedral conformation with sp^3-hybridozed boron atom (Dicesare and Lakowiez, 2001).

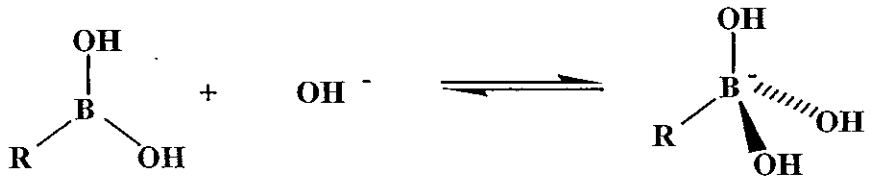
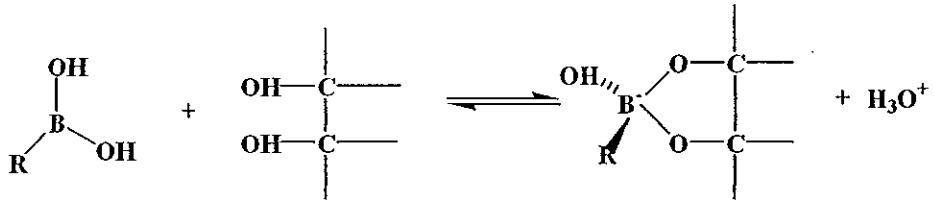


Figure 13 The change in coordination of boronic aid (trigonal to tetrahedral



R = *m*-nitrophenyl, phenyl, methyl, and n-butyl

Figure 14 Formation of boronic acid with cis-diol

Limitation of instrument

The HPLC system was operated by reverse phase mode that consists of water as the main polar mobile phase. But this 3-MCP-phenylboronate ester is stable in non-polar environment. Therefore, the mode of operation was not suitable for separation of phenylboronic acid and phenyboronate ester. Moreover, the HPLC system in the laboratory was operated only with isocratic elution which was ineffective to separate the sample containing components of widely different relative retention. The study showed that the isocratic elution gave poor resolution (Figure 12) and inconveniently long retention times. One way to solve this problem is applying by the gradient elution in which the composition of the mobile phase is varied throughout the separation so that it provides a continual

increase in solvent strength and thereby a more convenient elution time (Poole and Schuette, 1984)

Process of separation (Hamiton and Sewell, 1982)

Chromatography involves the separation of components of mixture by virtue of difference in the equilibrium distribution (K) of the components between two phases: the mobile phase and the stationary phase. If C_s and C_m are the concentrations of a component in stationary and mobile phases respectively, then:

$$K = C_s/C_m \qquad (1)$$

Migration of component molecules may be assumed to occur only when the molecules are in the mobile phase. The rate of migration of a component is then inversely proportional to its distribution coefficient, so components with a high distribution in the stationary phase will move more slowly through the column and hence be separated from the components with a lower distribution in the stationary phase. Without this difference in distribution and, by interference, a differential rate of migration, no separation can be achieved. Substances are separated in chromatographic column when their rates of different migration. For column chromatography the resolution (R_S) can be related to the capacity factor (k'), the relative retention (α) and the number of theoretical plate (N) in term of the second component of the pair by the relationship:

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1 + k'}\right)(N)^{1/2} \qquad (2)$$

The resolution can therefore be changed by altering the thermodynamic properties of the system (which alter α and k') or by alter the column conditions (flow rate, particle size, etc.) to change N.

In order to investigate further the effect that these terms have on the resolution it is useful to consider them as independent functions, thus:

Selective	$(\alpha\text{-}1)/\alpha$
Capacity factor	$k'/(1+k')$
Efficiency	$(N)^{1/2}$

Effect of selectivity on R_S

$$R_S = c_1(\frac{\alpha - 1}{\alpha})$$

where c_1 represents constant capacity factor and efficiency. When α =1 there will be no thermodynamic difference between the two components (K_1=K_2) and hence no separation.

Effect of capacity factor on R_S

$$R_S = c_2(\frac{k'}{1+k'})$$

where c_2 represents constant selectivity and efficiency. When $k' = 0$ there will be no separation.

Effect of efficiency on R_S

$$R_S = c_3 N^{1/2}$$

This resolution increases as N increases. Since R_S is proportional to $N^{1/2}$ and N is proportional to the column length.

From the study and results in 3.3, the limitation of the instrument and mode of operation of the HPLC system made it less favorable to determine 3-MCPD in derivative form. Therefore, gas chromatography was investigated as an alternative method for analysis the derivative of 3-MCPD.

3.4 Optimum conditions for derivatization reaction

The derivatization reaction of 3-MCPD with phenylboronic acid was carried out at optimum conditions. In this work, the derivatization reactions were investigated and the results are followed;

3.4.1 Temperature for derivatization reaction

Optimization of temperatures for derivatization reaction started at room temperature (~ 30 °C) to 110 °C. The results are shown in Table 4 and Figure 15, and the optimum temperature was at 90 °C

From the result it showed that the response of 3-MCP-phenylboronate ester derivative increased with increasing the temperature. Increasing the temperature of reaction improved the substrate solubility and enhanced the rate of reaction. Some difficult -to-derivatize functional group will only react to completion at elevated temperature (Poole. and Schuette, 1984). Moreover, temperature is a measure of kinetic energy of a system, so higher temperature implies higher average kinetic energy of molecules and more collisions per unit time. A general rule of thumb for most chemical reactions is that the rate at which the reaction proceeds will approximately double for each 10 °C increase in temperature. Once the temperature reaches a certain point, some of the chemical species may be altered and the chemical reaction will slow or stop (http://chemistry.about.com/ library/weakly/aa 100702a.htm).

Table 4 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various reaction temperatures

Temperature (°C)	Response*, pA*s
30	103.8
40	95.32
50	94.59
60	116.65
70	125.56
80	115.46
90	133.98
100	112.18
110	119.07

* 5 replication, RSD < 10%

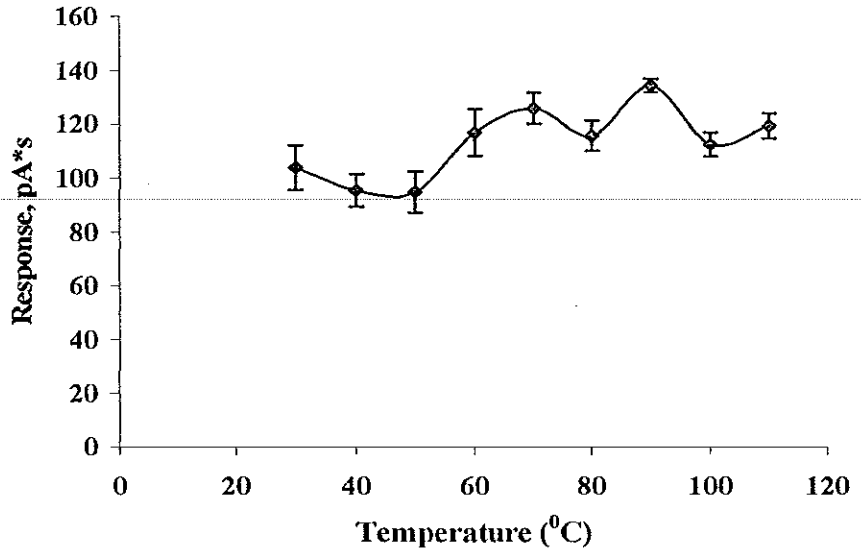


Figure 15 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various reaction temperatures

3.4.2 Time for derivatization reaction

The optimum time for derivatization reaction was investigated between 10 to 40 minutes (2.3.5.2) and the results showed in Table 5 and Figure 16.

Table 5 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various reaction times

Time (min)	Response*, pA*s (%RSD)
10	120.83 (3.57)
20	121.52(2.77)
30	139.36(4.85)
40	132.12(4.64)

*5 replications, %RSD < 10

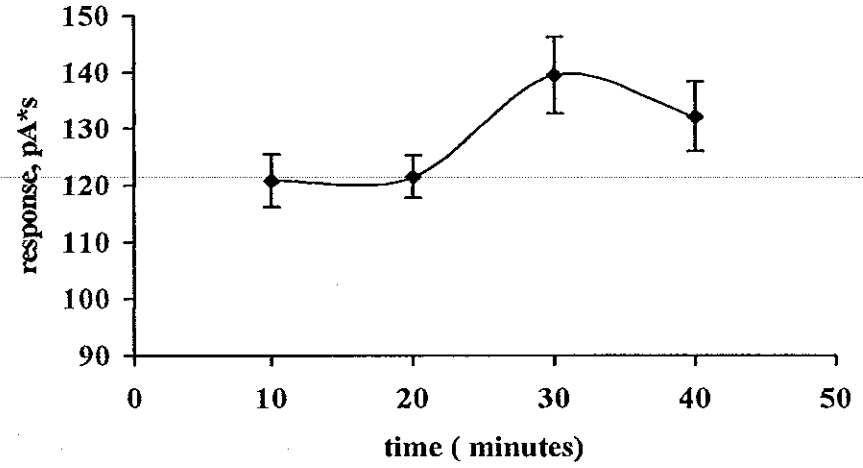


Figure 16 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various reaction times

The results showed that the response increased when the time increased from 10 to 30 minutes, after that the response slightly declined. This indicated that the completed reaction process occurred at 30 minutes. Thus the optimum time for derivatization reaction was chosen at 30 minutes with % RSD 4.8.

3.4.3 Extraction time

The optimum extraction time was investigated between 10 to 40 seconds (2.3.5.3) and the results are shown in the Table 6 and Figure 17.

Table 6 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various extraction times

Time(second)	Response*, pA*s(%RSD)
10	129.72(1.35)
20	138.46(0.72)
30	128.51(2.74)
40	126.45(2.15)

*5 replications, %RSD < 10

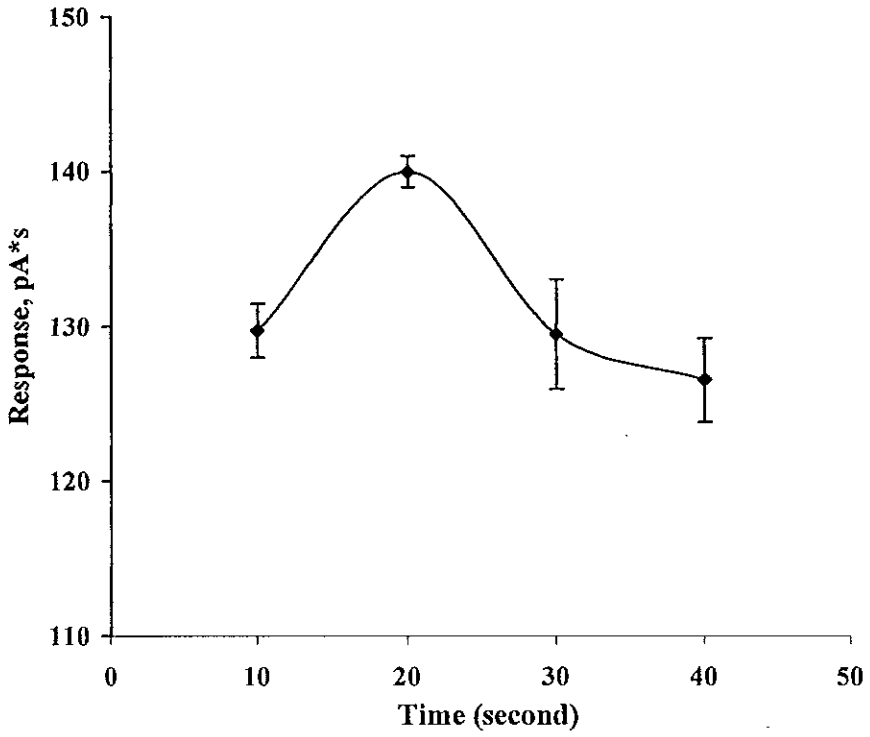


Figure 17 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various extraction times

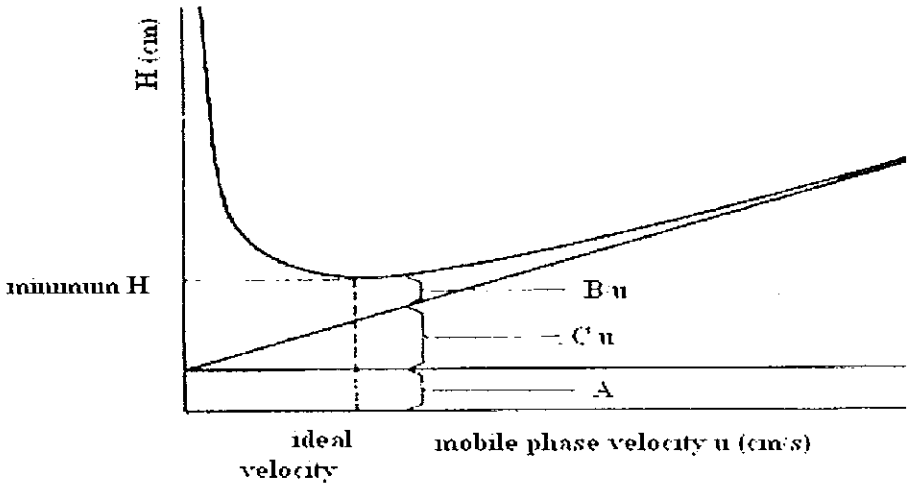
3-MCP-phenylboronate ester derivative was extracted and gave high response when time increased from 10 to 20 seconds after that the response slightly declined but the response was not significant different. So, 20 seconds was chosen the optimum extraction time

3.5. Optimization of gas chromatograph-flame ionization detector conditions

3.5.1 Optimum carrier gas flow rate, helium (He)

The carrier gas flow rate was optimized by considering from the relationship between the height equivalent to a theoretical plate (HETP) and carrier gas flow rate. The HETP can be calculated by the van Deemter equation (3) and determined from the Van Deemter plot (Figure 18).

$$HETP = A + B/u + Cu \qquad (3)$$



Van Deemter plot

Figure 18 The van Deemter plot

Where; A, Eddy diffusion

The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This caused broadening of the solute band, because different paths are of different length.

B/u, longitudinal diffusion

The concentration of analyte was less at the edges of the band than at the center. Analyte diffused out from the center to the edges. This caused band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion.

C, Resistance to mass transfer

The analyte take a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity with stationary phase, then the analyte in mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened by this process. The higher the velocity of mobile phase becomes the worse the broadening. The resistance to mass transfer (term C) has a greatest effect on band broadening, and its effect in capillary column is controlled by the mass transfer in mobile phase. Equation (4) take a different from for capillary column:

$$H=B/u + C_G u \qquad (4)$$

C_G refers to mass transfer of analyte in gas phase

In this work, a 30 m wall-coated open tubular (WCOT) column (0.32 mm i.d.) was used, so the A term is nonexisted because there is only one flow path and no packing material. The resistance-to-transfer term C has the greatest effect on band broadening, and its effect in capillary column is controlled by the mass transfer in gas phase C_G (Grob, 1985).

The above equation (4) showed that HETP is depends on the flow rate (u). It is also known that an optimum carrier gas flow rate give optimum column efficiency resolutions which the narrowest HETP.

The number used to describe the column efficiency is expressed in term of number of theoretical plate (N) and the length of column. To indicate column efficiency the HETP can be determined according to equation (5) and equation (8)

$$HETP = L/N \qquad (5)$$

where, L is the length of the column in centimeters

N is number of theoretical plates.

N can be calculated from the equation,

$$N = 16(T_R/W_b)^2 \qquad (6)$$

$$N = 5.54\ (T_R/W_{1/2})^2 \qquad (7)$$

In this thesis, a capillary column was employed and obtained the shaped peaks. It is difficult to measure the base peak width, thus, the plate number N can be calculated from the value obtained from a

chromatogram as shown in Figure 18 and the following equation (8) was used.

$$N = 2\pi(T_R h/A)^2 \qquad (8)$$

Where, T_R is the retention time

h is integral peak height and

A is integral peak area

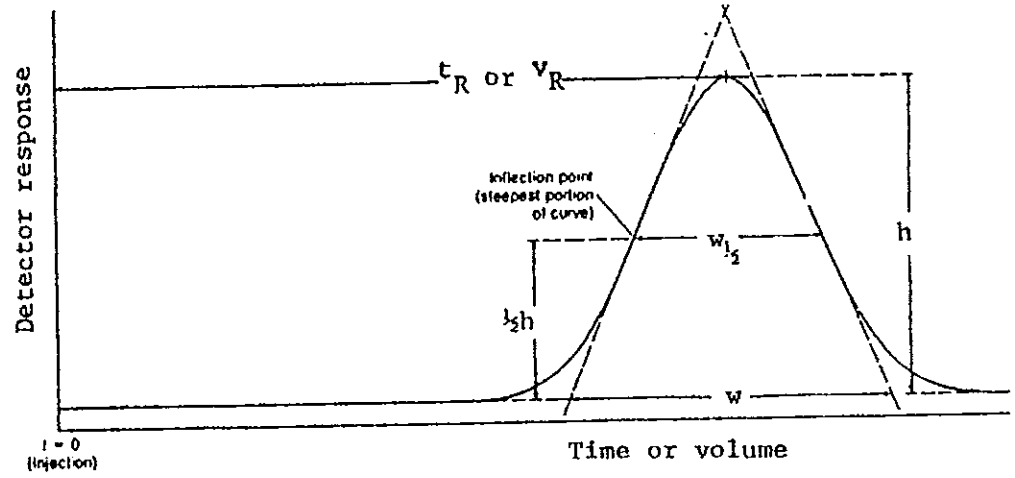


Figure 19 Measurement used in calculating total theoretical plate

N was calculated using equation (8) and substituted in equation (5), with know L term, to obtain HETP. The HETP and the carrier flow rates were plotted as the van Deemter graph. Table 7 and Figure 20 indicated that the narrowest HETP was at the carrier gas flow rate of 3.0 ml/min for 3-MCP-phenylboronate ester derivative.

Table 7 The Height Equivalent to a Theoretical Plate (HETP) of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative at various flow rates.

Flow rate	$N \times 10^5$	$HETP \times 10^{-2}$ (cm)*
1.0	1.58	1.90
1.5	1.67	1.80
2.0	1.66	1.81
2.5	1.79	1.68
3.0	1.91	1.57
3.5	1.82	1.65
4.0	1.65	1.82

*5 replications, RSD< 4 %

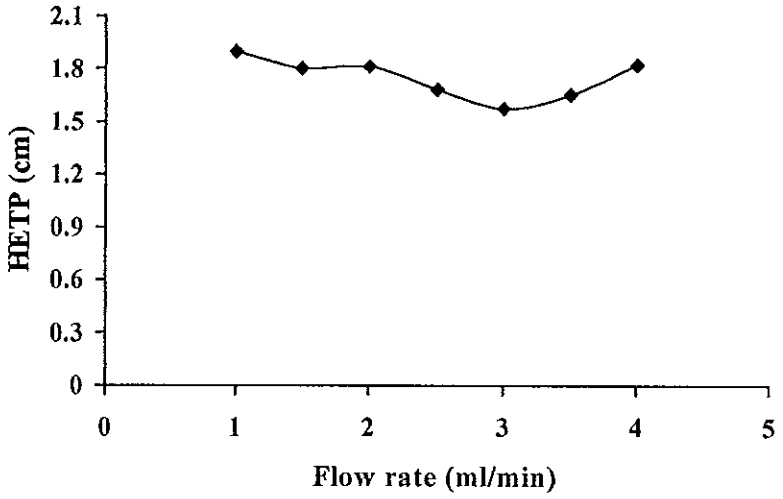


Figure 20 The van Deemter plot of 3-MCP-Phenylboronate ester derivative

3.5.2 Optimization of column temperature programming

In gas chromatography, column temperature is one of the important parameters besides the stationary phase, since it led to the peak resolution. Therefore, the optimum temperature program was studied as 2.3.6.2 and considered in balancing between the time and response.

Initially the column temperature programming was operated as: initial temperature 100 °C for 4 min, programmed to 280 at 10 °C/min (Rodman and Ross, 1986). The column temperature programming was then optimized to achieve a higher response. Therefore, the optimization steps were carried out in four steps as indicated in 2.3.6.2. The results of step *I*, the initial temperature optimizing, are shown in Table 8 and Figure 21. The results indicated that the analysis time decreased when the temperature increased. The highest response was at 90 °C, thus, optimum initial temperature was selected as 90 °C.

Table 8 The response and analysis time of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various initial temperatures

Temperature (°C)	Response* pA*s	Analysis time (min)*
60	392.38	26
70	393.10	25
80	391.21	24
90	399.70	23
100	394.49	22

*5 replications, RSD< 4 %

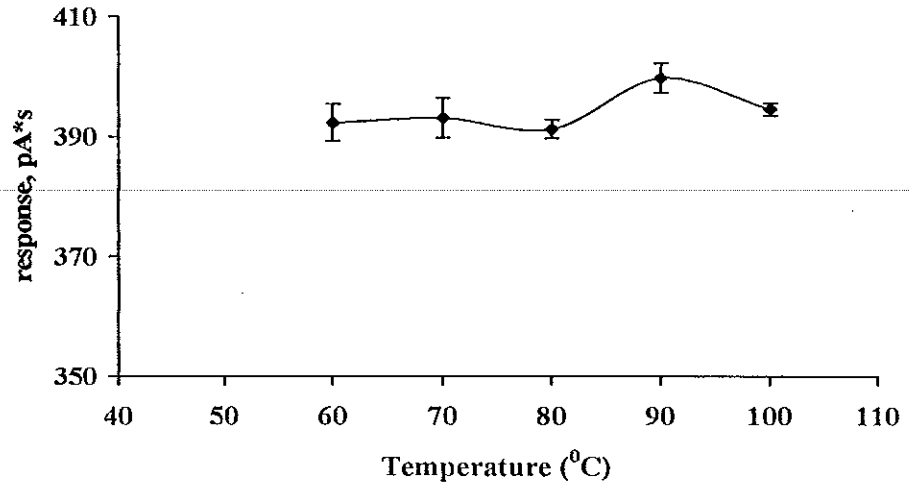


Figure 21 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various initial temperatures

The holding time of initial temperature was varied from 0 to 4 min in step ***II*** and the best response was selected. The results are shown in Table 9 and Figure 22.

Table 9 The response and analysis time of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various initial hold times

Hold time (min)	Response*, pA*s	Analysis time (min)*
0	364.49	19
1	399.53	20
2	404.16	21
3	405.76	22
4	412.67	23

* 5 replications, RSD< 4 %

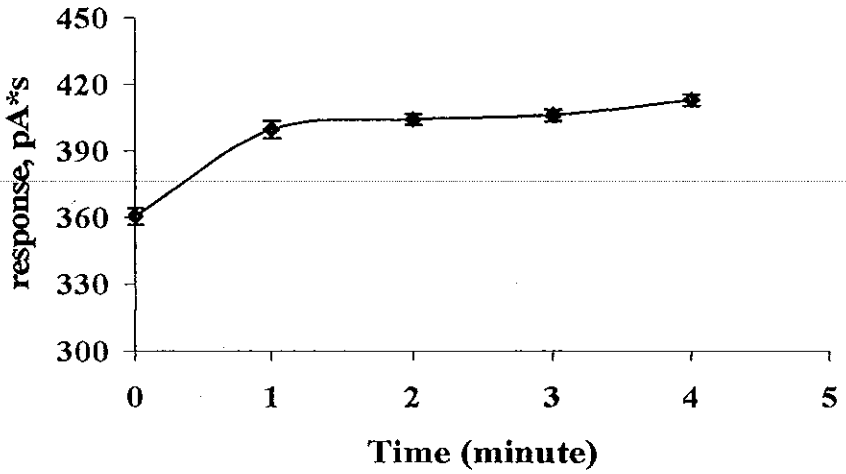


Figure 22 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution various hold times

Between the hold time of the initial temperature from 0 to 4 minutes the response to derivative ester at 1 to 4 minutes were higher than at zero minute. However, the responses at 1,2,3, and 4 minutes differed less than 10 %, therefore, the shorter hold time, 1 minute was chosen for optimum.

The final temperature was varied from 250 °C to 300 °C (2.3.6.2) in step ***III*** with the increment of 10 °C. The results were obtained as in Table 10 and Figure 23. The highest response was at 270 °C and the response was approximately the same when the temperature increased. So, the temperature at 270 °C was chosen to be optimum of final temperature, since after 270 °C the response was almost constant with difference less than 10 %.

Table 10 The response and analysis time of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various temperatures

Temperature (°C)	Response, pA*s	Analysis time* (min)
250	389.93	17
260	392.35	18
270	424.72	19
280	422.91	20
290	422.60	21
300	424.13	22

* 5 replications, RSD< 4 %

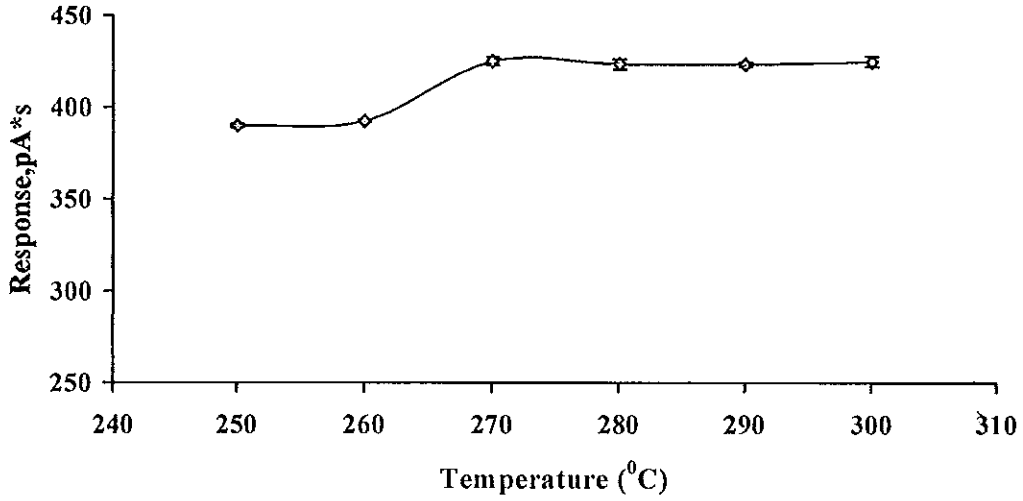


Figure 23 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution various final temperatures

The final temperature selected should be near the highest-boiling component present in the sample. In this work, the phenylboronic acid

which was a derivatizing reagent that was eluted after phenylboronate ester derivative. However, its boiling point had not been mentioned in the literature, thus, 270 °C was taken as optimum final temperature for this ester.

The ramp rate of temperature in step *IV* was obtained from experiment in 2.3.6.2. The results were shown in Table 11 and Figure 24.

Table 11 The response and analysis time of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester standard working solution at various ramp rates

Ramp rate (°C/min)	Response, pA*s	Analysis time*
5	399.93	39.0
10	398.07	21.0
15	415.08	15.0
20	416.65	12.0
25	410.86	10.2

* 5 replications, RSD< 4 %

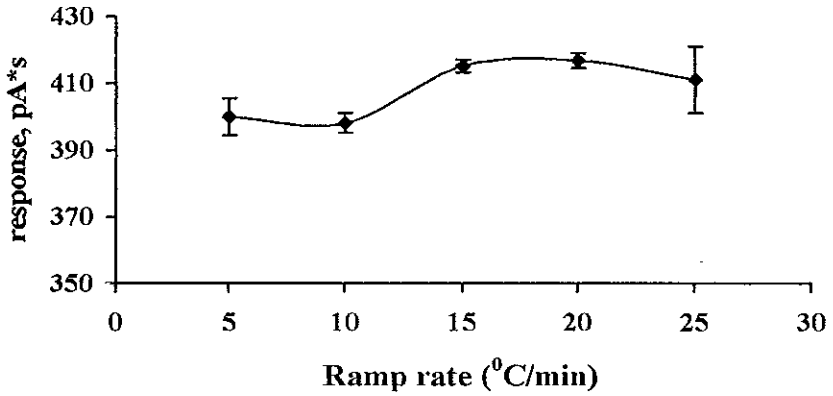
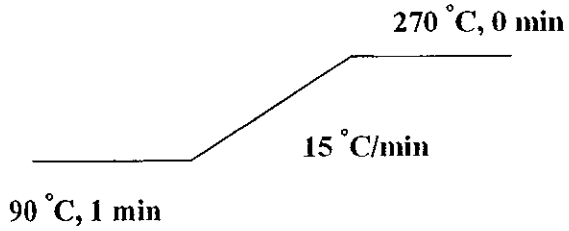


Figure 24 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution various the ramp rates

The ramp rate of 20 and 25 °C/min gave lower analysis time but the baseline drifted. Thus, ramp rate at 15 °C/min was chosen as optimum ramp rate because the response at 15 and 20 °C/min differed less than 10 %.

In summary the optimum conditions of the column temperature programming was 90 °C (initial temperature) and hold for 1 min then ramped at 15 °C/min to 270 °C. The signal took 2 minutes return to the baseline. The total analysis time was 13 minutes.



The proposed analysis time, 13 min was less than other methods, *i.e*, GC-MS, GC-FID and GC-FTIR showed in Table 12.

Table 12 The analysis time of different derivative of 3-MCPD

Chemical derivatization	Detector	Analysis time (min)	References
Phenylboronic acid	GC-FTIR, GC-FID, GC-MS	22.0	Rodman, 1986 Hamlet, 2002
heptafluorobutyrylimidazole (HFBI)	GC-MS	32.6	Bodén, 1997
N,O-bis-(trimethylsilyl) trifluoroacetamide(BSTFA)	GC-MS, GC-FID	19.0	Meierhans, 1998
Acetone	GC-MS	23.5	

3.5.3 Injector temperature

According to instantaneous vaporization requirement, the injector port should have temperature high enough to change the liquid sample to vapor. The results from experiment 2.3.6.3 are shown in Table 13 and Figure 25

Table 13 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution. at various injector temperatures

Temperature (°C)	Response, pA*s
200	333.01
225	339.67
250	447.02
275	441.24
300	446.98

*5 replication, % RSD <4

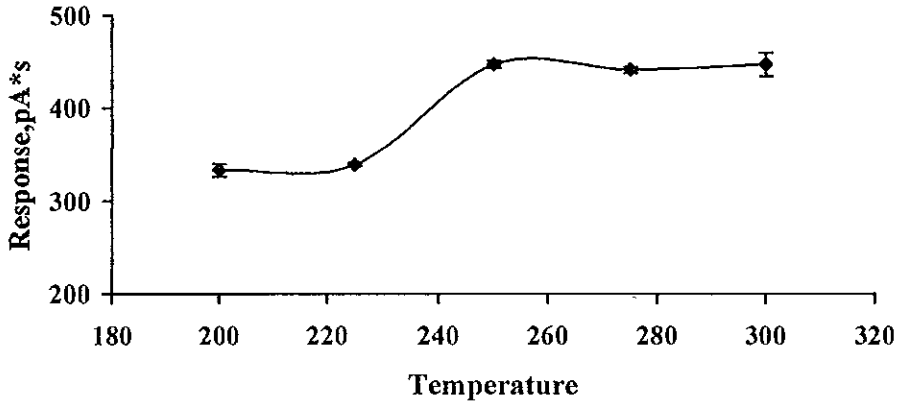


Figure 25 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various the injector temperatures

In Table 13 and Figure 25, the best detector response for 3-MCP-Phenylboronate ester derivative was at 250 °C.

3.5.4 Detector temperature

The detector temperature is always set above 100 °C to prevent moisture water formation in the combustion process inside the detector (Grob, 1985). The detector was the flame ionization detector (FID) that consisted of hydrogen (H_2)/air flame burning jet and collector plate. The effluent from the column is mixed with hydrogen and air that electrically ignited a small metal jet. Most organic compounds produce positive ions and electrons that can conduct electricity through the flame. There is an electrode above the flame to collect the ions formed at a hydrogen/air flame. The number of ions hitting the collector is measured and generated a signal (Baugh, 1993). In this work, the GC manual suggested that the detector temperature should be set at 150 °C, if the temperature was lower than the recommended temperature, flame would not lit. The detector temperature was optimized by varying the detector temperature between 200 and 300 °C as in 2.3.6.5.The results are shown in Table 14 and Figure 26.

Table 14 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various detector temperatures

Temperature (°C)	Response, pA*s
200	380.87
220	395.12
240	410.74
260	421.85
280	432.08
300	433.65

*5 replications, RSD< 4%

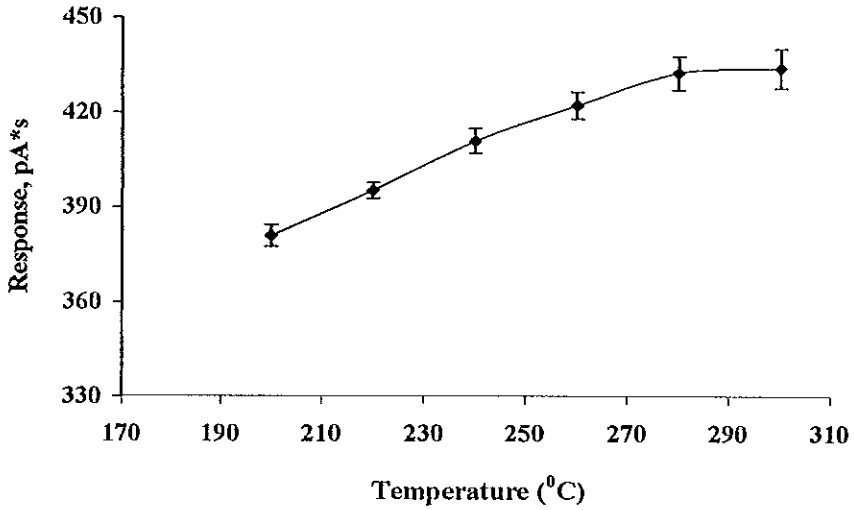


Figure 26 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution at various detector temperatures

From the result the response increased with temperature up to 280 °C where the response started to level off. Thus, the detector temperature at 280 °C was applied in this work.

3.5.4.1 Optimization of gases flow rate used in FID

In this experiment the flow rate of nitrogen, which was used as make up gas was applied at 30 ml/min. This condition was used as recommended by the manufacturer, *i.e.* the make up gas flow rate should be in the range of 10 to 60 ml/min, and suggested at 30 ml/min.

3.5.4.1.1 Fuel (hydrogen) flow rate

The detector consists of a small hydrogen-air diffusion flame burning produced from oxidant and fuel gas. The oxidant and fuel were air and hydrogen respectively. Therefore, flow rates of air and hydrogen would significantly influence the detector sensitivity and the noise level. The hydrogen flow rate was varied from 20 to 50 ml min^{-1} as in 2.3.6.5 and the results are shown in Table 15 and Figure 27

Table15 The effect of hydrogen flow rate on the response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution

Flow rate (ml/min)	Response, pA*s
20	351.43
30	474.26
40	370.06
50	180.81

* 5 replications, RSD< 4 %

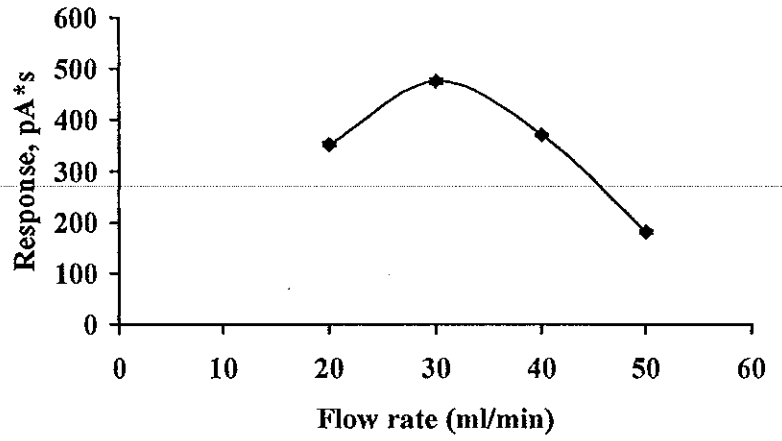


Figure 27 The response of 100 μg ml^{-1}, 1-μl 3-MCP-phenylboronate ester derivative standard working solution various the flow rates of hydrogen gas

The highest response was obtained at 30 ml/min and this was selected as the optimum flow rate.

3.5.4.1.2 Oxidant (air) gas

As in 2.3.6.5 the air flow rate was varied from 100 ml min^{-1} to 400 ml min^{-1} and the results are shown in Table 16 and Figure 28. The flow rate of 300 ml/min gave the highest response and was chosen as the optimum flow rate of air. From this study the optimum flow rate ratio of nitrogen gas, fuel gas and air was 1:1:10 respectively and this agreed with the ratio obtained by other worker (Jennings, 1987).

Table16 The effect of the oxidant (air) gas on the response of 100 µg ml^{-1}, 1-µl 3-MCP-phenylboronate ester derivative standard working solution

Flow rate (ml/min)	Response, pA*s
100	479.89
200	559.50
300	562.16
400	558.82

*5 replications, RSD< 4 %

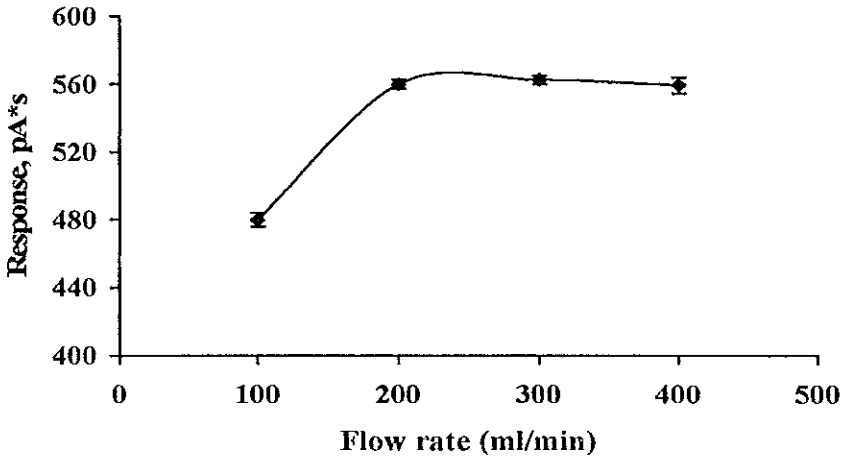


Figure 28 The response of 100 µg ml^{-1}, 1-µl 3-MCP-phenylboronate ester derivative standard working solution various the flow rates of oxidant (air) gas

3.5.5 Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in sample which can be detected but not necessary quantified as an exact value. Several approaches for determining the detection limit are possible, depended on the procedure either non-instrumental or instrumental. In this work, the detection limit was based on the signal-to-noise ratio, this approach can only be applied to analytical procedures which exhibit baseline noise. Determination of signal-to-noise ratio is performed by comparing measured signal from samples with low concentrations of analyte with those blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio of 3:1 is generally considered acceptable for estimating the detection limit (www.sac.accreditation.org.sg). In this thesis, the limit of detection of 3-MCPD which was 3-MCP-phenyboronate ester form was 0.5 $\mu g\ ml^{-1}$. The detection limit of 3-MCPD which derivatized with other derivatizing reagent were reported as shown in Table 17.

Table 17 The detection limit of different derivative of 3-MCPD

Chemical derivatization	Detector	Detection limit	Reference
heptafluorobutyric acid anhydride (HFBA)	GC-MS	5.0 μg kg^{-1}	Chung *et al.*, 2002
Acetone	GC-MS	1.0 μg kg^{-1}	(Meierhans *et al.*, 1998
N,O-bis-(trimethylsilyl	GC-MS	0.04 mg kg^{-1}	Bodén. *et al.*, 1997
trifluoroacetamide (BSTFA)	GC-FID	0.43 μg ml^{-1}	Kissa, 1992
heptabutyrylimidazole (HFBI)	GC-MS	50 μg kg^{-1}	Bergen. *et al.*, 1992
		0.01 mg kg^{-1}	Hamlet.*et al.*, 2002
butaneboronic acid	GC-ECD	0.1 μg ml^{-1}	Pesselman. and Feit., 1988.
phenylboronic acid	GC-FID	0.2 ppm	Plantinga., *et al.*, 1991

The limit of detection of 3-MCPD was nearly the same as Kissa (1992) and Plantinga *et al.* (1991) because the FID gave good sensitivity. However, in this work, the amount of phenylboronic acid concentration used for derivatization and the injected volume to GC-FID was less than reported by Plantinga *et al.* (1991) (250 mg ml^{-1} phenylboronic acid and 2

μl injected to GC-FID). Therefore, the limit of detection of 3-MCPD was very satisfactory for this work.

3.5.6 Linearity and range

Linearity should be evaluated by inspection of a plot signal as a function of analyte concentration or content. If there is a linear relationship, test result should be evaluated by appropriate statistical method. The correlation coefficient, y-intercept, slope of regression line and residual some of squares should be submitted. A plot of this data should be included. In this work, the linearity of 3-MCPD and 3-MCP-phenylboronate ester derivative were investigated. The results are shown in Table 18 and Figures 29 and 30 respectively

Table 18 The response of 3-MCP-Phenylboronate ester and 3-MCPD at various concentrations

Concentration (μg ml^{-1})	Response, pA*s $\times 10^2$	
	Dilution 3-MCP-phenylboronate ester	Dilution 3-MCPD
0.5	-	0.0127
0.6	-	0.0158
0.8	-	0.0185
1.0	0.178	0.0424
3.0	0.066	0.0969
5.0	0.131	0.170
10	0.266	0.319
20	0.552	0.579
30	0.857	0.892
50	1.45	1.51
100	3.25	3.25
300	8.67	8.80
500	14.41	15.27
600	17.53	18.34
700	19.47	21.49
800	21.69	23.12
900	24.05	25.83
1000	29.44	29.48

* 5 replication, RSD < 4%

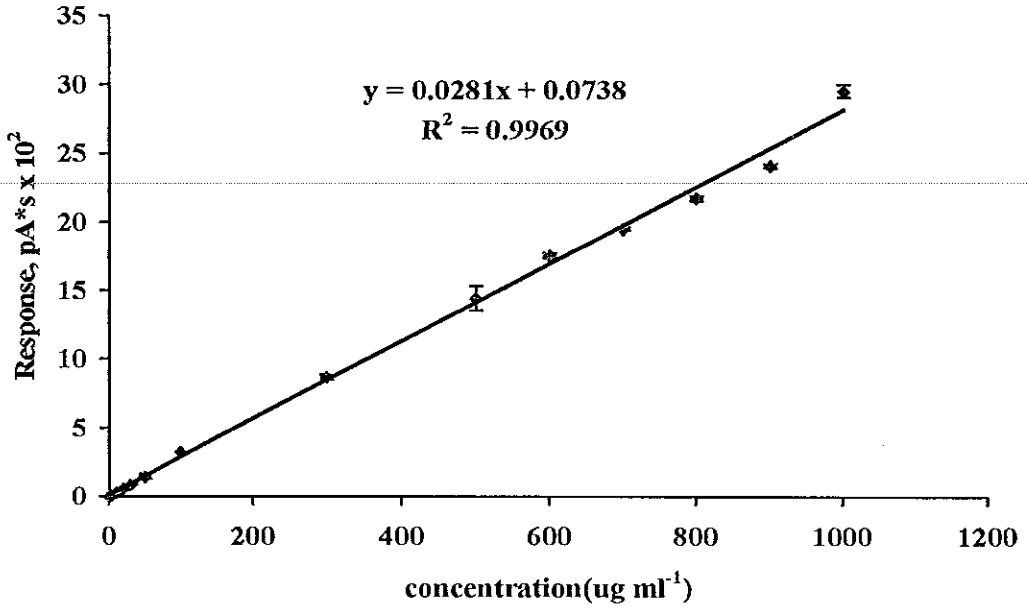


Figure 29 The response of dilution of 3-MCP-phenylboronate ester derivative at various concentrations

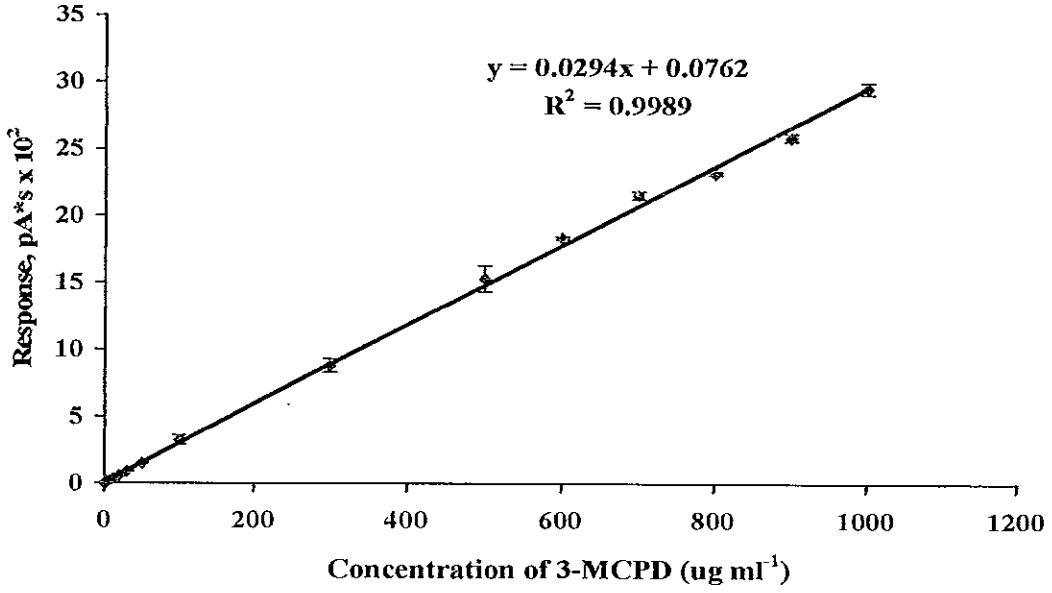


Figure 30 The response of dilution of 3-MCPD derivative at various concentrations

From the results, the linearity of dilution of 3-MCPD was 0.5-1000 μg ml^{-1} and the linearity of dilution of 3-MCP-phenylboronate ester derivative was 1-1000 μg ml^{-1}. Although, the linearity of FID detector is good for a range as high as 10^8 (Grob, 1985) but generally 3-MCPD concentrations in soy sauce were less than 1000 ug ml^{-1} so, this concentration is enough to analysis. In this work, the dilution of 3-MCPD was chosen, because it gave lower detection limit and better linear regression than 3-MCP-phenylboronate ester derivative.

Table 19 The linear dynamic range of different derivative of 3-MCPD

Chemical derivatization	Detector	Linear dynamic range	Refereces
heptafluorobutyric acid anhydride (HFBA)	GC-MS	10-1000 μg kg^{-1}	Chung *et al.*, 2002
N,O-bis-(trimethylsilyl trifluoroacetamide (BSTFA)	GC-MS	0.05-2.00 μg kg^{-1}	Bodén. *et al.*, 1997
heptabutyrylimidazole (HFBI)	GC-MS	0.1-500 mg L^{-1}	Bergen. *et al.*, 1992 and Feit., 1988.
phenylboronic acid	GC-FID	0.53-13.7 mg L^{-1}	Plantinga., *et al.*, 1991

Table 19 shows linear dynamic range of derivative of 3-MCPD reported by several workers. For GC-FID the linear dynamic range of 3-MCPD in

this work was better than the concentration range of the calibration curve reported by Plantinga *et al.*(1991). Therefore, these results indicated that the FID gave the wide sensitivity detection over wide range of concentration.

3.6 Sample preparation

3.6.1 Solvent flow rate

The rate at which solvent flows through the column is also significant for the effectiveness of a separation. In general, the time the mixture to be separated remains on the column is directly proportion to the extent of equilibration between stationary and moving phases. Thus, similar compounds eventually separate if they remain on the column long enough. The time a material remains on the column depend on the flow rate of solvent (www.ares.unitmet.edu.ev/quimica/bpqo02/ cromatog.pdf). The flow rate for sample preparation was varied rate as in 2.4.1 and the results are shown in Table 20 and Figure 31.

Table 20 The effect of flow rate on eluting 3-MCPD

Flow rate(ml/min)	Response, pA*s
4	23.50
6	24.28
8	29.19
10	22.54

* 5 replications, RSD< 10 %

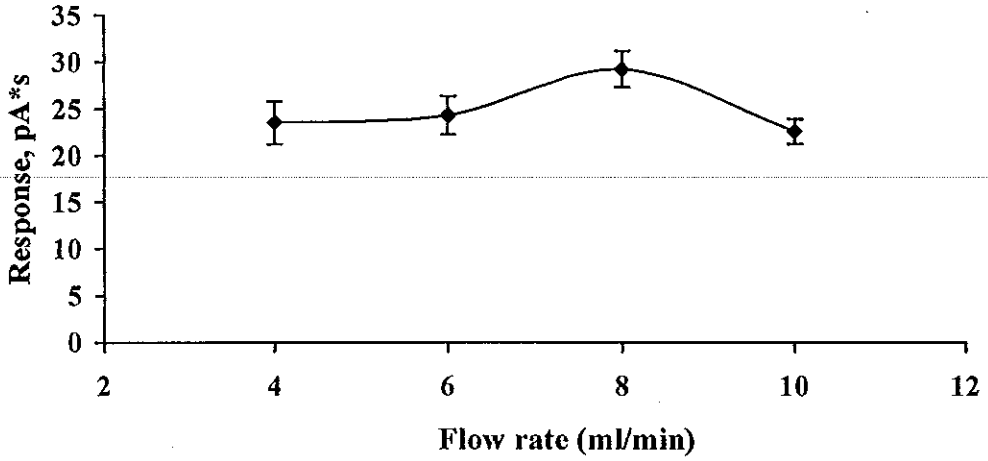


Figure 31 The response of 3-MCP-Phenylboronate ester derivative at various flow rates

From the result, the flow rate for sample preparation at 8 ml/min was used to elute 3-MCPD. In this work, the analyte are retained by silica gel. In silica gel (Figure 32), the adsorbent sites are oxygen atoms and silinol groups (-Si-OH) that easily form the hydrogen bonds with polar molecule (Fifield F.W. and Kealey D., 2000) i.e.the hydroxyl group of 3-MCPD. Therefore, the flow rate to elute 3-MCPD should be sufficiently strong to break the bond.

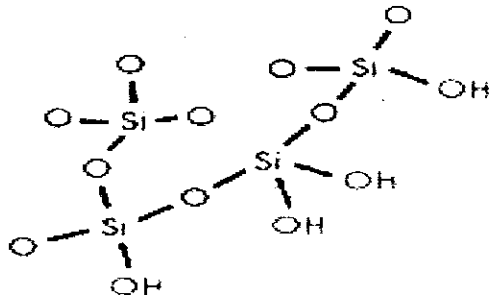


Figure 32 Silica gel structure

3.6.4 Extraction time

Extraction time is one of the factors affecting the efficiency of 3-MCPD extraction. Extraction times were varied from 0-20 minutes (2.4.2) at an interval of 5 minutes and the result are shown in Table 21 and Figure 33.

Table 21 The effect of extraction time on extracting 3-MCPD

Time (minute)	Response, pA*s
0	20.38
5	20.78
10	22.21
15	25.84
20	23.81

* 5 replications, RSD< 10 %

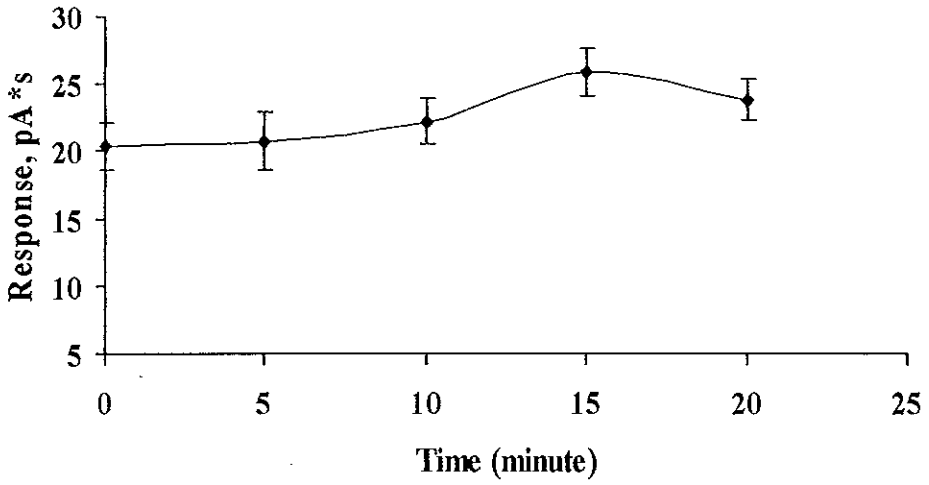


Figure 33 The response of 3-MCP-Phenylboronate ester derivative at various extraction times

From the results, when the extraction time increased, the response of analyte also increased, *i.e.*, extraction efficiency increase with time. The optimum extraction time was obtained at 15 minutes.

3.6.5 Eluting Solvent

Three solvents were used for eluting 3-MCPD as in 2.4.3 and the results showed in Table 22 and Figure 34. The some eluting solvents were selected from literature, such as ethyl acetate (Chung, *et al.*, 2002), and diethyl ether (Matthew and Anastasio 2000, and Mierhans, *et al.*, 1998).

Table 22 The effect of solvent on eluting 3-MCPD

solvents	Response, pA*s ±SD
Ethyl acetate	14.4±0.4
Diethyl ether	11.0±1.0
Dichloromethane	2.8±0.7

* 5 replications, RSD< 10 %

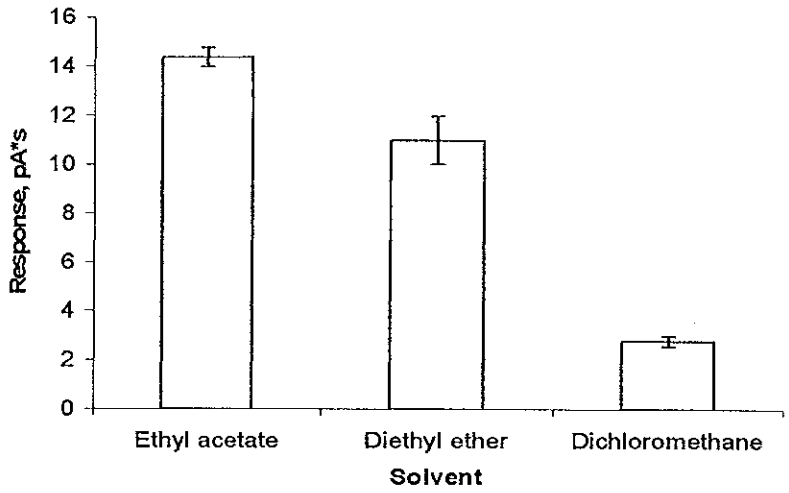


Figure 34 The response of 3-MCP-Phenylboronate ester derivative at various solvents

From the result, the appropriate solvent for eluting 3-MCPD was ethyl acetate which gave the highest response. Because 3-MCPD is polar, so, ethyl acetate has enough polarity to elute 3-MCPD. In addition, the extraction efficiency depends on polarity index, dipole moment, dielectric constant and solubility. These values were shown in Table 23

Table 23 The influent of polarity index, dipole moment, dielectric constant and solubility on solvents

Solvents	Polarity* Index	Dipole* moment	Dielectric* constant	Solubility*
ETOAC[1]	4.40	1.78	6.02	8.70
DET[2]	2.80	1.15	4.34	6.89
DCM[3]	3.10	1.60	8.90	1.60

[1] ETOAC = Ethyl acetate

[2] DET = Diethyl ether

[3] DCM = Dichloromethane

*Srichana, 2002

Normally, the organic solvents that have high polarity will have high dielectric constant and in contrast, solvent that have low polarity will have low dielectric constant. The dielectric constant of ethyl acetate, diethyl ether and dichromethane are shown in Table 23. In addition, the molecules that have high polarity index will have strong polarity and corresponded to dipole moment and solubility. The polarity index, dipole moment and solubility of ethyl acetate, diethyl ether and dichloromethane are shown in Table 23 respectively. Moreover, the structure of 3-MCPD was polar due to strong solubility in water. Thus, comparing to any

properties as described above, it showed 3-MCPD could partition in to ethyl acetate better than diethyl ether.

3.6.6 Volume of eluting solvent

The eluting solvent volume was an important parameter because it would affect the extraction efficiency. If the volume is too small, it may not be sufficient to elute the analyte. Column chromatographic technique required organic solvent volume large enough to completely elute 3-MCPD. The volume of organic solvent used in extraction process was optimized in 2.4.4 and the results are shown in Table 24 and Figure 35. The suitable solvent volume to extract 3-MCPD was selected as 150 ml.

Table 24 The effect of volume of solvent on eluting 3-MCPD

Volume	Response, pA*s
50	14.5
100	18.0
150	32.7
200	33.3

* 5 replications, RSD< 10 %

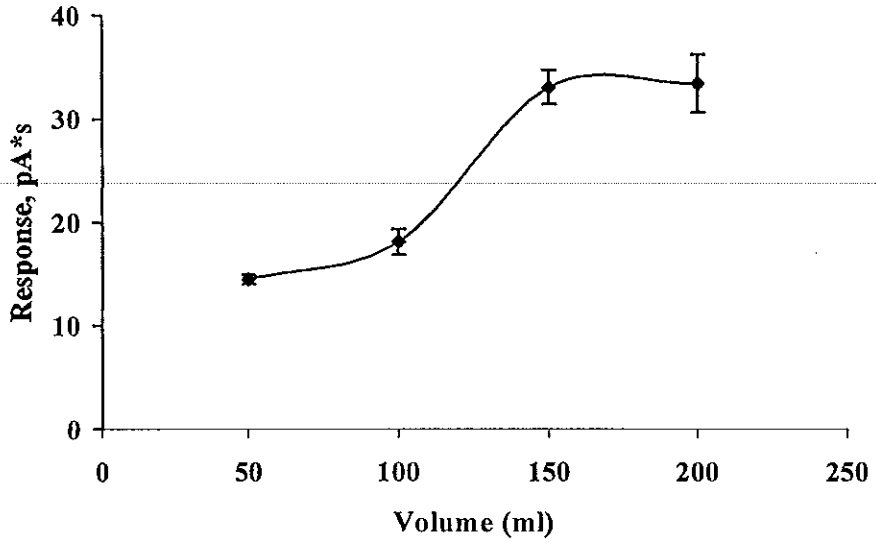


Figure 35 The response of 3-MCP-Phenylboronate ester derivative at various volumes of solvent

3.6.7 Clean up & Recovery

For clean up, the sample was eluted by ethyl acetate-hexane (10:90 v/v) to clean the non-polar compounds in the sample followed by the elution of 3-MCPD by ethyl acetate. The comparison between the clean up and non-clean up was done and the results are shown in Table 25 and Figure 36.

Table 25 Comparison of percentage recovery of non clean up and clean up method

Method	% recovery ±SD
Non clean up	95±9
Clean up	90±5

* 5 replications, RSD< 10 %

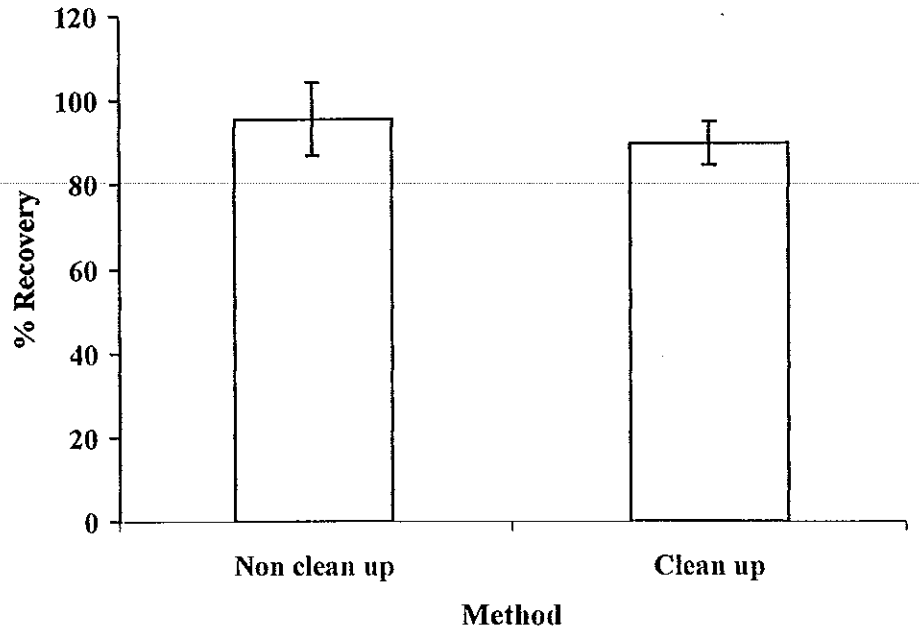


Figure 36 The effect of non clean up and clean up method to recovery of 3-MCPD

From the results, the percentage of recoveries of non cleaning up and cleaning up were 95±9% and 90±5% respectively. The recoveries were not different, thus, the non-cleaning up was applied for the analysis. Silica gel 60 (230-400 mesh) was compared with other adsorbent (silica gel 60 (60 mesh) and diatomaceous earth or Extrelute). The recoveries of all three materials were approximately the same (Table 26). Therefore, either of these can be used.

Table 26 Percentage of 3-MCPD recovery with various adsorbents

adsorbent	% recovery ±SD
Silica gel 60 (230-400 mesh)	94.5±8.8
Silica gel 60 (60 mesh)[4]	98.0±4.9
Diatomaceous earth[5]	96.6±0.2

[4] Chung *et.al.*, 2002

[5] Spyres , 1993

3.7 Qualitative and quantitative analysis of 3-MCPD in soy sauce

Five brands of soy sauces were bought from department stores. Soy sauce samples were extracted by column chromatography and analyzed by GC-FID at optimum conditions. The external standard method and internal standard method were applied for qualitative and quantitative analysis. The analysis of 3-MCPD in soy sauce, the effect of the matrix of soy sauce was investigated by applied the internal standard technique. This method used n-hexadecane as an internal standard and described 2.5.3. The results are shown in Table 27 and Figure 37.

The samples were analyzed by GC-FID at optimum conditions and the chromatogram of samples is shown in Figure 38. The results showed that 3-MCPD contaminated in five brands of soy sauce in range from non-detectable to 57.07 $\mu g\ g^{-1}$ in Table 28.

Table 27 The relation between the concentration of 3-MCPD and the ratio of peak area of analyte to internal standard (A_a/A_{is})

Concentration (μg/g)	$A_a/A_{is} \times 10^{-2}$
3	2.12
5	3.33
10	7.00
25	19.8

* 5 replications, RSD< 10 %

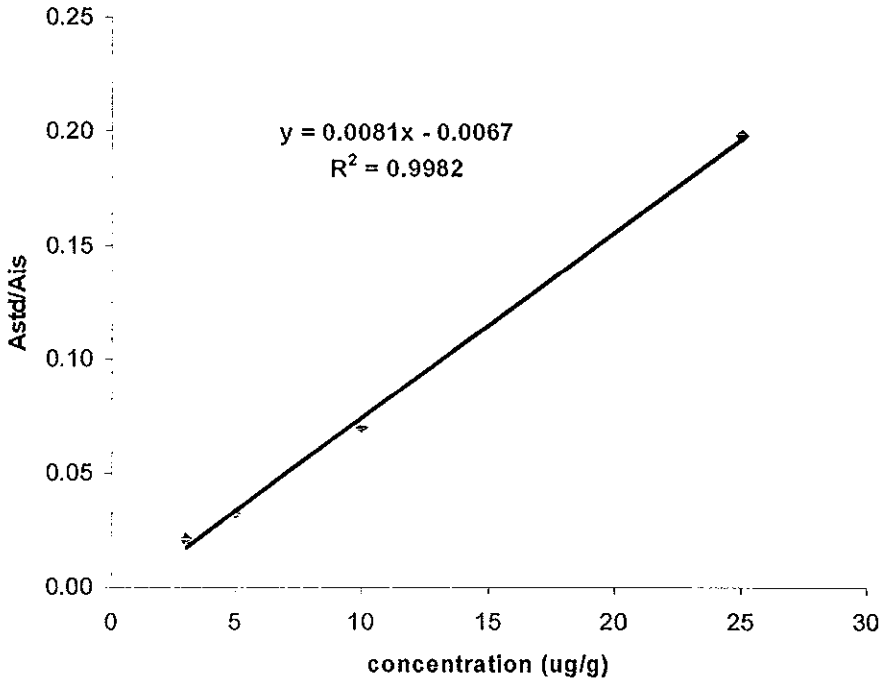
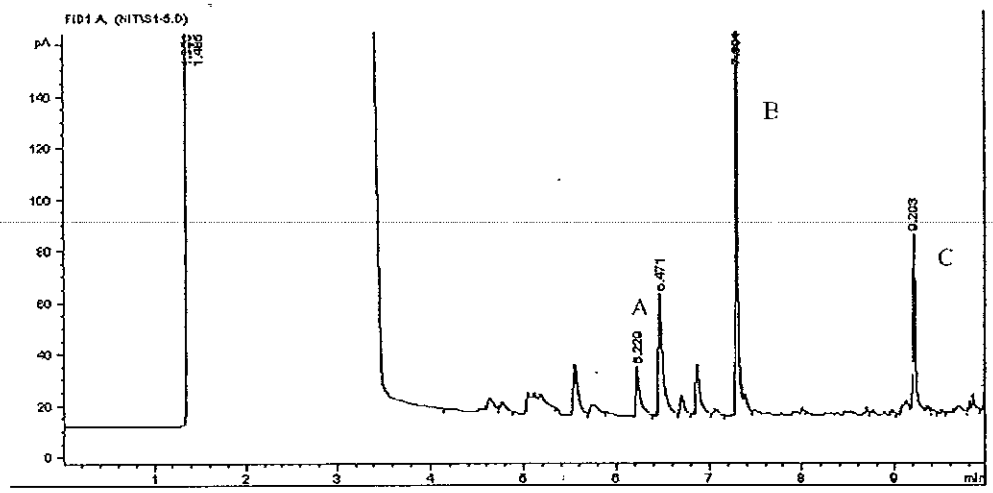


Figure 37 The relationship between concentration of 3-MCP-phenyboronate ester derivative and the ratio of peak area of analyte to internal standard



A = phenylboronate ester derivative B = internal standard, n-hexadecane

C = phenylboronic acid

Figure 38 Chromatogram of sample

Table 28 The concentration of 3-MCPD that contaminated in samples

Samples	Net. Volume	Date of Expire	Date of analysis	Concentration ($\mu g\ g^{-1}$)
Soy sauce1	100	10-12-04	05-03-04	57.07±8.03
	740	10-10-06	23-02-04	2.14±0.15
Soy sauce 2	700	12-01-07	23-02-04	2.31±0.45
Soy sauce 3	700	01-06-05	23-02-04	n.d.
Soy sauce 4	600	09-03-05	24-02-04	1.44±0.03
Soy sauce 5	700	01-12-06	24-02-04	n.d.

* 5 replications, RSD< 10 %

From Table 28, soy sauce 1, 2 and 4 were contaminated over legal limit (European Commission's Scientific Committee on Food (SCF) < 0.01 $\mu g\ kg^{-1}$, European Union (EU) ≤ 0.05 mg kg^{-1}, MAFF < 0.01 mg kg^{-1},

Finland and Austria ≤ 1.00 mg kg^{-1} (www.fda.moph.go.th/fda-net/html/chemical/News_ipcs6-3/mcpd.html)).

Chapter 4

Conclusions

3-Monochloropropane-1, 2-diol or 3-MCPD is a compound that found in acid-hydrolyzed vegetable protein (acid-HVP). It is formed as a result of high temperature chlorination of glycerol present in fat and oils include protein starting material. The analysis of 3-MCPD was performed by derivatization with phenylboronic acid as derivatizing reagent. The derivative of 3-MCPD was analyzed by GC-FID at optimum conditions. Samples preparation was carried out by column chromatography. The proposed method was suitable for qualitative and quantitative analysis of 3-MCPD in soy sauce.

The method used in this work consisted of three parts; derivatization reaction, gas chromatographic analysis, and sample preparation. The derivatizative of 3-MCPD with phenylboronic acid was confirmed by IR spectrometry and gas chromatography with mass spectrometry in SIM mode. Gas chromatography with flame ionization detector was to be the technique for separation these compounds (phenylboronic acid and 3-MCP-phenylboronate ester derivative).

In derivatization reaction step, the optimization conditions of temperature and time of derivative reaction were 90 $^{\circ}$C and 30 minutes. After the reaction completed, the analyte was extracted to organic layer for 20 seconds.

In optimization conditions for GC-FID, the 3-MCP-phenylboronate ester derivative was separate by HP 5 capillary column (30 m $\times$ 0.32 mm

I.D., 0.25 film thickness of 5% phenyl 95% dimethylpolysiloxane). Optimum conditions for GC techniques were, carrier gas (He) flow rate at 3 ml/min, the column temperature programming was achieved as: initial temperature 90 °C for 1 min, ramped to 270 °C at 15 °C/min. The optimum temperature for injector and detector were 250 °C and 280 °C respectively. For FID detector, make up gas (N_2) flow rate was 30 ml/min and fuel (H_2) gas flow rate: oxidant (air) gas flow rate was 30:300 ml/min. These optimum conditions provided low detection limit (0.5 μg ml^{-1}) and wide linear dynamics range (0.5 -1000 μg ml^{-1}) with a linear regression (R^2) of greater than 0.99.

In sample preparation technique, the column chromatographic extraction was applied. 3-MCPD was extracted by ultra sonic bath for 15 minutes and adsorbed on silica gel 60, 230-400 mesh, in column of 20 mm I.D. × 400 mm length. The analyte was eluted by ethyl acetate at 150 ml with a flow rate at 8 ml/min. In this study, the sample preparation had no need for the clean up step and gave a high percentage recovery (94.5±8.8%).

For qualitative and quantitative analysis of 3-MCPD in soy sauce, five brands of soy sauces were sampling from the department stores in Hat Yai, Songkhla. The concentrations were found in the range of non-detectable to 57.07±8.03 μg g^{-1}.

Although some of the derivatives still need a rather time-consuming procedure but the method it can solve some of the problems such as increasing the volatility and decreasing the polarity of polar compound, improving both selectivity and chromatographic efficiency,

and enhancing detectability. For column chromatographic extraction, this method is simple, cost effective and can be used for routine analysis. Moreover, this method could provide useful information for advance analysis of 3-MCPD in other foods.

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