DEVELOPMENT OF ANALYTICAL METHOD FOR BISPHENOL A DIGLYCIDYL ETHER (BADGE) IN OIL PHASE FROM CANNED TUNA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) TECHNIQUE

SUPAWADEE NORADEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (INDUSTRIAL HYGIENE AND SAFETY) FACULTY OF GRADUATE STUDIES MAHIDOL UNIVERSITY 2006

ISBN 974-04-7945-6

COPYRIGHT OF MAHIDOL UNIVERSITY

Copyright by Mahidol University

Thesis
Entitled

DEVELOPMENT OF ANALYTICAL METHOD FOR BISPHENOL A DIGLYCIDYL ETHER (BADGE) IN OIL PHASE FROM CANNED TUNA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) TECHNIQUE

....
Miss.Supawadee Noradee
Candidate

....
Assoc.Prof.Pornpimol Kongtip,
Ph.D. (Occupational Health Science)
Major-Advisor

....
Assoc.Prof.Witaya Yoosook,
D.Eng (Process Engineering)
Co-Advisor

....
Asst.Prof. Jaranit Kaewkungwal
Ph.D. (Biostatistics)
Co-Advisor

....
Lect.Suttinun Chantanakul,
M.D.
Co-Advisor

....
Prof. M.R. Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies

....
Asst.Prof. Preecha Loosereewanich,
Ph.D.(Occupational Health Science)
Chair
Master of Science Programme in
Industrial Hygiene and Safety
Faculty of Public Health

Copyright by Mahidol University

Thesis
Entitled

DEVELOPMENT OF ANALYTICAL METHOD FOR BISPHENOL A DIGLYCIDYL ETHER (BADGE) IN OIL PHASE FROM CANNED TUNA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) TECHNIQUE

was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Master of Science (Industrial Hygiene and Safety)
on
29 November, 2006.

....
Miss.Supawadee Noradee
Candidate

....
Assoc.Prof. Chintana Saiwan,
Ph.D. (Chemistry)
Chair

....
Lect.Suttinun Chantanakul
M.D.
Member

....
Assoc.Prof. Pornpimol Kongtip,
Ph.D.(Occupational Health Sceince)
Member

....
Asst.Prof. Jaranit Kaewkungwal
Ph.D.(Biostatistics)
Member

....
Assoc.Prof.Witaya Yoosook,
D.Eng (Process Engineering)
Member

....
Prof. M.R. Jisnuson Svasti, Ph.D
Dean
Faculty of Graduate Studies
Mahidol University

....
Assoc.Prof.Phitaya Charupoonphol,
M.D., Dip. Thai Board of Epidemiology
Dean
Faculty of Public Health
Mahidol University

Copyright by Mahidol University

ACKNOWLEDGEMENT

The success of this thesis is of strong work which I would like to attribute for the extensive understanding and support by many people. It is a pleasant aspect that I have the opportunity to express my heartfelt gratitude to all of them. I think this is a good possibility that I wish to thank to all of sincere help and earnest participation in my study.

First of all, I would like to express my deepest and sincere gratitude to my kindsupervisor, Assoc. Prof. Dr. Pornpimol Kongtip, for her guidance, valuable advise, encouragement throughout this study in which enable me to carry out of this thesis successfully.

I am grateful to my Co-advisor, Assoc. Prof. Dr. Wittaya Yoosook, Lect. Suttinun Chantanakul for their valuable advice, suggestion, discussions and comments. I also would like to give sincere thanks my Co-advisor, Assist. Prof. Jaranit Kaewkungwan for his guidance and support with the respect of statistical analysis.

I am also grateful to Assoc. Prof.Chintana Saiwan who was the external examiner for my thesis defense, and who made suggestions and recommendations to support me in this thesis.

I would like to express my special thanks to Ms. Sukrauthai Wongsubin, Ms. Pradup Mesawat, Mr. Sirichai Kosittarat and staff of Central Instrument Facility for their kindly help, advice and encouragement and providing all laboratory facilities during the study.

Finally, I would like to take this opportunity to express my gratitude to my company to allow me to concentrate in analysis of the data. Deepest thanks to my family and my friends for giving me so much support, entirely care, love and power to this success of my thesis.

Supawadee Noradee.

Copyright by Mahidol University

DEVELOPMENT OF ANALYTICAL METHOD FOR BISPHENOL A DIGLYCIDYL ETHER (BADGE) IN OIL PHASE FROM CANNED TUNA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)TECHNIQUE

SUPAWADEE NORADEE 4536285 PHIH/M

M.Sc. (INDUSTRIAL HYGIENE AND SAFETY)

THESIS ADVISORS: PORNPIMOL KONGTIP, Ph.D.(Occupational Health Science), WITAYA YOOSOOK, D.Eng. (Process Engneering), SUTTINUN CHANTANAKUL, M.D., JARANIT KAEWKUNGWAL, Ph.D.(Biostatistics)

ABSTRACT

Bisphenol A Diglycidyl Ether (BADGE) is one of chemical substance which can be migrated into food from internal coating. The most popular interior can coating often uses epoxy resin and vinyl organosol. The purpose of this research was to develop an analytical method for quantification of BADGE in oily phase of canned tuna from collected canned samples in markets of Thailand by using High Performance Liquid Chromatography (HPLC) and evaluate the reliability of analyzing method.

The oily phase from canned sample was extracted by liquid-liquid extraction by using n-hexane and acetonitrile. The separation condition of BADGE analysis was carried out by reversed-phase high performance liquid chromatography (RP-HPLC) using isocratic condition with fluorescence detection. Symmetry C_{18} column used as an analytical column and isocratic system was acetonitrile and water. The suitable flow rate was 1.0 ml/min. The results of extraction, recovery efficiency is ranged between 98.23 % to 101.90%, the accuracy of analysis ranged between 100.22 % to 102.82%. BADGE concentrations released from canned tuna which was in storage for less than 6 months were 68.073 µg/l. For the canned tuna store longer than 6 months, BADGE concentration was 36.454 µg/l. The limit of detection of this analysis is 0.4141µg/l. The overall accuracy and precision of analysis method of BADGE were 0.17% and 0.85%, respectively. They were acceptable to the NIOSH an analytical criteria.

The results showed that this analytical method can be used for analyzing BADGE in oily phase. Furthermore, this method can be use as a guideline to develop method for analyzing BADGE in different types of canned food.

KEY WORDS: BISPHENOL A DIGLYCIDYL ETHER (BADGE) / HPLC / CANNED TUNA

67 P. ISBN 974-04-7945-6

Copyright by Mahidol University

การพัฒนาวิธีวิเคราะห์สาร บิสฟีนอล เอ ไดไกลซิดิล อีเทอร์ ในตัวกลางที่เป็นน้ำมันจากปลาทูน่ากระป๋องตัวอย่าง ด้วยเทคนิค HPLC (DEVELOPMENT OF ANALYTICAL METHOD FOR BISPHENOL A DIGLYCIDYL ETHER (BADGE) IN OIL PHASE FROM CANNED TUNA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) TECHNIQUE.

สุภาวดี โนระดี 4536285 PHIH/M

วท.ม. (สุขศาสตร์อุตสาหกรรม และ ความปลอดภัย)

คณะกรรมการควบคุมวิทยานิพนธ์ : พรพิมล กองทิพย์, Ph.D.(Occ. H.Sci.), วิทยา อยู่สุข D.Eng.(Process Engineering), สุทธินันท์ ฉันท์ธนกุล, M.D., จรณิต แก้วกังวาล Ph.D.(Biostatistic)

บทคัดย่อ

สารบิสฟีนอล เอ ไดไกลซิดิล อีเทอร์ เป็นสารชนิดหนึ่งที่สามารถปลดปล่อยออกมาจากสารเคลือบด้านในของกระป๋องมาปนเปื้อนในอาหารที่บรรจุอยู่ภายในกระป๋องได้โดยสารเคลือบผิวด้านในที่นิยมใช้ คือ สารอีพอกซีเรซิน และสารไวนิลออร์กอนอซอล การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการวิเคราะห์เพื่อหาปริมาณสารบิสฟีนอล เอ ไดไกลซิดิล อีเทอร์ ในตัวกลางที่เป็นน้ำมันของปลาทูน่ากระป๋องซึ่งใช้ตัวอย่างกระป๋องที่มีจำหน่ายในตลาดของประเทศไทย โดยวิธีการวิเคราะห์ด้วยเทคนิค การแยกสารโดยใช้แรงดันของของเหลวที่มีค่าสูง รวมถึงได้ศึกษาความเชื่อถือได้ของวิธีการวิเคราะห์สารบิสฟีนอล เอ ไดไกลซิดิล อีเทอร์นี้ด้วย ชั้นของน้ำมันจากตัวอย่างที่ใช้ในการวิเคราะห์นั้นผ่านการสกัดด้วยวิธีใช้ตัวทำละลายที่เป็นของเหลว ใช้ตัวทำละลายเป็นเอ็น-เฮกเซน และอะซิโตไนไตร์ สภาวะของการแยกสารบิสฟีนอล เอ ไดไกลซิดิล อีเทอร์ ทำได้โดยใช้ เทคนิค การแยกสารโดยใช้แรงดันของของเหลวที่มีค่าสูง โดยระบบของตัวทำละลายมีค่าคงที่ตลอดระยะเวลาที่ทำการวิเคราะห์ มีการใช้ดีเทคเตอร์แบบฟลูออเรสเซนส์ คอลัมน์ที่นำมาใช้ในการวิเคราะห์คือ คอลัมน์ คาร์บอน C_{18} แบบสมมาตร อัตราการไหลที่เหมาะสม มีค่า 1.0 มิลลิลิตรต่อนาที ผลของการวิเคราะห์พบว่า ประสิทธิภาพในการสกัดมีค่าอยู่ระหว่างร้อยละ 98.23-101.90 และค่าความถูกต้องของการวิเคราะห์มีค่าอยู่ระหว่างร้อยละ 100.22-102.88 ปริมาณ สารบิสฟีนอล เอ ไดไกลซิดิล อีเทอร์จากตัวอย่างกระป๋องที่มีอายุการจัดเก็บน้อยกว่าหรือเท่ากับ 6 เดือน มีค่า 55.235ไมโครกรัมต่อลิตร ที่อายุการจัดเก็บมากกว่า 6 เดือนมีค่า 36.454 ไมโครกรัมต่อลิตร ปริมาณสารที่วิเคราะห์ได้น้อยที่สุด คือ 0.4141ไมโครกรัมต่อลิตร และค่าความถูกต้องแม่นยำเท่ากับร้อยละ 0.17 และ 0.85 ซึ่งเป็นไปตามเกณฑ์ของ NIOSH

ผลการศึกษาในครั้งนี้ จะเห็นว่าวิธีการวิเคราะห์สารนี้สามารถใช้ได้กับการวิเคราะห์สารบิสฟีนอล เอ ไดไกลซิดิล อีเทอร์ในตัวกลางที่เป็นน้ำมันได้และนำไปประยุกต์ใช้กับการวิเคราะห์สารบิสฟีนอล เอ ไดไกลซิดิล อีเทอร์ในตัวอย่างกระป๋องประเภทอื่นได้

67 หน้า ISBN 974-04-7945-6

Copyright by Mahidol University

CONTENTS

		Page
ACKNOWLEDGEMENT		iii
ABSTRACT		iv
LIST OF TABLES		viii
LIST OF FIGURES		x
CHAPTER I	**INTRODUCTION**	
	Background and rationale	1
	Research question	4
	Objective	5
	Hypothesis	5
	Scope and limitation of study	5
	Variables	6
	Glossary term and Definitions	6
CHAPTER II	**LITERATURE REVIEW**	
	Bisphenol A diglycidyl ether (BADGE)	8
	Toxicokinetics	11
	Regulations	13
	Source of exposures	14
	Coatings	15
	High Performance Liquid Chromatography	23

Copyright by Mahidol University

CONTENTS (cont.)

		Page
	Literature cited	33
CHAPTER III	**MATERIALS AND METHODS**	
	Apparatus and chemical reagents	36
	Solution preparation for analysis	37
	Sample treatment	39
	HPLC condition for BADGE analysis	39
	Reliability of the method	40
	Subjects	41
	Statistical for analysis	42
CHAPTER IV	**RESULTS**	
	The calibration curve of BADGE analysis	44
	Recovery results of BADGE analysis	45
	Accuracy and Precision of the method	46
	Determination of limit of detection and limit of quantification	46
	Analysis of samples	48
CHAPTER V	**DISCUSSION**	
	Chromatographic separation and reliability of the method	50
	Canned tuna samples	52

Copyright by Mahidol University

CONTENTS (cont.)

		Page
	The comparison of BADGE concentration between Storage duration	53
	Other factors influencing BADGE concentration	54
CHAPTER VI	**CONCLUSION AND RECOMMENDATION**	
	Conclusion	55
	Recommendation	56
REFERENCES		57
APPENDIX		61
BIOGRAPHY		67

Copyright by Mahidol University

LIST OF TABLES

Table		Page
1-1	The Industrial Statistical of summarized all consuming product of canned tuna in 1999-2003 in Thailand. (ISIC:151210-010)	3
1-2	The Industrial Statistical of summarized all consuming product of canned sardine in 1999-2003 in Thailand. (ISIC:151220-020)	4
2-1	The detail of popular interior can coating	22
2-2	The properties of HPLC Mobile Phases	27
3-1	HPLC system for analysis (HPLC 2695)	39
4-1	Percentage recovery of BADGE extraction from oil in canned tuna	45
4-2	The accuracy of BADGE extraction from oil in canned tuna	46

Copyright by Mahidol University

LIST OF TABLES

Tables **Page**

1-1 The Industrial Statistical of summarized all consuming product of canned tuna in 1999-2003 in Thailand. (ISIC:151210-010) 3

1-2 The Industrial Statistical of summarized all consuming product of canned sardine in 1999-2003 in Thailand. (ISIC:151220-020) 4

2-1 The detail of popular interior can coating 22

2-2 The properties of HPLC Mobile Phases 27

3-1 HPLC system for analysis (HPLC 2695) 39

4-1 Percentage recovery of BADGE extraction from oil in canned tuna 45

4-2 The accuracy of BADGE extraction from oil in canned tuna 46

Copyright by Mahidol University

LIST OF FIGURES

Figure		Page
2-1	Chemical structure of BADGE	8
2-2	Synthesis process of Bisphenol A type resin	9
2-3	The picture of degradation of Polyvinylchloride polymer (from vinylorganosol coatings) at temperature of curing (A) , Reaction between BADGE and HCl (B)	9
2-4	Reaction of BADGE	10
2-5	Chemical Structures of BADGE and its derivatives	11
2-6	The picture of oxirane ring	19
2-7	Synthesis of epoxy resin	21
2-8	Vinylchloride vinyl acetate-copolymer	24
2-9	Schematic diagram of typical HPLC instrument	24
2-10	The structure of stationary phase	25
2-11	The solvent triangle	28
2-12	Schematic diagram of a loop injector in the (a) load (b) inject positions	30
2-13	Block diagram of a fluorescence detector	31
4-1	The Chromatogram of standard of BADGE	43
4-2	The Chromatogram of Blank oil	44
4-3	The calibration curve of BADGE in oil from canned tuna	45
4-4	Linear regression curve for determined LOD and LOQ	47
4-5	The chromatogram of BADGE in oil sample from canned tuna	48
4-6	The migration of BADGE in function of storage of time	49
5-1	The hydrolysis structure of BADGE	54

Copyright by Mahidol University

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Food is one of the main factors of human being life, such as growth, warmth, and prevention of diseases, brain development and intelligence to support working efficiency. Meanwhile it should be fresh food, cleanliness, pasteurization and safe. The contaminated food can cause illness or diseases to human being. The principle of Food Safety is H-A-C-C-P standing for Hazard Analysis Critical Control Point (HACCP), which is a system of preventive approach to identify potential food safety at various stage of food processing(4). It can be used to assess the related risks and to control food process effectively.

Each season crop from Fishery and Agriculture has more quantity than actual consumption. The preservation of food, therefore, plays an essential role to preserve food for consumption in other seasons and makes the advantage in keeping foods longer than their actual shelf life. One of the ways to preserve food is canned food(2) in which foods is kept in hermetically sealed container and vacuum stage sterile process in appropriate temperature and duration to control its product. Normally, shelf life of canned food is not more than 1-2 year but some kinds may have shorter temperature for keeping canned foods is 25°C (2).

Nowaday, Thailand is a country which has continual growth in both import and export market of canned foods. The laws of packaging is focus on appearance on packaging such as body, lid, opening method, testing method and control method after pasteurization (at 116-121°C). Thailand Industrial Standard controls chemicals releasing inside containers (3). The controlled chemicals are tin, cadmium, arsenic, phenol, formaldehyde, vinyl chloride monomer, epichlohydrin and other residue after evaporated by distilled water, citric acid, ethanol and heptane.

Copyright by Mahidol University

The raw material which used for making can is tin. The processes of can coating both inside and outside are to prevent defected can. (scratch, rust and corrosive from chemical inside or any contents) (4).

The inner coating is originally viscous liquid, which is changed to be adherence dry film coating on a surface of metal. The coating processes are composed appearance (5,6) of fixation and curing process that need suitable temperature to form coating film (5). Coating's types are divided into two parts: internal coating and external coating. In internal coating, the coating material contacts with foods directly, while the resistances of film and nutrition inside should be maintained. Internal coating is highly cross-linked to produce a thermosetting polymer and to prevent oxidation of metal. For external coating, the surface of can will resist to the scratch and make good appearance.

Bisphenol A diglycidyl ether (BADGE) is a monomer which is the product of reaction between Bisphenol A and Epichlohydrin (Fig.2-2)(5,7,8,9,10). It is also used as intermediate of epoxy resins, additive for polyesters, and scavengers for hydrochloric acid formed by degradation of PVC-based lacquers.(Fig.2-3) (5,7,8,9,10).

BADGE is listed in the EC Plastics Directive 90/128/EEC as a substance with toxic potential. The lipophilic character of BADGE is found in high concentrations and in oily foods (9,10).

In 1997, MAFF (Ministry of Agriculture Fisheries and Food) carried out the survey on migration of BADGE from canned food in United Kingdom. The results showed that the quantity of BADGE migrated from canned food over the limit of 1 mg /kg, recommended by the European Scientific Committee on Food (11,12). The results obtained also demonstrated that samples in oily phase gave the greatest proportion of BADGE. The toxicity of BADGE is related to cytotoxic effects in tissues with a high rate of cell division. It is listed as a mutagen, and primary irritant by the U.S. National Institute for Occupational Safety and Health. The European Commission considers extending the current legislation on plastics for food contacting

Copyright by Mahidol University

to surface coating in cans which the specify migration limits for BADGE of 1 mg/kg (11,12).

For the previous time, the safety of use BADGE (Bisphenol A diglycidyl ether) by European Legislation (Directive 2002/16/CE specified that the sum of the migration levels of BADGE, BADGE.H_2O, BADGE.HCl, BADGE.2HCl, BADGE.H_2O.HCl and BADGE.2H_2O shall not exceed the limit of 1mg/kg in foodstuffs or in food simulants (12,13,14,15). Recently, this regulation is repealed and changed the detail of migration limit into two groups; the sum of BADGE migration and hydrolysis form shall not exceed 9 mg/mg for the first group. The second group is the sum of migrations of chlorohydrins form shall not exceed 1 mg/kg (16).

The consumption of canned food was growing in Thailand, the statistical data from Industrial Committee in Domestic and up-country (from 1999-2005) which presents consuming data of both domestics and foreign country of canned tuna and sardine canned. (Table 1-1 and Table 1-2) (17)

Table 1-1 : Industrial Statistic of Conserved exported product of Canned Tuna in 1999-2005 in Thailand. (ISIC:151210-010) (17)

Year	Domestic consumption (mt ton)	Export consumption (mt ton)	Total consumption (mt ton)	Value of sale (Billion bath)
1999	3,937.39	138,345.76	142,283.15	11,486,245.81
2000	7,798.77	171,007.39	178,806.16	12,107,768.31
2001	13,239.15	266,407.01	279,646.16	17,784,431.11
2002	14,869.81	290,647.97	305,517.78	20,513,648.98
2003	16,917.28	311,916.23	328,833.58	23,634,746.87
2004	21,044.93	296,363.30	317,408.23	25,060,051.47
2005	9,653.93	389,536.75	399,190.65	42,623,563.67

(The Office of Industrial Economics)

Copyright by Mahidol University

Table1-2 : Industrial Statistical of Conserving export product of Canned Sardine in 1999-2005(ISIC:151220-020) (18)

Year	Domestic consumption (mt ton)	Export consumption (mt ton)	Total consumption (mt ton)	Value of sale (Billion bath)
1999	36,279.05	12,963.61	49,243.26	1,847,367.96
2000	37,815.07	24,744.56	62,559.63	2,193,824.07
2001	36,884.32	17,802.35	54,686.67	2,064,211.39
2002	33,086.56	18,361.64	51,448.20	2,071,289.81
2003	31,113.19	15,469.64	46,582.83	1,788,099.06
2004	28,309.02	13,004.83	41,313.85	1,726,615.99
2005	20,367.00	13,514.83	38,881.83	1,397,710.68

(The Office of Industrial Economics)

After reviewed the statistical information, the growth of canned food in Thailand was increasing significantly. Therefore, this research is interested to evaluate the quantity of BADGE in oily phase of tuna canned food.

The aim of this work is to develop a method for analysis of BADGE by using reverse-phase high performance liquid chromatography with fluorescence detection (8,10,11). The separation under gradient elution is initially studied to find the approximated gradient elution in order to improve resolution of samples. Liquid-liquid extraction will be employed with sample to decrease interference. The percentage of BADGE recovery and detection limit of method will be measured accordingly.

1.2 Research question

1. How much of BADGE residues in oil phase from canned tuna?
2. What is the suitable condition to extract BADGE from oil phase?
3. Is their any different BADGE quantity after storage of canned tuna for 6

Copyright by Mahidol University

month and over 6 months?

1.3 General objectives

To develop an analytical method for quantification of Bisphenol A Diglcidyl Ether (BADGE) in oily phase of canned tuna using High Performance Liquid Chromatography (HPLC).

1.4 Specific objectives

1. To study the suitable condition for analysis of BADGE by HPLC Technique.
2. To quantify the accuracy, precision and detection limit for analysis method of BADGE by HPLC.
3. To find out BADGE quantity in oily phase of canned tuna after storage for less than and equal to 6 and over 6 months.

1.5 Hyphothesis

1. The recovery of the extraction of BADGE from oily phase in canned tuna at various concentrations are greater than or equal to 75%.
2. BADGE quantity after storage of tuna canned food for more than 6 months is higher than that for less than equal to 6 months. .

1.6 Scope and Limitations

1. Inner coating type used for identification in this study is lacquer vinylic organozol type.
2. This study analysed BADGE quantity in vegetable oil from canned tuna (soybean oil).
3 Storage durations of tuna canned food are less than 6 months and over 6

Copyright by Mahidol University

months to 1 year.

1.7 Variables

Independent variable : Storage duration condition.

Dependent variable : Quantity of BADGE in canned tuna.

1.8 Definitions

Bisphenol A Diglycidyl Ether (BADGE) is the major component in commercial liquid epoxy resins which are manufactured by co- of proteins reacting of bisphenol A with epichlorohydrin. Liquid epoxy resins are either used as a binder in cured epoxy systems or further advanced with bisphenol A to higher molecular weight solid epoxy resins (5,8).

BADGE also used as additive to stabilize vinyl chloride and vinyl acetate in baking process of organosol lacquer.

Epoxy resin: It was one kind of thermosetting resin which was produced came from polymerization reaction of epoxide (in ethylene oxide or epichlohydrin) which good in adherance, flexibility and good chemical resistance(5,8).

Hydrolase: There are enzyme which catalyzes the hydrolysis proteinnases and peptidases of substance.

Accuracy: A measurement of the correctness of analytical results, as given by the difference between the analytical results and the true a known target. It is the difference between a physical quantity's average measurements and that of value, which give results in terms of percent accuracy. It refers to clustering of data about a known standard (19).

Precision: The extent of agreement between repeated, independent measurements of the same quantity of an analyte. Precision can be measured in terms of the standard deviation of the measurement system, which gives less than or equal to 10 percent coefficient of variation(19).

Copyright by Mahidol University

Percent of recovery (% recovery) : An estimation of the bias of an analytical Method(19). The percent recovery is calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Measured concentration}}{\text{Added concentration}} \times 100$$

Percent coefficient of variation (%CV) : The coefficient variation (%) for expression of dispersed statistical parameter, consisting of the standard deviation of the set of measurements divided by the mean (arithmetic or geometric) of the measurement, times 100 (19). The coefficient of variation is calculated using the following equation:

$$\% \text{ Coefficient of variation} = 100\,(SD/\overline{X})$$

Where; SD = standard deviation

$\overline{X}$ = mean value

Detection limit: The minimum quantifiable concentration, with a stated probability, measured by an analytical method giving and analytical precision less than or equal to 10 percent coefficient of variation. Using a predefined level of confidence, this is the lowest measured value at which some of the measured material is likely to have come from the sample (19).

Preservation: The process of packing products in the hermetically seal package. Pass the pasteurization process to destroy the microorganism (4,6).

Canned tuna : The canned tuna which supplied in Thailand.

Copyright by Mahidol University

CHAPTER II

LITERATURE REVIEW

The reviewed literature comprised BADGE, General Physical properties, reaction of BADGE, structure of derivatives of BADGE, toxicity of BADGE, regulations, coating knowledge and HPLC technique. The details are follows;

2.1 BADGE

2.1.1 General Information of BADGE (20,24)

BADGE (Bisphenol A Diglycidyl Ether)

CAS No.	: 1675-54-3
EINECS	: 216-823-5
Synonyms (NIOSH)	: 2,2-((1-Methyethylidene) bis(4,1phenylenemethylene)) Bisoxirane
Synonyms (IUPAC)	: 2,2-bis(4-hydroxyphenyl)propane bis(2,3epoxypropyl) ether
RTECS	: # TX3800000
EINECS Number	: 216-823-5
Chemical Formula	: $C_{21}H_{24}O_4$
Molecular weight	: 340-700
Chemical Structure	:

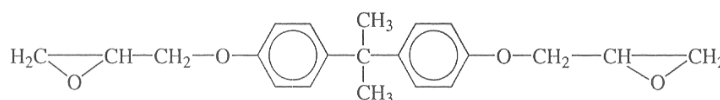


Fig.2-1 Chemical structure of BADGE

Copyright by Mahidol University

The reaction to form BADGE molecule (11).

BADGE is intermediate products of epoxy resin which is reacted from Bisphenol A and Epichlohydrin (Fig.2-1). BADGE molecule which is not completely reacted can migrate from epoxy-based packing material into the food. But in other ways, BADGE, PVC lacquers BADGE is added purposively to scavenge releasing hydrochloric acid during the heat treatment of the coating procedure. (Fig.2-2) (Elva M.Munguia-Lopezand Herlinda, 2001).

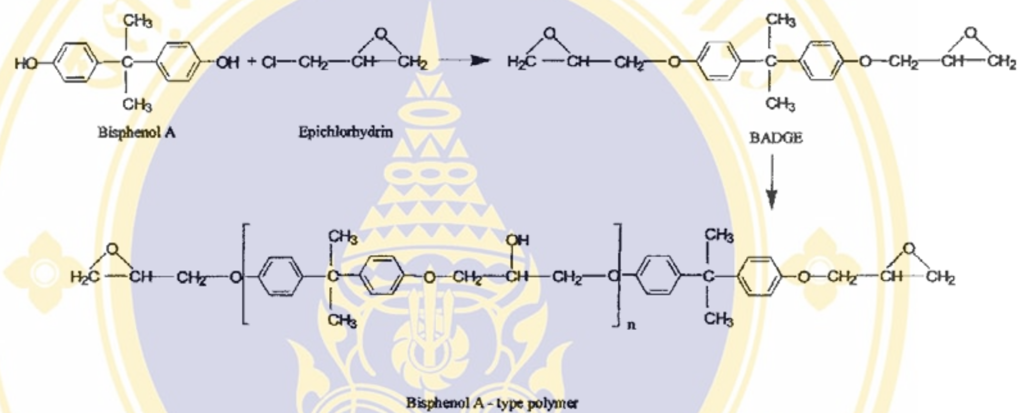


Fig 2-2. Synthesis process of Bisphenol A resin

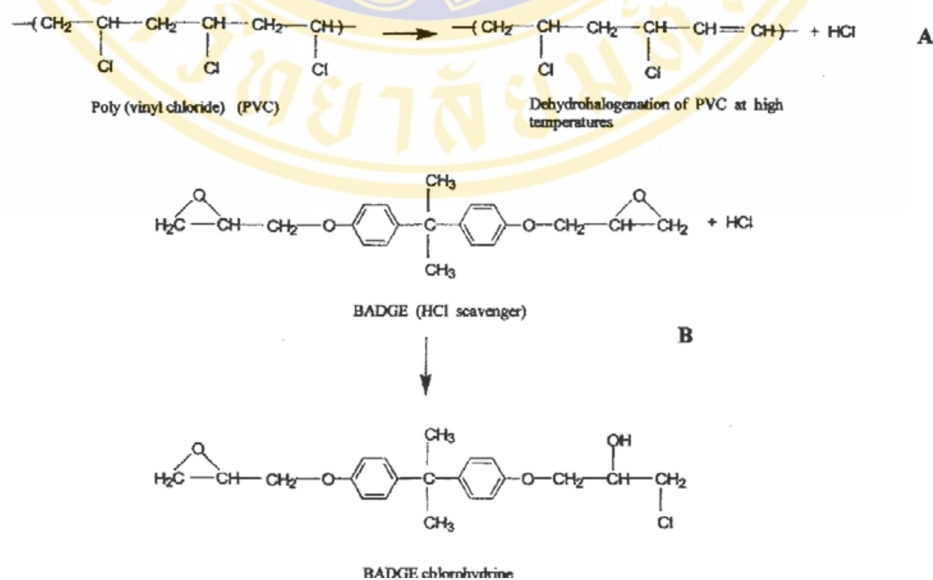


Fig.2-3.The degradation of Polyvinylchloride polymer (from vinyl organosol coatings) at temperature of curing (A) ; Reaction between BADGE and HCl (B)

Copyright by Mahidol University

The derivatives of BADGE were separated in two groups;

1. Hydrolyzed (BADGE·H_2O, BADGE·$2H_2O$, BADGE·HCl·H_2O).
2. Chlorinated compounds (BADGE·HCl, BADGE·2HCl). The reactions to get each derivative structure are as follows;

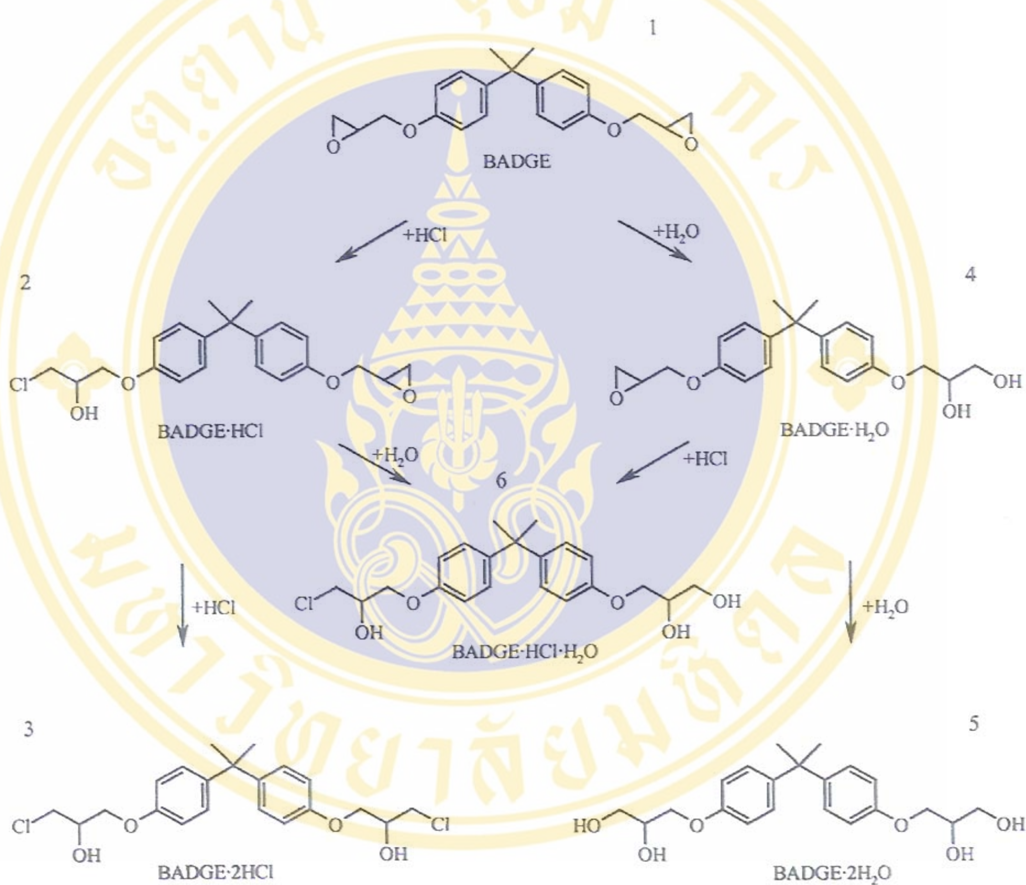


Fig.2-4 Reaction of BADGE (23)

Copyright by Mahidol University

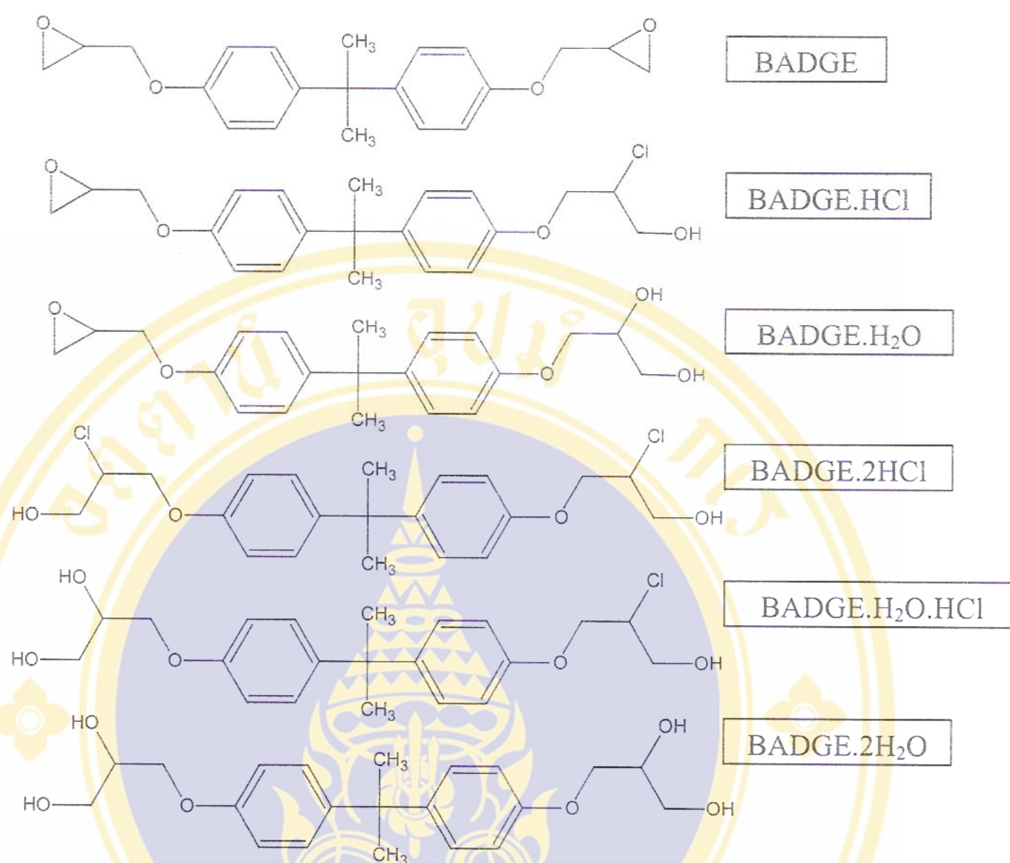


Fig.2-5 Chemical Structures of BADGE and its derivatives dihydroxypropyl ether) (8).

2.2 Toxicokinetics, metabolism and bioaccumulation (20)

Toxicokinetics and metabolism

Animal study

The toxicokinetics and metabolism of BADGE in intact animal systems and in tissue fractions have been studied in detail. Bentley et al (1989) investigated the hydrolysis of BADGE epoxide by the microsomal and cytosolic fractions of mouse liver and skin. It was reported that BADGE was rapidly hydrolyzed by the epoxide hydrolase of both tissues, with skin microsomal activity being about 10 times greater than that found in the cytosol of skin. In the other experiments using in vitro systems of liver fractions obtained from mouse, rat and rabbit, the two epoxide groups of

Copyright by Mahidol University

BADGE were very rapidly hydrolyzed to form the corresponding bis-diol of BADGE (bisphenol Abis(2,3-dihydroxypropyl ether). Further experiments showed no changes in the metabolism under conditions, which inhibited the epoxide hydrolase activity or promoted other breakdown mechanisms (for example oxidative metabolism). Formation of Bisphenol A from BADGE was not observed in any of these in vitro experiments. The metabolism of five different glycidyl ethers, including BADGE has been examined in human, rat and mouse liver, lung, and skin preparations (Boogaard et al 2000). The results of the metabolic studies found no evidence for any significant formation of Bisphenol A in incubations of BADGE with human or rodent liver or lung microsomal or cytosolic fractions, nor incubations with intact viable human or rodent skin. In intact animal systems, the fate of BADGE was studied in mice following oral or dermal administration (Climie et al 1981). Upon oral administration, BADGE was rapidly and extensively excreted with the faecal route being the major route of elimination while a small fraction of dose was recovered in the urine. The pattern of the metabolites identified in the urine and the faeces was consistent with the results of the in vitro studied and confirmed that the primary product of the metabolism of BADGE was the formation of the corresponding diol, which was further conjugated and/or converted to the corresponding carboxylic acids. Bisphenol A could not be detected in the excreta. Additional evidence indicating the inability of the mammalian biotransformation systems to convert BADGE to Bisphenol A was obtained in a similar experiment with BADGE diol, where Bisphenol A was not found in the urine or the faeces. Conveney (1983) reported that pathways in the rabbit appeared similar to those in the mice. Subsequently metabolic studies with Bisphenol A in the rat after oral or parenteral administration showed that Bisphenol A was eliminated primarily as unchanged material or as the mono-glucuronide conjugate of unchanged Bisphenol A (Pottenger et al 2000). Nolan et al (1981) reported routed-dependent difference in plasma 14C concentration-time profiles, tissue/plasma [14C] ratios, and urinary excretion following intra-veneous or oral administration of [14C] BADGE to rats. The [14C] BADGE was labeled at that isopropylidene methylene carbon. The plasma radioactivity resulted from intra-venous administration. The available data demonstrated that mammalian metabolic systems were unable to transform BADGE into Bisphenol A and it could be concluded that human

Copyright by Mahidol University

consumption of food containing low levels of BADGE would not lead to systemic exposure of Bisphenol A.

Human study (7,26)

Climie et al explained that BADGE rapid metabolized occurring by hydrolysis of BADGE by hydrolytic ring-opend 2 epoxide ring to form diol group.

The biomarker for hydrolysis product of BADGE (BADGE-4OH) in human Blood was β – glucuronides enzyme (Koichi Inoue et al, 2001).

2.3..Regulations (12,13,14,15,16)

In 1996, the committee provides 3 years more studied in DNA Binding on tissue of upper gastrointestinal tract with pure BADGE. The temporary restriction for specific migration of BADGE & its hydrolysis form will have the limit of 1 mg/kg food.

In 1999, The committee also concluded the temperate limit of restriction of present at 1 mg/kg food for the specific migration of BADGE and its hydrolysis products.

In 2002, Statement of the Scientific Committee on Foods (SCF) on BADGE examined the new mutagenicity on BADGE·2HCl, it was negative results obtained with BADGE·2HCl for mutation in mammalian cells in vitro. Negative results in assay for structural chromosomal aberrations in vitro. The positive response of BADGE·2HCl in vitro micronucleus might effect to chromosome segregation. The toxicology studied on mutagenicity test continue for 2 years in chronic toxicity/ carcinogenicity study.

In 2004, Information from European Food Safety Authority (EFSA); EFSA-9-2003-178, the new toxicological data of BADGE & BADGE·2HCl of SCF concerning the carcinogenic potential of BADGE and the genotoxicity of BADGE chlorohydrin was studied. The Scientific Panel on Food Additives, Flavoring, Processing Aids and Materials in contacts with Food (AFC)'s opinions concluded that new chronic toxicity/ carcinogenicity study in rat demonstrated following oral

Copyright by Mahidol University

administration BADGE had no potential in gastrointestinal tract in other tissues. The results of previous in vitro mutagenicity tests, concluded that BADGE·2HCl was not genotoxic in vivo.

Commission Regulation (EC) NO. 1895/2005 (18 Nov. 2005) specified migration limit for BADGE & certain derivatives by separation into 2 groups.

- Sum of the migration of BADGE & hydrolysis form shall not exceed 9 mg/kg in food/ food simulates.
- Sum of chlorohydrins form shall not exceed 1 mg/kg in food or in food simulates.

2.4 Sources of Exposure (20,23,24,25)

Bisphenol A diglycidyl ether (BADGE) is an epoxy resin monomer obtained by a reaction between Bisphenol A and epichlorohydrin, which mainly used for interior coating in food preserving. BADGE also used as additives in organosol coating to prevent thermal degradation of the hydrochloric acid apparently formed during curing process (Natchanun) (25)

Dermal exposure

Epoxy resin can be divided into two types: liquid and solid epoxy resin. The worker in manufacturing process of liquid epoxy resin potentially exposed to approximately 80-85% of BADGE with dermal route of exposure. Epoxy resin is classified as skin / eye irritant and skin sensitizers.

The solid epoxy resin workers in manufacturing process exposed to dust in each step of process. Skin contact during manufacturing process was the greatest potential route of exposure.

Oral exposure BADGE from food and drink can link with the epoxy based coating. Also the easy open The major consumer exposure to end can (EOE) which

Copyright by Mahidol University

coated with non completely cured film of PVC organosols affected on BADGE migration.

The information from European Consumption, United Kingdom FSA market survey (Dioni and Oldring 2002) calculated the per capita exposure to BADGE is 3-8 μg per person per day.

International Life Science Institute (ILS) calculated the maximum exposure of BADGE was to be 0.19 μg kg /body weight /day for body individual while the minimum exposure of BADGE was calculated as 0.000005 μg kg /body weight/day for body individual(20).

2.5. Coating

Definition of coatings (5):

The viscous liquid material that is applied to a substrate, the resultant dry film and the process application.

Purpose of the coating (22):

There are many purpose of coating, there are;

- Protection of the metal from the contents
- Maintenance of product quality by minimizing contamination of the product by metal ions or other materials in packaging
- Manufacturing facilitator
- Providing a basis for decoration or product identification
- Providing a barrier against external corrosion and abrasion
- Protection from metal absorption from the container.
- Protection from the environment within which it is used or stored.

When used for decorative purposes of coatings, they may be used in conjunction with printing inks and so on. It is important to recognize that a particular coating system may be required to perform several functions; these may conflict with each other and consequently the formulation may be compromise.

Copyright by Mahidol University

The composition of coating:(22)

Organic coatings are complex mixtures of chemical substances than can be group into four categories.

1. Binders
2. Volatile compounds
3. Pigments
4. Additives

Binder : are the materials that form the continuous film that adheres to the "substrate", binds together with the other substance in the coating to form a film.

Volatile compounds : are included in a large majority of all coatings. They use as a major role in the process of applying coatings. They commonly called "solvent" that make the coating fluid enough for application and they evaporate during application.

Pigments: are insoluble solid that are dispersed and suspended in the binder after film formation. Generally, the primary purpose of the pigments is to provide color and opacity to the coating film. They also have the important effects on application characteristic and film properties.

Additives : are materials that are includes in small quantities to modify some property of the coating. Examples are catalyst, stabilizers and flow modifier.

Then coatings are applied by blushing, rolling, spraying, dipping and other method which could change to be adherent film called film formation. The coatings are thermosetting polymers which can be crosslink on the substrate after achieving suitable heat condition.

Type of coating (22):

Coating can be divided in two mainly types by the majority in application.

1. Exterior coating : This coating purpose is to decorate the packaging , to protect surface and to endure in application.

Copyright by Mahidol University

2. Interior coating : This coating purpose is to protect packing material inside can which directly contact with substrate and it must be maintained the nutrition for the whole shelf life.

3 Types of can responsible for highest migration level :

- Deep-drawn two pieces cans.
- Easy open lid.
- Cans for aggressive foodstuffs.

These 3 types of canned which corresponding internal coatings consisted mostly of vinylic organosols. Experimented with human gastric simulation showed BADGE and it monoepoxy hydrolysis converted to the corresponding mono and bisdiols.

The two chlorohydrins (BADGE·H_2O, BADGE·2HCl) formed with chloride ions present during curing of vinylic coatings & especially when over-curing coatings.

Definition in coating term (22)

- *Internal lacquer* – a protective coating used on the insides of all types of metal containers which may be pigments.
- *Sanitary enamel* – an internal protective coating or lacquer used for process food cans which may be pigmented.
- *Size- or size coat* –a coating applied either internally or externally (usually in a tin film) to promote adhesion of coatings applied subsequently
- *Varnish* – a clear (transparent or translucent) coating applied externally to provide gloss, abrasion resistance, chemical resistance or lubricity
- *Follow on varnish* – a clear varnish applied over wet (unstoved) printing ink and sometimes called trailer or trailing varnish
- *White coating*- a white pigmented (usually with titanium oxide) coating applied externally, usually to provide a basis for subsequent decoration

Copyright by Mahidol University

by printing on any type of container, although requirements and formulations are different for heat processed and non-processed containers; similar coatings are also available in other colors.

Materials involved in the construction of metal packaging are as follows(22):

- Metals
- Protective lacquers and decorative coatings
- Printing inks
- Gasket materials, such as lining compounds for ends, and linings for vacuum closures
- Metal-polymer laminates

Metal

Type of metal for can coating mainly composes of 3 kinds of substrate:

- ET (Electrolytic tin plate)
- TFS (Tin free steel)
- AL (Aluminium)

Resins used in surface coating

The formulation of a dry film of coating involves a number of stages:

- Application of substrate.
- Elimination of solvent, to form an adherent film.
- Curing (or simply drying in the case of thermoplastic system).

Most coatings used in the metal packing industry are of the thermosetting type, however some notable exceptions, which include unmodified solution of vinyl coatings, certain polyester powder and organosols, which each have a substantial degree of thermoplasticity. The characteristics of the dry film depend upon the resins

Copyright by Mahidol University

used in the formulation of the coating. With few exception, resins are not used singly but in combination with other resins with which they react to form a cross-linked structure Thus, the properties of the cured film depend upon the resins used and the proportion of each component.

There are 2 important coating types which often used for internal coating. There are epoxy resin type and vinylorganosol type (9, 22, 27, 28, 29).

Epoxy resins. There are two different groups of epoxy resins, each of which contains the highly oxirane ring.

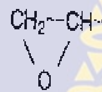


Fig.2-6 The picture of oxirane ring

The second group, formed by the condensation reaction between epichlohydrin and bis-phenol A, are used more generally and form the basis for a wide range of surface coatings.

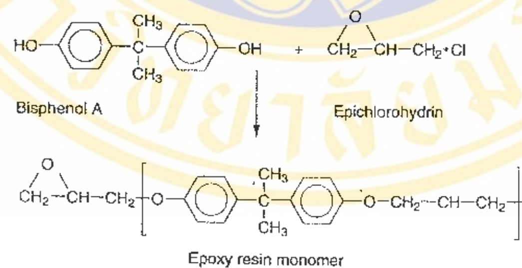


Fig.2-7 Synthesis of epoxy resin

Epoxy resins are used widely in the formulation of coatings in the metal packaging industry and in other industries. In the case of metal packaging, epoxides are used in conjunction with a range of resin types to produce coatings for many types of application, including the following:

- Phenol formaldehyde.

Copyright by Mahidol University

- Polyesters.
- Polyamides.
- Amino resins.
- Acrylics.

Currently, coatings based on epoxy chemistry represent the most important and versatile materials for metal packing, which is the reason that recent concern about their toxicological status has become such an important issue in the industry. Basically, almost any type of food-can pack can be protected by using epoxy-phenolic lacquer, organosol or a combination of both.

Vinyl resins.

The basic building blocks for vinyl resins are polyvinyl chloride (PVC) and vinyl chloride-vinyl-acetate co-polymers, and these are used to produce a wide range of solution and dispersion coatings. PVC is also used in the manufacture of high-solids plastisols, including lining compounds for vacuum closures.

Material based on PVC shares an important characteristic, which is that they show some level of thermoplasticity that ranges from the totally thermoplastic solution vinyl and plastisol types of material to thermally stabilized organosols, which retain a significant level of this characteristic. This need to be born in mind in metal-forming operations where, for example, low temperature may results in reduced flexibility, and excessively high temperature may cause the coating to stick to the tool.

Thermal degradation with the liberation of free hydrochloric acid, especially in the present of iron, can also be a problem when using vinyl-based material.

Organosols

This important group of high-solid (50%-70%) can coatings is also one of the most complexes in terms of application and storing. Organosols are mad by

Copyright by Mahidol University

swelling high molecular weight PVC particles in an organic solvent and dispersing the swollen particles in the second solvent. Additional materials are added, of which the most significant are BADGE (bisphenol A diglycidyl ether) or epoxy-novolac resins to impart thermal stability. Other resins, such as polyesters and acrylics, which enhance chemical resistance and adhesions, and suitable plasticizers, may be added to complete the formulation.

Organosols have the characteristic properties of vinyl materials, that is, high flexibility, good flavor characteristics and reasonable adhesion plus improved thermal stability and chemical or product resistance from addition of modifying resins. They do, however, remain prone to staining and have only modest resistance to sulphur staining. They are consequently often pigmented with aluminium powder.

The major applications for organosols are the manufacture of Draw Redraw (DRD) cans, because of their inherent flexibility, and for easy-open ends, where a high film thickness of a flexible material is required to resist damage to the internal coating from the external scoring operation. Much of the success of organosols as a lacquer for food cans derives from it being used in thick rather than its inherent product resistance.

The drying and curing process is divided into three stages:

1. Solvent evaporation
2. Fusion of the PVC particles into a coherent film
3. Cross-linking, to give a modest degree of chemical resistance.

Solution vinyl coatings

In the simplest case, solution vinyl coatings (sizes, white coatings and lacquers) are made by dissolving vinyl chloride-vinyl acetate co-polymer in a suitable solvent of blended solvents(mixtures of ketonic and aromatic hydrocarbons are typical example)

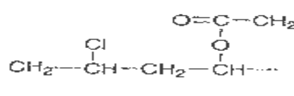


Fig.2-8 Vinylchloride vinyl acetate-copolymer

Copyright by Mahidol University

Such materials have all the characteristic properties associated with vinyl resins and need only be dried-there is no curing reaction-the solvent has poor resistance. The major disadvantage of these materials is their low-solids content, which can be problematical when roller-coating sheets at high production speeds.

Canned food can be kept to consume in the range of timing for 1-2 year because of pasteurized process can kill bacteria (***Clostridium nitrificants***)(2,4). Although the quality of can also affect to the nutrition of food when keeping for all their shelf life. The suitable temperature for keeping canned foods is 25°C.

The raw material which is used for producing can almost was Tin that the process of coating both inside and outside to prevent any condition of quality (more scratch resistant, rust and corrosive from chemical inside or any contents).

Coatings are viscous liquid have to pass coating process to get adherence dry film on metal. They can be improved content resistant in any situation. The coating process is composed of fixation and curing process that need suitable temperature to form coating film. Types of coating are interior and exterior coatings; for interior coating, the coating will contact with foods then the resistances of film on different contents need more consideration which can be maintained film appearance or nutrition inside. Internal coating will be highly cross-linked to get thermosetting polymer and prevent oxidation of metal to any reaction from inside contents and pasteurized temperature. The internal coating type in market is shown in Table 2-1

Table 2-1 : The detail of popular interior can coating.

Resin System	Description	Main uses	Typical Products Packed	Flexibility	Pack Resistance
Epoxy Phenolic	High molecular weight epoxy resins cross-linked with phenolic resins	-Universal gold lacquer for 3 piece cans/ends -Shallow drawn can	-Non aggressive/ medium aggressive products -Vegetables, meat, fish, soups, tomatoes, fruits	Good	Very Good
Organosol	PVC dispersed in thermosetting binder solution	-Deep drawn cans -Easy open ends -Metal closures (Often used over epoxy phenolic	-Aggressive products -Pickled foods, ready meals, some fruits	Very Good	Very Good

Copyright by Mahidol University

Lacquer used for the interior coatings of food cans is frequently based on epoxy resins or vinylic organosols (polyvinylchloride; PVC) (Josef Lintschinger, 2000). The epoxy resin contains bisphenol A diglycidyl ether (BADGE) as the cross-linking agent. It is well known that unreacted BADGE can migrate from epoxy-based packing material into the food. In the PVC lacquers BADGE are added purposively to scavenge the release of hydrochloric acid during heat treatment of the coating procedure. (Fig.2-3B). The other factor which affect to the migration are the heating temperature, can content, type of container, filling condition and the processing of canned product. In production process of lacquer, the technical mixture of BADGE is polymerized with Bisphenol A. Its is well known that all these compounds and various oligomers of them can migrate from the inner coatings of cans into food easily soluble in edible oil and food lipids and migration into oily food. (Urs Berger Michael Oheme.Line Girardin, 2001). In some cases, these are analytical techniques, often based upon infra-red(IR) spectroscopy, for measuring degree of cure, but these tend to be coating-specific and too long for on-line application. Consequently, the can-maker may rely on good process control or subjective tests, such as resistance to solvent. The intention here is to give the reader an outline of the various drying and curing mechanism associated with the different resin and coating systems encountered in metal packaging.

2.6 High Performance Liquid Chromatography (21)

Principle of HPLC (High Performance Liquid Chromatography)

The chromatography is the technique used for separation of chemical substance on two phases. The first phase called stationary phase and the second one was mobile phase. The stationary phase has a small particle adsorbent diameter less than 150 µm which has the porous surface. Some of them were liquid coated on the solid support and packed on column. The mobile phase may be liquid or gas phase which moves the chemical substance into the chromatographic system. When chemical sample dissolves in the phase and moves in stationary phase. The sample distributes between stationary phase and mobile phase by the physical and chemical

Copyright by Mahidol University

property of the sample. The liquid sample or a solid sample dissolved in a suitable solvent which carried through a chromatographic column by a liquid mobile phase. Separation is determined by solute/stationary phase interactions, including liquid-solid adsorption, liquid-liquid partitioning and by solute/mobile phase interactions. A schematic diagram of a typical HPLC instrument is shown in figure.2-9

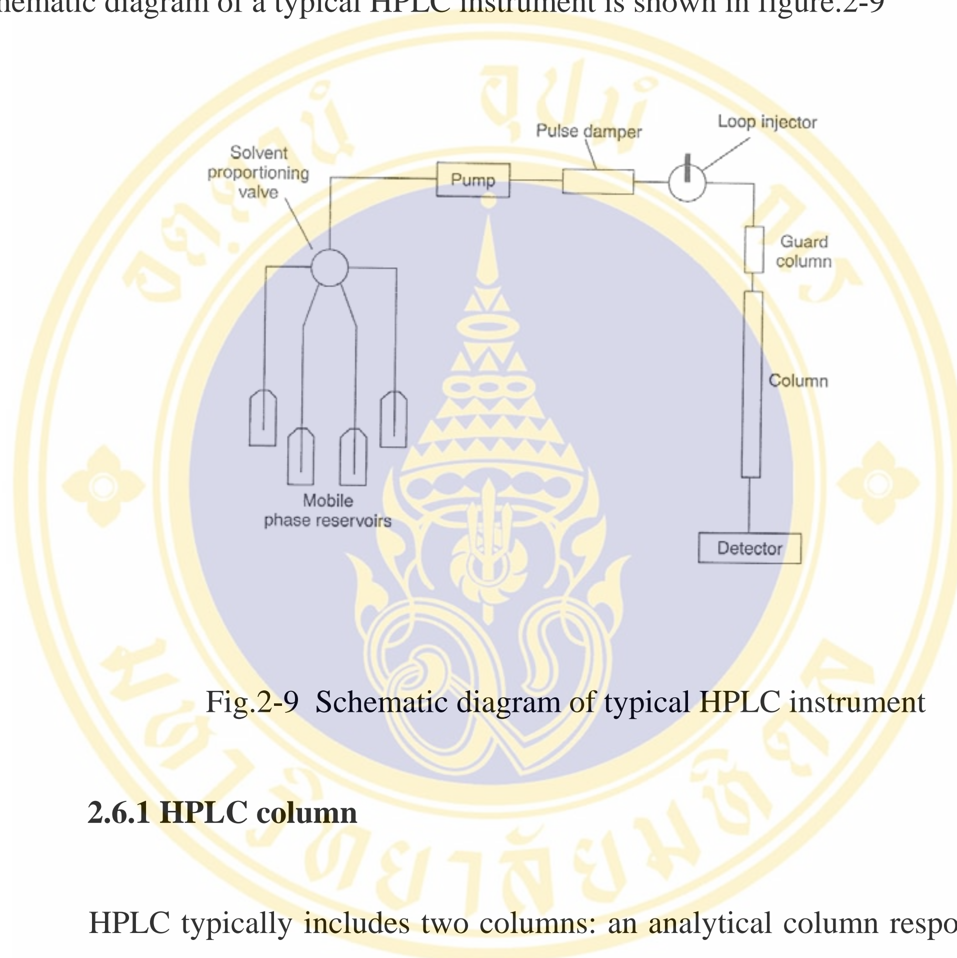


Fig.2-9 Schematic diagram of typical HPLC instrument

2.6.1 HPLC column

HPLC typically includes two columns: an analytical column responsible for the separation and a guard column. The guard column is placed before the analytical column, protecting it from contamination.

Analytical column: The most commonly used column for HPLC are constructed from stainless steel with internal diameter between 2.1 mm. and 4.6 mm. and lengths ranging from approximately 30 mm. to 300 mm. These columns are packed with 3-10 μm porous silica particles that may have an irregular or spherical shape. Typical column efficiencies are 40,000-60,000 theoretical plate/m. Assuming Vmax/Vmin is approximately 50, a-25 cm. column with 50,000 plates/m has 12,500 theoretical plates and a peak capacity.

Micro columns use less solvent and because the sample is diluted to a lesser

Copyright by Mahidol University

extent introduce larger signals at the detector. These columns are made from fused silica capillaries with internal diameters of 44-200 µm and length of up to several meters. Micro columns packed with 3-5 µm particles have been prepared with column efficiencies of up to 250,000 theoretical plates.

Open tubular micro columns also have been developed, with internal diameters of 50 µm and length of approximately 1m. These columns, which contain no packing material, may be capable of obtaining column efficiencies of up to 1 million theoretical plates.

2.6.2 Guard column

Two problems tend to shorten the lifetime of an analytical column. First, solutes binding irreversibly to the stationary phase degrade the column's performance by decreasing the available stationary phase. Second, particulate material injected with the sample may clog the analytical column. To minimize this problem, a guard column is placed before the analytical column. Guard column usually contains the same particulate packing material and stationary phase as the analytical column, but the significantly shorter and less expensive; a length of 7.5 mm and a most one-tenth of that for the corresponding analytical column is typical.

2.6.3 Stationary Phase

The stationary phase is a liquid film coated on packing material consisted 3-10 µm porous silica particles. The stationary phase may be partially soluble in the mobile phase, causing it to 'bleed' from the column over time. To prevent the loss of stationary phase, it is covalently bound to particle. Bonded stationary phase are attached by reacting the silica particles with an organochlorosilane of the general form, $Si(CH_3)_2RCl$, where R is an alkyl or substituted alkyl group.

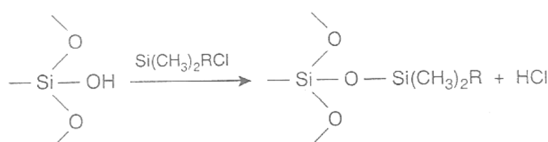


Fig 2-10 The structure of stationary phase

Copyright by Mahidol University

To prevent unwanted interactions between the solutes and any unreacted -SiOH groups, the silica frequently is 'capped' by reacting it with $Si(CH_3)_3Cl$; such columns are designated as end capped.

The properties of a stationary phase are determined by the nature of the organosilane's alkyl group. If R is polar functional group, then the stationary phase will be polar. Examples of polar stationary phase which R group contains a cyano ($-C_2H_4CN$), diol ($-C_3H_6OCH_2CHOHCH_2OH$), or amine ($-C_3H_6NH_2$) functional group.

The reverse phase chromatography which is more common encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane for which the R group is an n-octyl (C_8) or n-octyldecyl (C_{18}) hydrocarbon chain.

To prevent unwanted interactions between the solutes and any unreacted–SiOH groups, the silica frequently is "capped" by reaction with $Si(CH_3)_3Cl$.The properties of a stationary phase are determined by the nature of the organosilane's alkyl group. If R is a polar functional group, then the stationary phase will be polar. Since the stationary phase is polar the mobile phase is nonpolar or moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called normal-phase chromatography.

2.6.4 Mobile phase

The elution order of solutes in HPLC is governed by polarity. In a normal-phase separation, the least polar solute spends proportionally less time in the polar stationary phase and is the first solute to elute from the column. Retention time controlled by selection of mobile phase, with a less polar mobile phase leads to longer retention time. When two solutes are adequately resolved, switching to a more polar mobile phase which provide an acceptable separation with a shorter analysis time. In reverse-phase separation the order of elution is reversed, with the most polar solute being the first to elute. Increasing the polarity of the mobile phase leads to longer retention times, whereas shorter retention times require a mobile phase of lower

Copyright by Mahidol University

polarity.

Several indices have been developed to assist in selecting a mobile phase, the most useful of which is the polarity index. (Table 2-4) provides values for the polarity index, P'

Table 2-2 The properties of HPLC Mobile Phases

Mobile Phase	Polarity index (P')	UV Cutoff (nm)
cyclohexane	0.04	210
n-hexane	0.1	210
carbon tetrachloride	1.6	265
i-propyl ether	2.4	220
toluene	2.4	286
diethyl ether	2.8	218
tetrahydrofuran	4.0	220
ethanol	4.3	210
ethyl acetate	4.4	255
dioxane	4.8	215
methanol	5.1	210
acetonitrile	5.8	190
water	10.2	-

Changing the mobile phase's polarity index, by changing the relative amounts of two solvents, provides a means of changing a solute's capacity factor. Such changes are not selective; thus two solvents that significantly overlap may continue to be poorly resolved even after making a significant change in the mobile phase's polarity.

To achieve a better separation between two solutes it is necessary to improve the selective factor, α or second changing in the selective factor for a pair of solutes which is to change one or more of the mobile-phase solvents. In a reverse-phase separation, it is accomplished by changing the solvent mixed with water. Besides methanol, other common solvents for adjusting retention times are

Copyright by Mahidol University

acetonitrile and tetrahydrofuran.(THF). A common strategy for finding the best mobile phase is to use the solvent triangle.

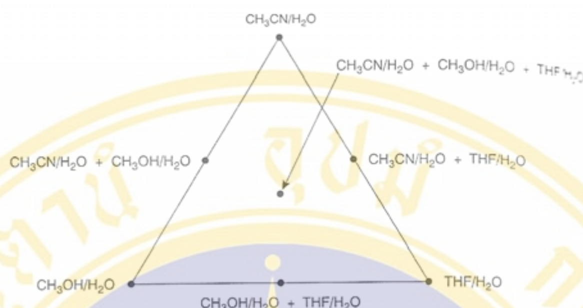


Fig.2-11 The solvent triangle

The advantages of the reversed-phase techniques are numerous. The most outstanding being more extreme simple operating conditions. There is no need for long equilibration time with the solvent and gradients are particularly easy to perform and to reproduce. The column gives very reproducible results over long periods with no need for special care of storage. Increasing numbers of separation are now being performed using the reversed-phase mode. In reversed-phase liquid chromatography, two techniques related to the selection and composition of the compounds of mobile phase are widely used. These are the isocratic elution mode and the gradient elution mode.

2.6.4.1 Isocratic elution

In isocratic elution mode, the various parameters such as the composition of mobile phase, flow rate and temperature remain constant during the analysis time.

The major advantages of isocratic elution are:

1. Simplicity
2. Compatibility with all detectors
3. Higher resolution
4. Convenience in repeated use for similar samples

Copyright by Mahidol University

The disadvantages are :

1. Limited to sample with 0.5<k’<15
2. Decreased sensitivity at higher k’ values

2.6.4.2 Gradient elution

Important factors for using gradient elution program are when the reaction range is too wide or when two or more peaks are not separated from each other. In gradient elution mode the following variables may be programmed.

1 Pressure or flow programming.

2 Temperature programming.

3 Programming the stationary phase either by variation of adsorbent activity or using coupled columns.

4 Solvent programming or gradient elution.

A gradient elution is preformed by changing the composition of the mobile phase by adding a strong solvent with increasing concentration to a week initial solvent. Gradient elution mode will have at least two solvents, a weak solvent 1 and a strong solvent 2. The chromatogram is started with a mobile phase weak enough to give a good resolution of the solution with low “k’ ” values eluting at the start of the chromatogram. The addition of solvent 2 to solvent 1 increase the eluent strength until a concentration of solvent 2 is reached. Solvent 2 must be strong enough to elute the strongly retained solute with large “ k’ “ values in a reasonable time.

The basic requirements of a gradient elution system are:

1. The gradient must be reproducible and consistent regardless of the solvent used.

2. The flow rate through the column must be constant since not only will the flow rate affect the retention time but it will also affect quantitative results because it affects peaks areas.

In order to get reproducible results, the factors that affect the retention time must be controlled, i.e. column equilibration, temperature, flow rate and mobile phase.

The major advantages of gradient elution are the fact that it provides the maximum resolution per unit time. It solves the problem of sample having a very

Copyright by Mahidol University

broad range of " k' " values. It improves the detection of peaks eluting late and is useful for the rapid survey of unknown samples.

The major disadvantage of gradient elution is that it cannot be used with the refractive index or conductivity detectors.

2.6.5 HPLC Plumbing

From Fig 2-15 present the several reservoirs to control mobile phase's polarity which plays an important role for improving liquid chromatographic separation. It is essential when using a gradient elution, for which the mobile composition is systematically changed from a weaker solvent to a stronger solvent.

The mobile phase solvents must be treated to remove dissolved gas, such as N_2 and O_2. Dissolves gases often lead to formation of gas bubbles when the mobile phases enter the detector, resulting in a distortion of the detector's signal. The most common is to accomplish degassing process using vacuum pump and then placed in their reservoir.

The mobile phase solvents are pulled from their reservoirs by the action of the pump. Most HPLC instruments use a reciprocating pump consisting of a piston host back-and-forth movement capable both of maintaining a constant flow rate of up to several milliliters per minute and obtaining the high output pressure needed to push the mobile phase through the column. A solvent proportioning valve controls the mobile phase's composition, making the necessary change in the mobile's phase composition possibly when using a gradient elution.

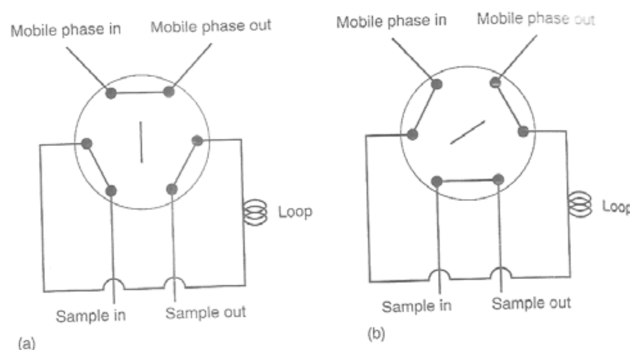


Fig. 2-12 Schematic diagram of a loop injector in the (a) load (b) inject positions.

Copyright by Mahidol University

2.6.6 Sample introduction

The typical operating pressure of an HPLC is sufficiently high, the sample is introduced using the loop injector (Fig 2-12). Sampling loops are interchangeable, the available volumes ranging from 0.5 μl to 2 μl.

2.6.7 Detector for HPLC

Fluorescence Detector

Principle of Fluorescence detector

When light is absorbed by a molecule, and an electron is promoted to a higher energy state, there are a number of pathways by which this energy can be dissipated, allowing a return to the ground state. Most commonly, energy is lost by transfering to surrounding molecules and contributes only to an overall heating of the environment. However, some molecules may lose only a part of their energy in this way, reverting to the lowest vibrational state of the electronic excited state. The return to the electronic ground state then occurs with energy being lost by emission of a photon, this process being called fluorescence. Because some energy is lost to general heating in the return to the vibrational ground state, the emitted photon has a lower energy and thus longer wavelength than the excite light. Only a relatively small percentage of molecules lost a significant proportion of their excitation energy by fluorescence, and thus give strong fluorescence signals.

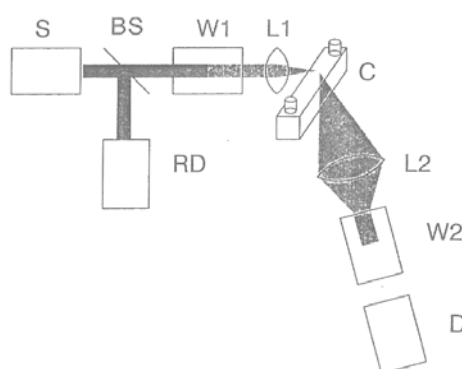


Fig.2-13 Block diagram of a fluorescence detector.

Copyright by Mahidol University

In the Fig.2-13, light from a source S is focused through an excitation wavelength selection device, WI, and into flow cell, C. Lens I.2 collects fluorescent emission from the flow cell, and the collected light passes through the emission wavelength selection element, W2. The light then passes to a photodetector, D, the signal which is passed to an amplifier and smoothing circuitry. A second light pat is produced by the beam splitter BS, passing to the reference photodiode, RD.

The block diagram of a fluorescence detector for HPLC (Fig.2-13). The source of fluorescence is commonly a broad spectrum lamp such as a deuterium lamp, or a xenon lamp. Occasionally high intensity line sources such as mercury (254nm) or zinc (214nm). Highest sensitivities can be achieved with intense laser sources; disadvantage is the need to find a suitable type of laser with an output which matches the fluorophore of interest. When using a continuous excitation source, the wavelength selection devices might be monochromators or filters. With the line sources the excitation wavelength selection is often by a matching filter. The flow cell is rather different in design than that UV absorbance detector; the requirement is to effectively illuminate the cell volume with the excitation light, while collecting the maximum possible fluorescent emission from the same volume and minimizing the amount of extraneous light arriving at the photodetector. This extraneous light comprised scattered light from flow cells. If the scattering of the excitation beam occurs, greater emphasis must be placed on the removal of this light by filtering before the photodetector. Because of fluorescent emission is usually very weak, and under optimum conditions the background light levels should be zero, the requirements for photodetector are for high sensitivity, and low background noise from the photodetectors itself. These requirements are usually met by use of a photomultiplier tube, rather than photodiode commonly found in absorbance detectors. The output signal of fluorescence detectors is processed in the same way as the signal from the absorbance detector with various degrees of filtering being provided to smooth the output signal.

Copyright by Mahidol University

Literature Cited.

C.Simoneau et al was analyzed BADGE concentration from canned fish samples from all 15 Member States and Switzerland. Samples was extracted with n-hexane and acetonitrile, analyzed with Spherisorb ODS-2 column. The mobile phase for elution sample consisted of acetonitrile and water (60/40; v/v). Separation condition used isocratic mode for 20 minute by flow rate of mobile phase at 1 ml/minutes. The results of the recovery was ranging from 86.4 % to 92% with standard deviation 2.3%. The BADGE concentration in canned fish in oil showed higher than specific migration limit of 1 mg/kg for 3%.

H.Petersen studied a method for the simultaneous identification and quantification of bisphenol A-diglycidyl ether (BADGE) and their precursor bisphenol A in food, bisphenol A di-(3-hydroxypropyl) either as an internal standard. Analysis carried out by using RP-HPLC with fluorescence detection and additional information using RP-HPLC with mass selective detection. The described method is validated for the analysis of foodstuffs as well as fatty food stimulants. The limit of detection was between 10 and 30 μg/kg of food.

Josef Lintschinger studied a method for the simultaneous determination of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their hydrolysis and chlorohydroxy derivatives in canned food is presented. Oily and aqueous food samples were extracted with tert-butyl methyl ether and acetonitrile. The compounds were extracted and determined by using reverse-phase gradient high performance liquid chromatography with fluorescence detection. The validation of the method was analyze various canned food samples. In none of samples were significant amounts (>1μg/g) of BADGE or BFDGE, whereas most samples BADGE/ BFDGE chlorohydroxy compounds were detected.

Rauter determined Bisphenol A diglycidyl ether (BADGE) in oily food from Austria by using a new simplified HPLC method. Samples are extracted with pentane, back extracted with methanol, and finally dissolved in the mobile phase (cyclohexane/tert-butyl methyl ether). Separation was performed on a normal phase HPLC column using fluorescence detection. Verification of BADGE- containing peak was carried out by using GC-MS. Additionally, the synthesis and determination of

Copyright by Mahidol University

BADEG hydrolysis products, Bisphenol A bis(2,3 dihydroxypropyl) ether (BADGE.2H_2O) and Bisphenol A diglycidyl (2,3-dihydroxypropyl) ether (BADGE.H_2O) were presented. From 67 analyzed cans, containing various fatty meat or fish products, 16% were above the maximum quantity of 1 μg/g tolerated by the European Community, 45% were in the range between 0.1-1 μg/g, 24% between 0.02 and 0.1 μg/g, and in 15% the BADGE concentration were below detection limit of 0.02 μg/g. The hydrolysis product of BADGE.H_2O was not detected in any samples up to a concentration of 0.5 μg/g.

Natchanun et al studied a gradient reverse-phase liquid chromatography with fluorescence detection method for simultaneous identification and quantification of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE), and their 10 derivatives in food matrixs was developed and validated for the analysis of oil-in-water and aqueous base foodstuffs. The method linearity range was 0.016-10 ppm which are hundred-fold below and tenfold above the EU restriction at 1 ppm (mg/kg). The method detection limits range 0.72- 4.20 ppb and the method quantitation limit ranged 2.40-14.85 ppb, respectively. The validation data indicated excellent precision, acceptable recovery, and good robustness, all supporting a good potential to further develop the method as a standard method for the determination of migration from interior can coatings into foodstuff.

Brede C. et al studied straightforward method was established for determination of migration contaminants in olive oil with a special focus on the two can coating migration compounds bisphenol A diglycidyl ether (BADGE) and bispenol F diglycidyl ether (BFDGE). The preferred sample preparation was extracted in a single liquid-liquid extraction of compounds from the oil into 20% (v/v) methanol in acetonitrile, followed by clean-up with solid-phase extraction on aminopropyl bonded to silica. This purification procedure selectivity removed all free fatty acids from the extracted medium without removing phenolic compounds of interest. The solid-phase extraction columns were used many times by implementing a procedure of washing out the strongly retained fatty acids with 2% acetic acid in methanol. Gas chromatography couple with full scan (m/z 33-700) electron ionization mass spectrometry was used for determination of several model compounds in olive oil samples. BADEG and BFDGE could be determined in the 0.05-2 mg/kg range in oil

Copyright by Mahidol University

sample with a relative SD of <6% (six replicates). The method was used in as enforcement campaign for the Norwegian Food Control Authority to analyze vegetable oil samples from canned fish-in-oil.

Copyright by Mahidol University

CHAPTR III

MATERIALS AND METHOD

3.1 Introduction

The aim of this study was to develop a method for determination of Bisphenol A diglycidyl ether (BADGE) residue in oil phase from canned tuna by High Performance Liquid Chromatography (HPLC) with Fluorescence detector. The method was then used for analyzing canned tuna after storage for less than 6 months and longer than 6 months. Reverse Phase Liquid Chromatography was used for analysis of BADGE. This study is an experimental design.

3.2 Apparatus and chemical reagents

3.2.1 Equipment

3.2.1.1 High Performance Liquid Chromatography (HPLC2695, Water, Germany) with fluorescence detector (Water 2475) and analytical column, symmetry C18 (3.9mm x 150 mm i.d., 5 µm. particle size) with a guard column (Water T 04225 Water, Germany).

3.2.1.2 Ultrasonic bath (sonicator: DSC 321TH) Sonicor Instrument Cooperation Copiague N.Y.).

3.2.1.3 Super speed refrigerated centrifuge (Beckman Center: JA20-MC).

3.2.1.4 Speedfuge (Savant HSC 10K) ; (Rotor: HSR 24).

3.2.1.5 Analytical Balance (Presica model 40SM-200A, 0.1 mg Switzerland).

3.2.1.6 Centrifuge tube(Nalgene company, Rochester, New York, U.S.A).

3.2.2 Chemical Reagents

3.2.2.1 n-hexane 95 % (Fisher Scientific : AR grade).

Copyright by Mahidol University

3.2.2.2 Acetonitrile (Fisher Scientific : HPLC grade).

3.2.2.3 NANOpure Analytical Water NANOpure Analytical Deionization System, Grade 3A for HPLC from Central Instrument Facility (CIF) of Faculty of Science, Mahidol University.

3.2.2.4 Standard of BADGE (> 97% ; YD-128).

3.2.3..The preparation of BADGE stock solution

3.2.3.1 Mixture of acetonitrile and water an equal volume (50/50 V/V) of acetonitrile and water was mixed and used as solvent of preparation of each BADGE concentration.

3.2.3.2 BADGE stock solution concentration of 1000 mg/l. For the first stock solution, 25 mg of BADGE's standard weighed and put it in 25-ml volumetric flask. Mixture of acetonitrile and water 50/50, V/V) were added to a total volume of 25- ml. The concentration of this stock solution was 1000 mg/l.

3.2.3.3 BADGE stock solution concentration of 1000 µg/l An aliquot of 25-µl of BADGE stock solution in 3.2.3.2 was pipetted into 25- ml volumetric flask. Mixture of acetonitrile and water were added to this total volume of 25-ml. The concentration in this stock solution was 1000µg/l.

3.2.3.4 BADGE stock solution for recovery analysis.

1. BADGE stock solution concentration of 1000 µg/l from section 3.2.3.3.
2. BADGE solution concentration of 600µg/l.

An aliquot of 3.0-ml of BADGE stock solution concentration of 1000 µg/l in section 3.2.3.3 was pipetted into 5-ml volumetric flask. Mixture of acetonitrile and water were added to total volume of 5- ml. The concentration in this stock solution was 600µg/l.

3. BADGE solution concentration of 300µg/l.

An aliquot of 1.5- ml of BADGE stock solution concentration of 1000 µg/l in section 3.2.3.3 was pipetted into 5- ml volumetric flask. Mixture of acetonitrile and water were added to total volume of 5-ml. The concentration in this stock solution was 300µg/l.

Copyright by Mahidol University

3.2.3.5 BADGE stock solution for analysis of accuracy.

1. BADGE solution concentration of 800µg/l.

An aliquot of 4.0- ml of BADGE stock solution in 3.2.3.3 was pipetted into 5-ml volumetric flask. Mixture of acetonitrile and water were added to total volume of 5-ml. The concentration in this stock solution was 800µg/l.

2. BADGE solution concentration of 400µg/l.

An aliquot of 2.0-ml of BADGE stock solution in 3.2.3.3 was pipetted into 5-ml volumetric flask. Mixture of acetonitrile and water were added to total volume of 5-ml. The concentration in this stock solution was 400µg/l.

3.2.3.6 BADGE stock solution for analysis of detection limit.

1. BADGE solution concentration of 500µg/l.

An aliquot of 2.5-ml of BADGE stock solution in 3.2.3.3 was pipetted into 5-ml volumetric flask. Mixture of acetonitrile and water were added to total volume of 5-ml. The concentration in this stock solution was 500µg/l.

2. BADGE solution concentration of was 100 µg/l.

An aliquot of 1.0 ml of BADGE stock solution in 3.2.3.6 (1) was pipetted into 5- ml volumetric flask. Mixture of acetonitrile and water were added to total volume of 5- ml. The concentration in this stock solution was 100µg/l.

3.2.4 The preparation of BADGE solution for calibration curve.

The calibration curve was consisted of 10 concentrations of BADGE solution. The BADGE solution concentration in section 3.2.3.6(2) was pipetted for 50µl, 250µl, 500µl, 1.0ml, 2.0ml, 3.0ml, 4.0ml, 5.0ml, 6.0ml, and 7.0ml into 5- ml volumetric flask. Mixture of acetonitrile and water were added to be total volume of 5-ml. These 10 points of calibration curve concentrations of BADGE solution were 1,5,10,20,40,60,80,100,120 and 140 µg/l, respectively.

3.2.5 The preparation of BADGE solution for detection limit.

The analyzed **BADGE** standard concentration for detection limit

Copyright by Mahidol University

ranged from 0.065-12.0 µg/l. The BADGE solution concentration in section 3.2.3.6 (2) was pipetted for 3.25, 6.25, 12.5, 25.0, 50.0, 100, 200, 300, 400 and 500 µl into 5- ml volumetric flask. Mixture of acetonitrile and water were added to total volume of 5- ml. The concentration of this stock solution was 0.065, 0.125, 0.250, 0.500, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 µg/l.

3.2.6 Sample treatment: Liquid – liquid extraction.

10.0000 g. of oil from canned tuna was weighed and put into the 200- ml glass bottle and 5.0- ml of n-hexane was added. Then, 10-ml. of acetonitrile were added and the bottle was put into an ultrasonic bath for 10 minutes at room temperature. Then sample was centrifuged at 6300 RPM at 25°C for 10 minutes by Super speed refrigerated centrifuge. The mixture was separated into two layers. Oil and hexane formed the viscous lower layer. The acetonitrile extracted (upper layer) was removed and diluted with an equal volume of water and then centrifuged again at 6500 RPM for 5 minutes. All this solution were filtered through 13mm, 0.45µm Nylon filter and 20µl was injected to the HPLC equipped with fluorescence detector. The fluorescence was recorded at an excitation wavelength of 225 nm and emission wavelength of 305 nm.

3.3 HPLC Condition for BADGE analysis

The standard BADGE and extracted badge sample from canned tuna were analyzed by HPLC at the condition in table 3-1.

Table 3-1 HPLC system for analysis of BADGE (HPLC 2695)

Instrument Company	Model
Injector Waters	Waters (WA T046980)
Column Waters	Waters (W21111V 007)
	Symmetry C18;(3.9x150mm) i.d.,5µm, 100A^{o}

Copyright by Mahidol University

Guard column Waters	Waters (WA T04225)
Detector Waters	Waters 2475 Fluorescence
	λexcitation = 225nm.
	λemission = 305nm.
Data system Waters	Millenium[32]Chromatography NT workstation version 3.2

The mobile phase for analysis of BADGE used isocratic system comprised acetonitrile and water at the ratio of 65:35.

3.4 Reliability of the method.

The reliability of the method was performed for limit of detection, limit of quantification, accuracy and precision.

3.4.1 Determination of LOD and LOQ

The limit of detection is the lowest concentration of an analyzed. The ten set of low concentration of BADGE was prepared for determination of LOD and LOQ.The standard stock solution of BADGE in section 3.2.3.6 was pipetted for 3.25, 6.25, 12.5, 25.0, 50.0, 100, 200, 300, 400 and 500 µl (stock solution of standard concentration of 100µg/l). Mixture of acetonitrile and water were added to total volume of 4.996- ml, 4.993-ml, 4.985-ml, 4.975-ml, 4.950-ml, 4.900-ml, 4.800-ml, 4.700-ml, 4.600-ml and 4.500-ml. The concentrations of BADGE were 0.065, 0.125, 0.250, 0.500, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 µg/l.The ten LOD concentrations were analyzed by HPLC at the condition In section 3.5.4 and the response was plotted as a function of mass of BADGE determined and peak area of the chemical. The standard error of estimate (SEE) for regression curve was calculated by a formula below.

$$SEE = \sqrt{\frac{\Sigma (Y_{obs} - Y_{est})^2}{n-k}}$$

Y_{obs} = observe response

Copyright by Mahidol University

Y_{est} = estimated response from regression curve

n = total number of data point

k = 2 for a linear regression curve

The LOD and LOQ were calculated by the equation below;

LOD = 3 x standard error of the regression / slope

LOQ = 10 x standard error of the regression / slope

3.4.2 Determination of recovery, accuracy and precision

3.4.2.1 The preparation of BADGE solution for recovery.

One ml of known concentration of BADGE from stock solution in section 3.2.3.4 (3,2,1) at the concentrations of 15, 30 and 50 µg/l spiked to 10.0000 g blank oil. Then, all samples were extracted according to the method in section 3.3.1. Finally, 20 µl of each sample of was injected into the HPLC. The experiment was carried out for three replications.

3.4.2.2 The preparation of BADGE solution for accuracy.

One ml of known concentration of BADGE from stock solution in section 3.2.3.5 (1,2) at the concentrations of 20 and 40 µg/l spiked to 10.0000 g blank oil. Then all samples extracted according to the method in section 3.3.1. Finally, 20-µl of each sample was injected into the HPLC. The experiment was carried out for three replications. The analysis was repeated for between assays for three days.

3.5 Analysis of canned tuna

60 cans of tuna were collected from market for both storage duration less than 6 months and longer than 6 months. Oil samples from canned tuna were drained out, weighed for 10.0000 g and put it into the beaker. The oil sample was extracted following the section 3.2.6 and analysis following the section 3.3.

The storage duration less than 6 months and equal to 6 months of can was the canned tuna bought in October to November 2005. The storage duration longer than 6 months of canned tuna was the canned tuna produced in January 2006.

All samples were randomized sampling from 5 department stores in Bangkok. It was classified into two groups of storage duration; less than 6 months and longer

Copyright by Mahidol University

than 6 months. Each group contained 30 cans and they were sampling in the same brand which came from only one source of organosol coating. It is because it can reduce the variation in the quantity of BADGE at the manufacturing process. This brand was famous in the market.

The storage duration of analyzed canned tuna can be classified into 4 groups; group 1: storage duration for 7 months and 4 days, group 2: storage duration for 6 months and 3 days, group 3: storage duration for 6 months and 2 days and group 4: storage duration for 4 months and 16 days.

3.6 Statistical for analysis.

3.6.1 Descriptive statistics

The general characteristics of all data were analyzed in terms of percentage, mean ($\overline{X}$), standard deviation (SD) and coefficient of variation.

3.6.2 Analysis statistics

Studied in relation of storage duration with quantity of BADGE was performed by Pearson Correlation.

$$r = \frac{\Sigma xy - (\Sigma x) - (\Sigma y) \ / \ n}{\sqrt{\Sigma x^2 - (\Sigma x)^2 / n} \sqrt{\Sigma y^2 - (\Sigma y)^2 \ / \ n}}$$

Copyright by Mahidol University

CHAPTER IV

RESULTS

This study was to develop a method for determination of Bisphenol A diglycidyl ether (BADGE) residue in oil phase from canned tuna by High Performance Liquid Chromatography (HPLC) with fluorescence detector. Test results were as follows:

4.1 The chromatogram of BADGE at concentration 20 µg/l

The stock solution of BADGE at concentration 20µg/l were injected and analyzed by HPLC. The chromatogram of standard BADGE at concentration 20µg/l is shown in Fig 4.1.

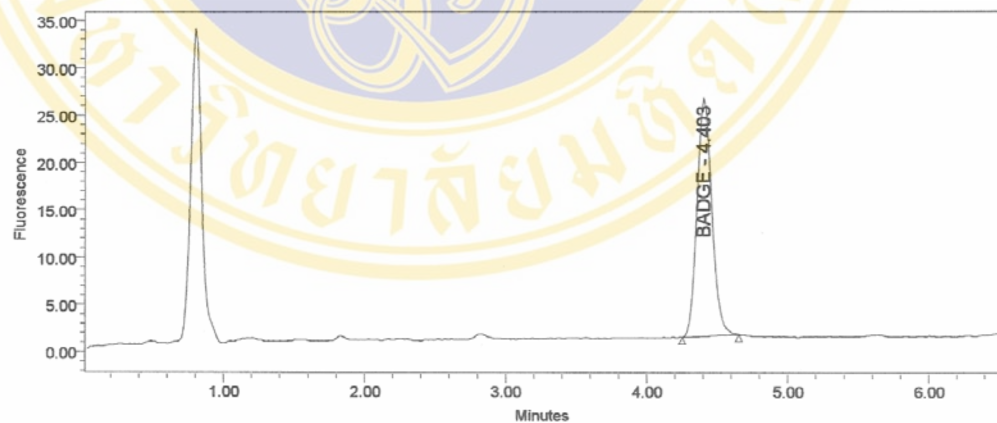


Fig.4-1 The Chromatogram of standard of BADGE at concentration 20µg/l

4.2 The chromatogram of Blank oil.

The chromatogram of blank oil which extracted and analyzed by HPLC. The chromatogram of blank oil is shown in Fig 4-2.

Copyright by Mahidol University

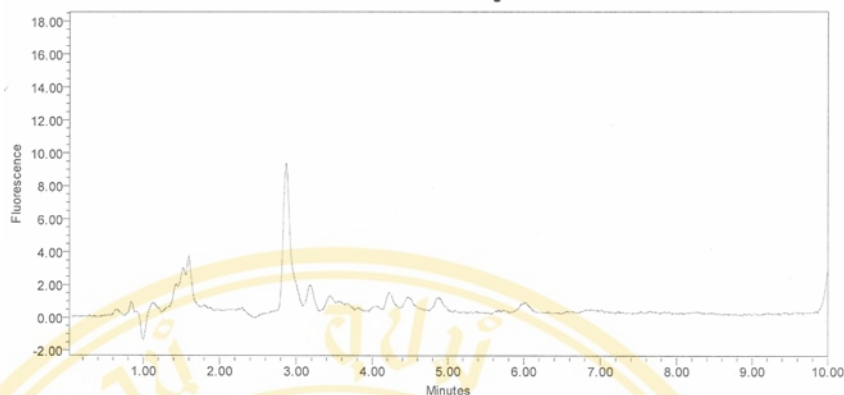


Fig.4-2 The chromatogram of Blank oil.

The chromatogram of Blank oil contained some interference peaks, but they have different retention time from the standard of BADGE peak.

4.3 The calibration curve of BADGE solution.

The known concentrations of BADGE solution were prepared at the concentration ranging 1.0 - 140 µg/l and injected into the HPLC. Linear relationship of this calibration curve was obtained by plotting the peak area of BADGE against the BADGE concentration in µg/ml. Fig 4-3 presented the calibration curve was Y = 6198.7X – 6501.3. The coefficient of determination of this calibration curve was (R^2 = 0.9986). This calibration curve was used for determining BADGE concentration in oil.

Copyright by Mahidol University

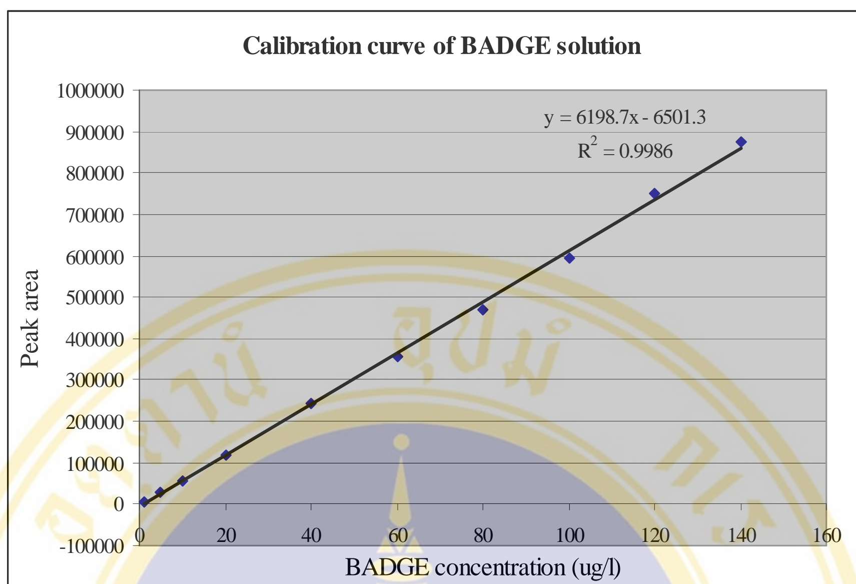


Fig.4-3 The calibration curve of BADGE solution

4.4 Recovery of the method

The blank oil was spiked with three concentrations of BADGE standard, extracted and analysis by the studied method. The recovery and coefficient of variation are presented in Table 4.1

Table 4-1 Percentage recovery of BADGE extraction from oil in canned tuna.

Spiked concentration (μg/l)	Analyzed concentration (X±SD)	Recovery results (%) (X±SD)	Coefficient of Variation (%)
15	15.024 ± 0.024	99.27 ± 0.02	1.47
30	31.152 ± 0.398	101.90 ± 0.40	1.22
50	50.001 ± 0.001	98.32 ± 0.01	0.87

Note : Recovery = Mean ± standard deviation

The results of average percent of recoveries of BADGE in oil were ranging from 98.32% to 101.90% (Table 4-3), the average recovery was 99.83%. In this study, all results was followed NIOSH recommendation with recovery of an analysis must be higher than 75%.

Copyright by Mahidol University

4.5 The accuracy and precision of the method.

The blank oil was spiked with two concentrations of standard solution of BADGE and extracted by the same method as the sample of tuna oil. The results of percentage recovery and coefficient of variation are shown in Table 4.4

Table 4-2 The accuracy and precision of BADGE analysis (between day accuracy of the method)

Spiked concentration (µg/l)	Day of analyzed	Analyzed concentration (X ± SD)	Recovery (%)	Coefficient of Variation (%)
20	1	20.233 ± 0.077	101.17 ± 0.07	0.38
	2	20.563 ± 0.161	102.82 ± 0.16	0.78
	3	20.406 ± 0.352	102.03 ± 0.35	1.72
40	1	41.084 ± 0.762	102.71 ± 0.76	1.85
	2	40.086 ± 0.067	100.22 ± 0.07	0.17
	3	40.306 ± 0.254	100.77 ± 0.25	0.63

Note : Recovery = Mean ± standard deviation

The accuracy of the method for BADGE analysis in between day was carried out at the concentrations of 20.400 ± 0.197 µg/l and 40.492 ± 0.361 µg/l gave the percent recovery ranging from 100.22 ± 0.07 to 102.82 ± 0.16. The percent coefficient of variation of BADGE analysis was ranging from 0.17% to 1.85%. This percent coefficient of variation was less than 10%. It was indicated that the random error from each replication of the extracted method of BADGE was acceptable.

4.6 The determination of Limit of Detection and Limit of Quantification

Calibration of BADGE standard concentrations ranging from 0.065 to12 µg /l were prepared for the determination of the LOD and LOQ. A linear of regression curve is presented in figure.4-4 The linear regression analysis was used to determine the relationship between the peak area and the low level of BADGE concentrations. The equation of linear regression was Y= 4467.2X + 737.46 The coefficient of determination of this curve was 0.9995 (R^2= 0.9991). The results shown that the

Copyright by Mahidol University

lowest detection limit of analytical method was 0.4141 µg/l and the limit of quantification of the analytical method was 1.380 µg/l.

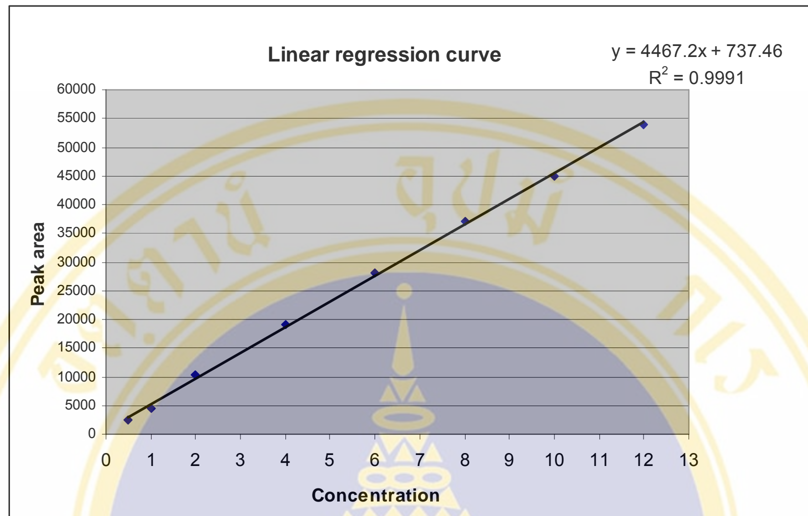


Fig 4-4 Linear regression curve for determination of LOD and LOQ

Linear regression equation : Y = 4467.2X + 737.46

LOD = 3 x standard error of the regression / slope

Standard error of the regression = SEE

$$SEE = \sqrt{\frac{\Sigma (Y_{obs} - Y_{est})^2}{n-k}}$$

Y_{obs} = observe response

Y_{est} = estimated response from regression curve

n = total number of data point

k = 2 for a linear regression curve

SEE ; when

n = 30

k = 2

SEE = 616.29

LOD = 3 x 616.29 / 4467.2 = 0.4141 µg/l

LOQ = 10 x standard error of the regression / slope

= 10 x 616.29 / 4467.2 = 1.380 µg/l

Copyright by Mahidol University

4.7 Analysis of oil samples from canned tuna

The analyzed canned tuna was randomized sampling for one brand from the market. Oil from canned tuna was extracted following the extraction method in section 3.2.5. Chromatogram of **oil samples from canned tuna** is shown in Fig 4-5.

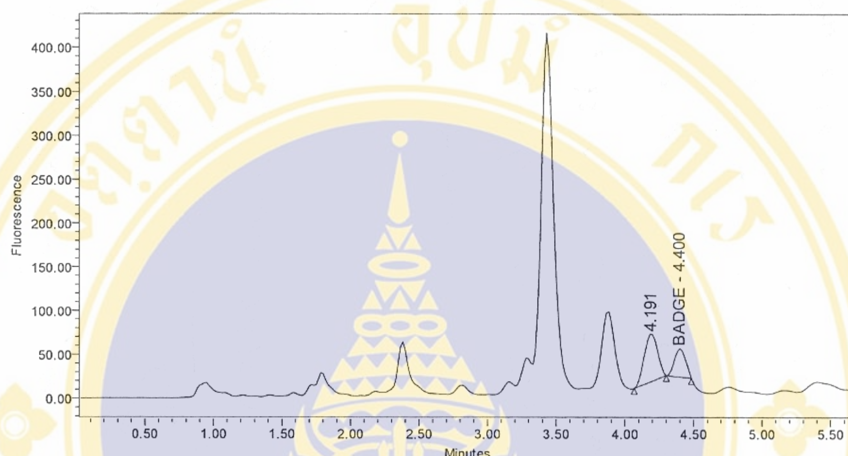


Fig.4-5 The Chromatogram of BADGE in oil sample from canned tuna.

The chromatogram of BADGE in oil sample from canned tuna was shown the same retention time as retention time of standard of BADGE. The analyzed of BADGE concentration from canned tuna which the storage duration both of storage duration less than 6 months was ranging from 24.017 to 128.296 µg/l. The storage duration longer than 6 months, BADGE concentration was ranging from 9.096 to 75.298 µg/l.

4.8 Analysis of average quantity of BADGE in oil from canned tuna.

After analyzed the BADGE quantity data from all of sixty canned of sample.

Copyright by Mahidol University

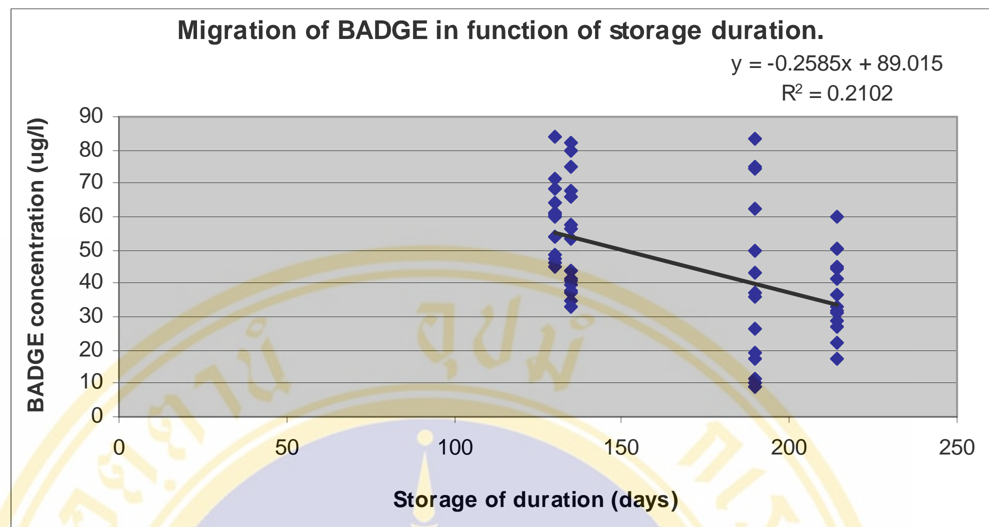


Fig 4-6 The migration of BADGE quantity in function of the storage duration.

From the analyzed results, the average of BADGE concentration which the storage duration less than 6 months was 68.073 µg/l. The BADGE concentration which the storage duration longer than 6 months was 36.454 µg/l.

From Fig 4-6, results shown that BADGE quantity was decreased with longer of storage duration of canned tuna. This means BADGE molecule was changed to be the hydrolysis derivatives form.

Copyright by Mahidol University

CHAPTER V

DISCUSSION

Most interior coating of canned food used epoxy resin and vinyl organosol type which BADGE molecule can be released into the food (11). BADGE was considered to be genotoxic, cytotoxic and mutagenic (7). The metabolized product of BADGE was mutagenic carcinogen. Then, BADGE was chosen in this study because it could remain as an un-reacted matter on the polymerization condition or incompletely curing in coating process. The lipophilic characterization of BADGE resulted in highly migration concentration of BADGE in oil medium.(9), BADGE was stable in oil medium against the degradation to hydrolysis form. Recently, the European Commission, the center of all information related to the controlled regulations in Europe, specified limit of total migration of BADGE and hydrolysis formed shall not exceed 9 mg/kg. The chlorohydrin form shall not exceed 1 mg/kg. Thailand market of canned tuna was grown up every year both for domestic and export market, BADGE was considered to be the one of significant source of migrated substance to the human.

The purpose of this present study was to develop a reliable analysis method for determination of BADGE by HPLC using fluorescence detection and a symmetry C_{18} column and the relationship between storage duration and migrated quantity of BADGE.

5.1 The Chromatographic separation.

Most published methods for analyzing BADGE and other derivatives use reverse-phase HPLC (8,23) to determine BADGE concentration. The C_{18} column often used to improve the resolution of BADGE analysis.(10,23,27,29,31). The solvent system used for this analysis composed of acetonitrile and water. Most studied methods for quantification of BADGE concentration was published using fluorescence

Copyright by Mahidol University

detection.(7,8,9,23,25). The advantage of using fluorescence detection was the high selectivity and sensitivity of BADGE.

5.2 The calibration curve of BADGE

The calibration curve of BADGE was prepared at the concentration ranging 1.0-140 µg/l. The reliability of calibration curve was validated by its good linearity. The plotting of the peak area of BADGE against BADGE concentration in µg/l was shown in Fig 4-3. The linear relationship of the calibration curve was Y= 6168.7X – 6501.3. The coefficient of determination of this calibration curve was 0.9986.

5.3 Reliability of the Method

5.3.1 Accuracy and Precision

The present recovery and percent coefficient of variation of the experiment were carried out to analyze the accuracy and precision of the proposed method. As the results,the average recoveries of BADGE of the developed method were close to 100 % at the concentration ranging from 20.233 to 41.084 µg/l. The results indicated a good precision; the between-day coefficients of variation during the three days were in range of 0.17% to 1.85%, this was less than 2%. The accuracy of the method was ranging from 100.22% to 102.82% for BADGE analysis. Therefore, the results of this study indicated that the accuracy and precision of the method were satisfactory.

When compared with previous studied, it was found that the recovery was nearly the same as the previous studied method. The previous studied by Simoneau *et al*, 1999 reported the recovery ranging from 86.4 % to 92.1% and the precision of the method was less than 3%.

5.3.2 Detection limit

In this analyzed method, the limit of detection (LOD) was 0.4141 µg/l .The limit of quantification (LOQ) was 1.380 µg/l. The results of the previous studied by C.Simoneau *et al* , 1999 (Italy) reported the BADGE detection limit of 49 µg/l after monitored canned fish samples from different countries(34).The other

Copyright by Mahidol University

previous study which enhanced the selectivity of BADGE was studied by Raquel *et al*, they modified the method using a reverse phase high performance liquid chromatography combined with mass spectrometry detection (27).

5.4 Canned tuna samples

This study selected only one brand of canned tuna which was the second best selling and was well known in Thailand market. Each brand of canned tuna used different sources of can produce from different manufacturing process and might give various level of original BADGE concentration releasing from the can. That is why this research used only one type of canned food from one company. The selection of BADGE in oil medium for this study was due to the lipophilic characterization of BADGE and the high concentration of BADGE in oil medium (9).

The shelf life of canned food labeled on the can give the safe storage duration of canned food. Usually, the canned tuna can be kept for two years after production. This study selects only the canned tuna sold on the big supermarkets which has shelf-life for only six months. The longer shelf-life would be taken out of the supermarket. The canned tuna sold in the small shops may have different storage conditions and storage durations of longer than 6 months. The different storage conditions may lead to different BADGE concentrations releasing form the can. That is the reason why most canned tunas in this study had storage duration of 6 months. For that storage duration longer than 6 months, they were bought within 6 months and were kept for a short period of time before analysis.

5.5 BADGE analysis techniques

This study extracted BADGE with acetronitrile and analyzed by HPLC using fluorescence detector which was widely used method. Some analyzed BADGE using gradient system to separate different derivatives of BADGE. Other studied method improved recovery and detection limit of the method; for example, C.Nerin *et al* extracted BADGE and BADGE derivatives using solid phase microextraction to

Copyright by Mahidol University

increase the detection limit down to 0.7 ng/ml and to eliminate interference in the analysis.

Olga Pardo *et al* extracted BADGE and BADGE derivatives using pressurized liquid extraction (PLE) together with liquid-liquid extraction and solid phase extraction (SPE) and analyzed by reverse phase HPLC coupled to atomospheric pressure chemical ionization tandem mass spectrometry(APCI-MS-MS) detection to quantify the BADGE and BADGE derivatives concentration, to confirm the result and to lower the detection limit to 5 ng/g (29)

5.6 BADGE concentration from collected canned tuna.

The average BADGE concentration of canned tuna for storage duration less than 6 months was 68.073 µg/l ranging from 24.017 to 128.296 µg/l. The average BADGE concentration of canned tuna which storage duration longer than 6 months was 36.454 µg/l ranging from 9.096 to 75.298 µg/l. All of canned samples have the migrated level of BADGE lower than 9 mg/kg (9000 µg/g), which comply with the recent standard of the Commission Regulation from European Committee (NO.1895/2005). Concentrations of BADGE were greater than the previous study. The sampling cans from Mexico had migrated level ranging from 5.2 µg to 6.3 µg (32). The sample can exhibited concentrations exceeding the limit which was set by the European Commission's Scientific Committee on Food (SCF) (1mg/kg) for 3%. The other study surveyed BADGE migration from canned fish in Europe gave the average concentration of surveyed samples of 1.42 µg(34).

5.7 The comparison of BADGE concentration between storage duration longer than 6 months and less than or equal to 6 months.

The average migration of BADGE concentration after storage duration longer than 6 months showed lower level of BADGE concentration than the storage duration less than 6 months. It is because BADGE can react with water to form the hydrolysis form(7), as shown in Fig 5-1.

Copyright by Mahidol University

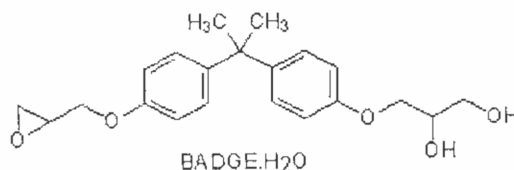


Fig 5-1 Hydrolysis derivatives of BADGE

5.8 The correlation of BADGE concentration and storage duration of canned tuna.

The correlation between storage duration of canned tuna and BADGE concentration from collected canned tuna was calculated. There was not significant correlation between storage duration and BADGE concentration. The correlation gave minus tendency of relationship. It means that longer storage duration of canned food will decrease BADGE quantity but the hydrolysis forms of BADGE may increase.

5.9 Other factors influencing BADGE concentrations in canned tuna

From previous studies, BADGE releasing concentrations in cans were different when studied food storage in different mediums such as in oil, brine, mineral water, etc. (8). The original BADGE concentration in organosol coating have different level of BADGE and lead to different concentrations of BADGE releasing into the medium. The different BADGE concentration was caused by completion of film formation process and baking temperature (5). The storage condition of canned tuna has an effect on the migration of BADGE into the medium. One study (34) showed that storage temperature of more than 60 °C leaded to higher concentration of BADGE than that of 25 °C storage temperature.

Copyright by Mahidol University

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The purpose of this research was to develop an analytical method for quantification of Bisphenol A diglycidyl ether (BADGE) in oily phase of canned tuna by using High Performance Liquid Chromatography. (HPLC)

The oil phase from canned tuna sample was extracted by liquid-liquid extraction using n-hexane and acetonitrile. The optimum condition for separation of BADGE was carried out by the reversed-phase high performance liquid chromatography (RP-HPLC) using isocratic condition with fluorescence detection. Symmetry C_{18} column (5 µm, 3.9 mm x 150 mm. i.d.) was used as an analytical column and a isocratic system was acetonitrile and water (63/35; by v/v) at flow rate of 1 ml/min. The separation showed that Symmetry C_{18} column (5 µm, 3.9 mm x 150 mm. i.d.) needed a separation time of 6.0 minutes. The limit of detection was 0.4141 µg/l. Fluorescence detection was employed using 225 nm as excitation wavelength and 305 nm for emission wavelength.

The accuracy of the method for target concentrations of 20 and 40 µg/l was carried out at the concentration of 20.400 $\pm$ 0.197 µg/l and 40.492 $\pm$ 0.361 µg/l , respectively. The average percent of recoveries of BADGE in oil was ranging from 98.23% to 101.90%.

The limit of detection and limit of quantification were estimated from the regression curve to be 0.4141 µg/l and 1.380 µg/l.

The analyzed BADGE concentration released from canned tuna which was storage for less than 6 months was 68.073 µg/l. The BADGE concentration of canned tuna storage longer than 6 months was 36.454 µg/l.

Copyright by Mahidol University

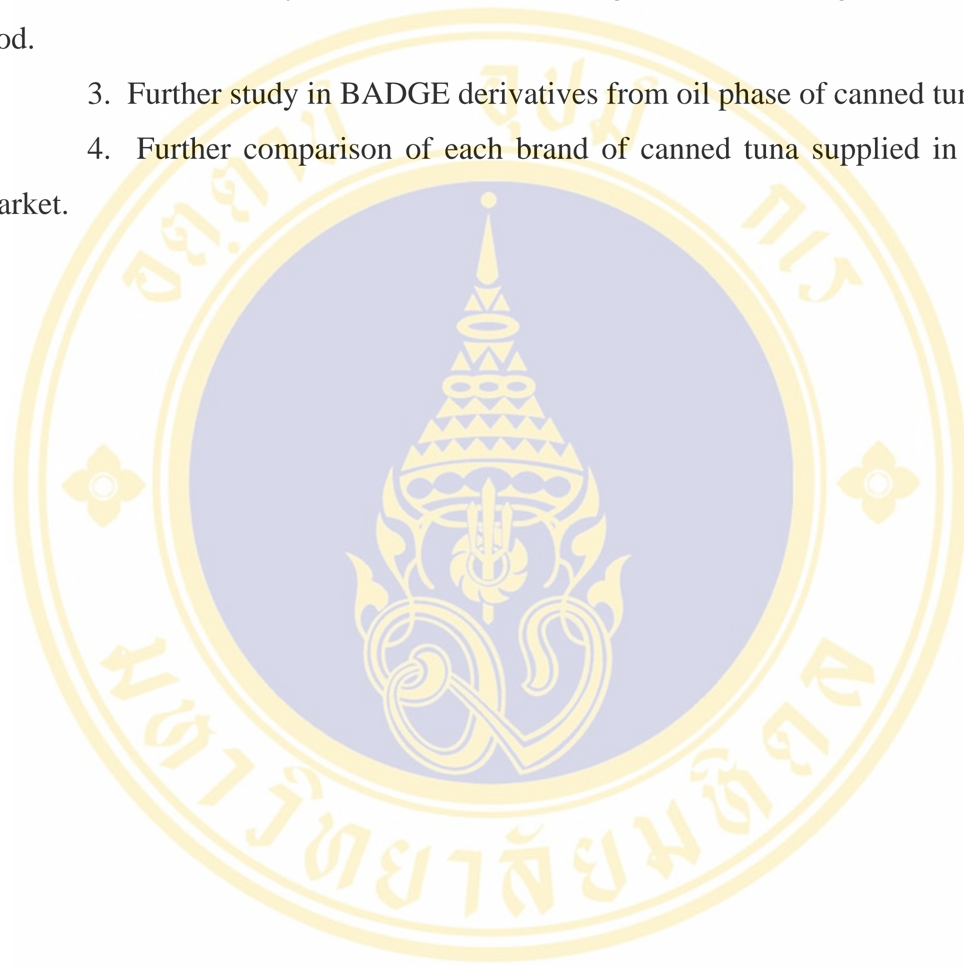
6.2 Recommendation

1. This study may be applied to develop method for analyzing BADGE in different types of canned food.

2. Further study in other factors affecting the BADGE migration into canned food.

3. Further study in BADGE derivatives from oil phase of canned tuna.

4. Further comparison of each brand of canned tuna supplied in Thailand Market.



Copyright by Mahidol University

REFERENCES

1. ดร.เยาวพา สุวัตถิ กลุ่มวิจัยอุตสาหกรรมเทคโนโลยีชีวภาพ, การถนอมอาหาร

2. http://www.food.gov.uk/foodindustry/Consultations/completed_consultations/ website of Michigan State University.

3. มอก.735-2533

4. www.cannedfood.org/quality.html

5. รศ.อ. อรอุษา สรวารี, สารเคลือบผิว (สี วาร์นิช และแลคเกอร์) ภาควิชาวัสดุศาสตร์ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย พิมพ์ครั้งที่ 1, / 2542

6. รศ.ศศิมน ปรีดา มหาวิทยาลัยสุโขทัยธรรมาธิราช, คุณภาพและมาตรฐานในอุตสาหกรรมอาหารกระป๋อง วารสาร Quality Insurance vol.9, no.64, 65, 66.

7. Koichi Inoue, Akiko Yamaguchi, Magumi Wada, Yoshihiro Yoshimura, Tsunehisa Makino, Hiroyuki Nakazawa. Quantitative detection of bisphenol A diglycidyl ether metabolites in human plasma by liquid chromatography electrospray mass spectrometry. J.Chromatography B, 2001; 75 :121-126.

8. R.Sendon Garcia, P.Paserio Losada, C.Perez Lamela. Determination of compounds from epoxy resins in Food Simulant by HPLC-Fluorescence. Chromatographia. 2003 ; 58: 337-342.

9. Josef Lintschinger, Walfried Rauter. Simultaneous determination of bisphenol A diglycidyl ether, bisphenol F diglycidyl ether and their hydrolysis and chlorohydroxy derivatives in canned food. Eur Food Res Technol , 2000 ; 211: 211-217.

10. Urs Berger, Michael Oehme, Line Girardin. Quantification of derivatives of bisphenol A diglycidyl ether (BADGE) and novolac glycidyl ether (NOGE) migrated from can coatings into tuna by HPLC/ fluorescence and MS detection. Fresenius J Anal Chem, 2001; 369:115-123.

11. Elvia M. Munguia-Lopez and Helinda Soto-Valdez. Effect of Heat Processing and

Copyright by Mahidol University

Storage Time on Migration of bisphenol A (BPA) and bisphenol A diglycidyl ether (BADGE) to aqueous food simulant from Mexican Can Coatings. J. Agri. Food Chem, 2001; 49 : 3666-3671.

12. Bisphenol A diglycidyl ether, Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment.,1996, p.8-11.
13. Statement on Bisphenol A diglycidyl ether (BADGE) in canned food, Committee on Toxicity of Chemicals in food, consumer products and the environment, 1997.
14. Opinion on Bisphenol A diglycidyl ether (BADGE) :SCF/CS/PM 3243 Final, 6/04/1999, Scientific Committee on Food, European Commission,1999.
15. Statement of the Scientific Committee on Food on Bisphenol A diglycidyl ether (BADGE) : SCF/CS/PM/GEN/13510/22112002 Final, 9 Dec 2002, Scientific Committee on Food, European Commission, 2002.
16. Commission Regulation (EC) No. 1895/2005 of 18 November 2005, on the restriction of use certain epoxy derivatives in materials and articles intended to come into contact with food, Official Journal of the European Union.
17. กรมส่งเสริมอุตสาหกรรม กระทรวงอุตสาหกรรม, สถิติอุตสาหกรรม ISIC 151210-010 ปลากระป๋อง ปลาทูน่า (ม.ค.1999 – ธ.ค. 2005)
18. กรมส่งเสริมอุตสาหกรรม กระทรวงอุตสาหกรรม, สถิติอุตสาหกรรม ISIC 151210-010 ปลากระป๋อง ปลาซาร์ดีน (ม.ค.1999 – ธ.ค. 2005)
19. NIOSH Manual of Analytical Methods.
20. WRc-NSF –November 2002 (BADGE) :European Commission.
21. W.J. Lough, I.W. Wainer, Fundamental Principles and Practice of High Performance Liquid Chromatography, Blackie Academic & Professional, 1996.
22. Geoff A. Giles and D.R.Bain, Canmaking for can Fillers, Sheffield Academic Press, 2000, 44-96.
23. H.Peterson, A.Schaefer, C.A.Buckow, T.J.Simat, H.Steinhart. Determination of

Copyright by Mahidol University

bisphenol A diglycidyl ether (BADGE) and its derivatives in food: identification and quantification by internal standard. Eur Food Res Technol , 2003; 216 : 355-364.

24. NIOSH, The registry of Toxic Effects of Chemical Substances (RTECS), MSDS.
25. Natchanun Leepipatpiboon, Ornthida Sae-Khow, Siripastr Jayanta. Simultaneous determination of bisphenol A diglycidyl ether, bisphenol F diglycidyl ether and their derivatives in oil-in water and aqueous-based canned food by high-performance liquid chromatography with fluorescence detection.
26. J.Climie, D.Hutson, Q. Stoydin, Xenobiotica. II, 1981;391-340.
27. Raquel Sendon Garcia, Perfecto Paserio Losada. Determination of bisphenol A diglycidyl ether and its hydrolysis and chlorohydroxy derivatives by liquid chromatography-mass spectrometry. J.Chromatography A, 2004 ; 1032:37-43.
28. Walfried Rauter, Gerald Dickinger, Rudolf Zihlarz, Josef Lintschinger. Determination of bisphenol A diglycidyl ether (BADGE) and its hydrolusis products in canned oily foods from the Austrian market. Z Lebensm Unters Forsch A. 1999; 208: 208-211.
29. Olga Pardo, Vicent Yusa, Nuria Leon, Agustin Pastor. Determination of bisphenol diglycidyl ether residues in canned food by pressurized liquid extraction and liquid chromatography-tandem mass spectrometry
30. L.Hammarling , H. Gustavsson, K. Svensson and A.Oskarsson., Migration of bisphenol A diglycidyl ether(BADGE) and its reaction products in canned food. Food Additives and Contaminants, 2000, vol.17, no.11, 937-943.
31. C.Nerin, M.R.Philo, J.Salafranca, L.Castle. Determination of bisphenol-type contaminants from food packaging materials in aqueous foods by solid-phase microextraction-high-performance liquid chromatography. J.Chromatography A.2002; 963 : 375-380.
32. C.Simoneau, A.Theobald, D.Wiltschko and E.Anklam. Estimation of intake of bisphenol A diglycidyl ether (BADGE) from canned fish consumption in Europe and migration survey. Food Additives and Contaminats. 1999 ;16: 457-463.
33. C.Simoneau, A.Theobald, P. Hannaert P.Roncari , A. Roncari, T.Rudolph and

Copyright by Mahidol University

Anklam. Monitoring of bisphenol A diglycidyl ether (BADGE) in canned fish in oil. Food Additives and Contaminats. 1999 ;16:,5 189-195

34. C.Simoneau, A.Theobald, P.Roncari, P. Hannaert and E.Anklam. Time-temperature study of the kinetics of migration of BADGE (bisphenol A diglycidyl ether) into a fatty medium. Food Additives and Contaminats. 2002 ;19: 73-78.

Copyright by Mahidol University

APPENDIX

Copyright by Mahidol University

APPENDIX A

Synonyms (NIOSH) : 2,2-((1-Methyethylidene) bis(4,1phenylenemethylene)) Bisoxirane

: 2,2- Bis(4-(2,3-epoxypropyloxy)phenyl) propane

: 2,2- Bis(4-hydroxyphenyl) propane, diglycidyl ether

: 2,2- Bis(p-glycidyloxyphenyl)propane

: 2,2- Bis(p-hydroxyphenyl)propane, diglycidyl ether

: 4,4'-Diydroxydiphenyldimethylmethane diglycidyl ether

: 4,4'-Isopropylidenediphenol diglycidyl ether

: Bis (4-glycidyloxyphenyl) dimethylmethane

: Bis (4-hydroxyphenyl) dimethylmethane diglycidyl ether

: Bpdge

: D.E.R.332

: Dian diglycidyl ether

: Dian-bis-glycidylether (Czech)

: Diglycidyl bisphenol A ether

: Diglycidyl ether of 2,2-bis(4-hydroxyphenyl) propane

: Diglycidyl ether of 2,2-bis(p-hydroxyphenyl) propane

: Diglycidyl ether of 4,4'-isopropylidenediphenol

: Diglycidyl ether of bisphenol A

: Diomethane diglycidyl ether

: ERL-2774

: Epi-Rez 508

: Epi-Rez 501

: Epotuf 37-140

: Epoxide A

: GY 6010

: Oligomer 340

: p,p'- Dihydroxydiphenyldimethylmethane diglycidyl ether

Copyright by Mahidol University

APPENDIX B

THE ANALYTICAL RESULTS

1. The recovery of BADGE's extraction from blank oil.

Table B-1 The calculated percent recovery of the analysis method of BADGE from blank oil.

Target Concentration	Extracted results (μg)	Percent Recovery (%)	Corrected results (μg/l)
15 μg/l	14.634	97.56	15.000
	15.024	100.16	15.048
	15.012	100.08	15.024
	X **SD** **% CV**		**15.024** **0.024** **1.47**
30 μg/l	30.383	101.28	30.772
	30.515	101.72	31.040
	30.881	102.70	31.643
	X **SD** **% CV**		**31.152** **0.398** **1.22**
50 μg/l	48.666	97.33	50.001
	49.432	98.86	50.002
	49.386	98.77	50.001
	X **SD** **% CV**		**50.001** **0.001** **0.87**

2. Table B-2 The calculated accuracy of the analysis method for BADGE extraction from canned tuna oil.

Rep.no	Concentration					
	Spiked concentration of 20 μg/l			Spiked concentration of 40 μg/l		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
1	20.228	20.442	20.328	41.899	40.132	40.127
2	20.159	20.500	20.100	40.389	40.118	40.597
3	20.313	20.746	20.790	40.965	40.008	40.193
X	20.233	20.563	20.406	41.084	40.086	40.306
SD	0.077	0.161	0.352	0.762	0.067	0.254
% CV	0.380	0.782	1.72	1.85	0.167	0.63

Copyright by Mahidol University

3. Table B-3 BADGE concentration after storage for less than 6 months

Sample no	Weight of oil (g)	Calculate value (µg/l)	Storage time (days)
1	10.0263	63.470	135
2	10.0056	38.480	135
3	10.0468	33.381	135
4	10.0539	73.611	135
5	10.0829	42.065	135
6	10.0428	47.796	135
7	10.1065	46.723	135
8	10.1984	24.017	135
9	10.3298	113.020	135
10	10.0474	41.080	135
11	10.0373	109.084	135
12	10.1512	124.701	135
13	10.0286	70.394	135
14	10.1347	33.900	135
15	10.1976	28.030	135
16	10.2681	90.635	135
17	10.4602	63.978	135
18	10.2186	66.283	130
19	10.1473	79.650	130
20	10.1150	50.215	130
21	10.2897	87.403	130
22	10.2618	128.296	130
23	10.2600	51.787	130
24	10.0297	81.272	130
25	10.0681	78.911	130
26	10.1025	54.504	130
27	10.0453	94.750	130
28	10.2176	101.923	130
29	10.0624	57.462	130
30	10.0383	65.356	130

4. Table B-4 BADGE concentration after storage for more than 6 month

Sample no	Weight of oil (g)	Calculate value (µg/l)	Storage time (days)
1	10.0115	17.473	214
2	10.0510	28.755	214
3	10.1335	50.285	214
4	10.1114	50.686	214
5	10.3196	44.860	214
6	10.1321	26.914	214

Copyright by Mahidol University

7	10.0613	32.076	214
8	10.0058	36.774	214
9	10.0095	41.294	214
10	10.0930	32.076	214
11	10.0786	36.774	214
12	10.0100	41.294	214
13	10.3252	32.943	214
14	10.1393	60.243	214
15	10.3437	62.281	190
16	10.0008	74.607	190
17	10.2478	49.688	190
18	10.1672	37.207	190
19	10.4020	83.512	190
20	10.0492	43.139	190
21	10.1625	19.116	190
22	10.1237	75.298	190
23	10.0344	17.230	190
24	10.6323	10.257	190
25	10.5832	9.096	190
26	10.0344	17.230	190
27	10.6323	10.257	190
28	10.5832	9.096	190
29	10.0224	35.771	190
30	10.2035	11.559	190

5. Table B-5 The data of limit of detection

BADGE concentration (µg/l)	Peak Area
0.50	2478
1.0	4450
2.0	10364
4.0	19000
6.0	28000
8.0	37000
10.0	44930

Copyright by Mahidol University

APPENDIX C

The calculation of accuracy and precision of the method.

(1) Recovery percentage

Observed value concentration = 15.012 μg/l

Target concentration = 15.000μg/l

$$\text{Percent recovery} = \frac{15.012}{15.000} \times 100$$

$$= 100.16\ \%$$

$$\text{Corrected mass} = \frac{100}{\text{Percent recovery}} \times \text{Observed concentation}$$

$$= \frac{100}{100.08} \times 15.012$$

$$= 15.024\ \mu g$$

(2) Relative standard deviation

$$S = \frac{SD}{X}$$

$$= \frac{0.024}{15.024} = 0.024$$

(4) Coefficient of variation percent (%CV)

$$\%CV = 100 \left(\frac{SD}{X}\right)$$

$$= 100 \times \frac{0.077}{20.233}$$

$$= 0.380\%$$

Copyright by Mahidol University

BIOGRAPHY

NAME	Miss Supawadee Noradee
DATE OF BIRTH	24 September 1978
PLACE OF BIRTH	Angthong, Thailand
INSTITUTION ATTENDED	King Mongkuth's University of, Technology Thonburi, 1998: Bachelor of Science (Chemistry) Mahidol University, 2005: Master of Science (Industrial Hygiene and Safety)
POSITION & OFFICE	Quality Assurance Technical Department, Toyo Ink Coating Thailand Co.,Ltd.
OFFICE	34 Moo.14 Bangchan Industrial Estate, Serithai Rd., Minburi, Bangkok 10510 Tel: 0-5178180-5 E-mail: nsupawadee@toyoink.co.th
HOME ADDRESS	119 Moo.2 S.S.P Apartment, Romklao Rd., Minburi, Bangkok 10510

Copyright by Mahidol University