Chapter 3

Research methodology

In this chapter, the procedures are divided into two sections. Section 3.1 describes the development of micellar liquid chromatographic with UV spectrometric method (MLC-UV method) for parabens determination in cosmetics. Section 3.2 is the development of liquid chromatography/mass spectrometry (LC-MS/MS) for perchlorate analysis in environmental samples. The instrumentations, optimal conditions, preparation of reagents and samples, and analytical features of the developed methods are described.

3.1 Development of MLC method for parabens analysis

3.1.1 Instruments

Instruments used in the developed method, preparation of reagents and samples clean-up are described below.

3.1.1.1 Micellar liquid chromatographic system

MLC analyses were carried out with Hewlett Packard 1090A liquid chromatograph (Agilent Technologies, USA) featuring a solvent delivery pump system, a diode array detector (DAD) and 20 µL loop injector, as shown in Figure B1 (see Appendix B). The guard column Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 µm), Agilent Technologies (USA), was used as analytical column (Figure B2, Appendix B). The chromatographic systems are given in Table 3.1.

3.1.1.2 Instruments used in the method

Instruments used in the preparation of reagents, standard solutions and samples are given in Table 3.2.

Table 3.1

Instrumentation of MLC-UV

Instrument	Model	Company
Liquid chromatograph	HP 1090A	Agilent Technologies (USA)
Diode array detector	79880A	Agilent Technologies (USA)
Manual injector	79835A	Agilent Technologies (USA)
Column	Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 µm)	Agilent Technologies (USA)
Data processing	HP ChemStation (A.08.03)	Agilent Technologies (USA)

Table 3.2

Instruments of the preparation of standard solutions and samples

Instrument	Model	Company
Analytical balance	TC-205	Denver Instrument (USA)
Vortex mixer	KMC-1300V	Vision Scientific Co., Ltd. (Korea)
Ultrasonic bath	CT-430G1	Wah Luen Electronic Tools Co., Ltd. (China)
Centrifuge	1040	Labquip International Co., Ltd. (England)
pH meter	FA 720	Orion (USA)
Autopipette (2-20, 20-200 and 100-1000 µL)	-	Eppendorf (Germany)
DI water purification system	ELGASTAT Option3A, 13172G series	Elga (England)
Nylon membrane filter (0.45 µm, 47 mm)	-	Lubitech Technologies Ltd. (China)
Nylon syringe filter (0.45 µm, 13 mm)	-	Lubitech Technologies Ltd. (China)

3.1.2 Chemicals

All chemicals and reagents used were of analytical reagent grade. All solvents used for chromatographic analysis were HPLC grade. Chemicals and solvents used in this work were listed in Table 3.3. Water was deionized and purified on DI water purification system and used to prepare all solutions.

Table 3.3
List of chemicals and suppliers

Chemicals	Formula	Suppliers
Methyl-4-hydroxybenzoate (MP)	$C_8H_8O_3$	Sigma (Germany)
Ethyl-4-hydroxybenzoate (EP)	$C_9H_{10}O_3$	Sigma (Germany)
Propyl-4-hydroxybenzoate (PP)	$C_{10}H_{12}O_3$	Sigma (Germany)
Butyl-4-hydroxybenzoate (BP)	$C_{11}H_{14}O_3$	Sigma (Germany)
Sodium dodecyl sulfate (SDS)	$C_{12}H_{25}SO_4Na$	Fluka (Switzerland)
Hexadecyltrimethylammonium bromide (CTAB)	$C_{19}H_{42}BrN$	Fluka (Switzerland)
Methanol	CH_4O	Fisher Scientific (Canada)

3.1.3 Cosmetic samples and sample preparation

In this section, the developed method was applied to determine parabens in cosmetic samples. Samples and sample preparation in this work are described below.

3.1.3.1 Cosmetic samples

The cosmetic products consisted of two types, which are commercial cosmetics (sample number 1-10) and Thai community cosmetics (sample number 11-64). All samples were listed in Table A1 and A2 (see Appendix A). Commercial cosmetics were purchased from department stores in Bangkok, Thailand.

They are consist of 10 samples, which are facial foam 1 piece, cleansing lotion 1 piece, toner 1 piece, facial cream 1 piece, body lotion 4 pieces, body scrub 1 piece and hand and nail lotion 1 piece. Thai community cosmetics were purchased from OTOP shops in the various provinces of Thailand. They are consist of 54 samples, which are soap 5 pieces, shower gel 12 pieces, facial foam 3 pieces, hand soap 1 piece, shampoo 13 pieces, hair conditioner 4 pieces, facial mask 1 piece, facial cream 8 pieces, body scrub 2 pieces, body lotion 4 pieces and armpit cream 1 piece. Placebo sample (shampoo) was obtained from Rajamangala University of Technology Thanyaburi, Pathum Thani provinces, Thailand.

3.1.3.2 Samples preparation

Two millilitres of methanol were added to approximately 0.1000-0.5000 g of each samples accurately weighed and vortexed for 2 minutes. After that, the solution was ultrasonicated for 5 minutes and centrifuged at 3000 rpm for 10 minutes. A 1.0 mL aliquot of the supernatant was transferred to a 10.0 mL volumetric flask. The sample was diluted to volume with mobile phase, and then filtered through a 0.45 µm nylon syringe filter for the analysis.

3.1.4 Preparation of standard solutions and other reagents

The preparation procedures of standard solutions and reagents employed in this work are following. Deionized water was used for preparation of all solutions.

3.1.4.1 Mobile phase

The developed method was carried out by using sodium dodecyl sulfate (SDS) at concentration of 0.046 mol L^{-1} as mobile phase. Mobile phase was prepared by diluting of 0.25 mol L^{-1} SDS, as described below.

Sodium dodecyl sulfate of approximately 36.0475 g was dissolved and diluted with DI water into 500.0 mL volumetric flask to give 0.25 mol L^{-1} SDS.

Mobile phase, 0.046 mol L^{-1} SDS, was prepared by pipetting of 0.25 mol L^{-1} SDS at exact volume of 92.00 mL, into 500.0 mL volumetric flask and making volume with DI water. This solution was filtered through a 0.45 µm nylon membrane filter before used.

3.1.4.2 Parabens standard solutions

a) Stock parabens standard solutions, 1 x 10^{-3} mol L^{-1}

Each stock standard solution of 1 x 10^{-3} mol L^{-1} methyl paraben, ethyl paraben, propyl paraben and butyl paraben were prepared by the following procedure.

Methyl paraben, ethyl paraben, propyl paraben and butyl paraben were accurately weighed at 0.0152, 0.0166, 0.0180 and 0.0194 g, respectively. Ten millilitres of methanol were added and diluted with DI water into 100.0 mL volumetric flask to give stock standard solutions of 1 x 10^{-3} mol L^{-1} for each paraben.

b) Working parabens standard solutions

Working mixture standard solutions of parabens were freshly prepared at concentration 1, 5, 10, 20, 30, 40, 50, 75 and 100 µmol L^{-1} by pipetting of the stock standard solutions of each paraben (1 x 10^{-3} mol L^{-1}) at 10.00, 50.00, 100.00, 200.00, 300.00, 400.00, 500.00, 750.00 and 1000.00 µL, respectively, and made to final volume of 10.0 mL of mobile phase. These solutions were further prepared to construct calibration curves that described in Section 3.1.5.4 (a).

3.1.5 Procedures and methods

In this part, micellar liquid chromatographic separation with UV spectrometric detection (MLC-UV) was developed, particularly in the analysis of parabens in cosmetics. Procedures and methods are described below.

3.1.5.1 Preliminary study

The suitable detection wavelength of all parabens was selected. The types of surfactants used as mobile phase were optimized, as followed.

a) Suitable detection wavelength

The suitable detection wavelength of all parabens was studied and selected by measuring of maximum absorption wavelengths (λ_{max}) of all parabens. Methyl paraben, ethyl paraben, propyl paraben and butyl paraben at concentration of 20 µmol L^{-1} in 0.046 mol L^{-1} SDS, by diluting stock standard solutions of 1 x 10^{-3} mol L^{-1} (200.00 µL of each stock standard solution in 10.00 mL of mobile phase), were measured maximum absorption using UV-visible spectrophotometer (UV-1700, Shimadzu, Japan).

b) Suitable micellar mobile phase

The types of surfactant as micellar mobile phase such as anionic surfactant (sodium dodecyl sulfate, SDS) and cationic surfactant (hexadecyltrimethylammonium bromide, CTAB) were optimized incorporating to guard column Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 µm) as analytical column by injecting mixture standard solution of parabens at concentration of 20 µmol L^{-1} (200.00 µL of each stock standard solution in 10.00 mL of mobile phase) at flow rate of 0.75 mL min^{-1} and UV detection at 254 nm.

3.1.5.2 Chromatographic behavior

The possibility of using C18 guard column as an analytical column for parabens separation in micellar liquid chromatography was investigated because of using short length column. Therefore, the chromatographic behavior was studied. The results will be corresponding to chromatographic behavior of solutes in micelle system with common analytical column C8 and C18 that proposed by Arunyanart and Cline-Love (Arunyanart & Cline-Love, 1984), as shown in equation 1.3 (see Chapter 1, Section 1.1.1.3).

For the study of the chromatographic behavior, concentrations of SDS were varied in the range of 0.025-0.150 mol L^{-1} which is higher than the critical micellar concentration (CMC of SDS is 8.27 mmol L^{-1}). The variations of concentrations of SDS as mobile phase were 0.025, 0.050, 0.075, 0.100, 0.125 and 0.150 mol L^{-1} (10.00, 20.00, 30.00, 40.00, 50.00 and 60.00 mL of 0.25 mol L^{-1} SDS in 100.00 mL of DI water). The system consisted of guard column Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 μm) as analytical column and UV detection at 254 nm at mobile phase flow rate of 0.75 mL min^{-1}. Standard mixture solutions of parabens at concentration of 10 μmol L^{-1} (100.00 μL of each stock standard solution in 10.00 mL of mobile phase) was injected into the system. Then capacity factors of each paraben, $k = (t_R - t_0)/t_0$, were calculated. The relationship between the reciprocal value of capacity factors of parabens (1/k) and concentrations of surfactant as micelle foam in mobile phase ([M]) were investigated.

3.1.5.3 Optimization of the MLC condition

In this method, MLC conditions for parabens separation were optimized by simplex optimization method. The simplex methods are based on an initial design of k+1 trials, where k is the number of variables. A k+1 geometric figure in a k-dimensional space is called a simplex as shown in Figure 3.1. The corners of this figure are called vertexes (or vertices).

For example, with two variables the first simplex design is based on three trials, for three variables it is four trials, etc. This number of trials is

also the minimum for defining a direction of improvement. Therefore, it is a timesaving and economical way to start an optimization project.

After the initial trials of the simplex process is sequential, with the addition and evaluation of one new trial at a time. The simplex searches systematically for the best levels of the control variables. The optimization process ends when the optimization objective is reached or when the responses cannot be improved further.

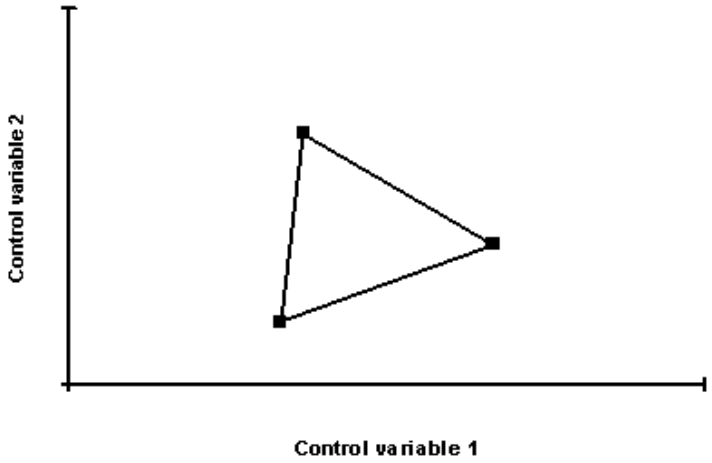

Figure 3.1 A simplex defined by three different trial conditions for two control variables (Grabitech Solutions AB, n.d.)

The advantage of simplex optimization method is timesaving for the case of many variables which is complicated. Therefore, the optimum conditions obtaining from simplex optimization method for simultaneous determination of four parabens was investigated. In this work, the resolution of methyl paraben, ethyl paraben, propyl paraben and butyl paraben as a function of flow rate and the concentration of micellar mobile phase were considered. Therefore, the number of variables (n) in this method is two and the number of trials (n+1) is three. The initial trials or initial vertexes (I) which used for starting of simplex optimization method are given in Table 3.4. The chromatographic conditions were guard column Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 μm) as analytical column and UV detection at 254 nm and mixture standard solutions of parabens at concentration of 40 μmol L^{-1}

(400.00 µL of each stock standard solution in 10.00 mL of mobile phase) was injected into all trials. The optimization process ended when the optimization objective was reached or when the responses cannot be improved further.

The targets of the MLC conditions for parabens separation obtaining from simplex optimization method were the retention time of the last peak (butyl paraben) not more than 7 minutes ($t_{BP} \approx 7$ min) and resolution factors not less than 1.5 ($R_S \geq 1.5$). The simplex optimization method was reached, as given in equation 3.1.

$$CRF = \sum_{i=1}^{L} R_i + L^a - b \mid T_A - T_L \mid + c\,(T_1 - T_0) \qquad \text{…….(3.1)}$$

Where CRF is chromatographic response function, R_i is resolution factor between the closed peak (four peaks give three values which are R_1, R_2 and R_3), L is numbers of detection peaks (four peaks, L=4). T_A and T_L are retention time of the last peak (butyl paraben) which is assigned and is given from the experiment, respectively. T_1 is retention time of the first peak (methyl paraben) which is given from the experiment. T_0 is dead time in the experiment ($T_0 = 1.20$). a, b and c are constant values which is assigned following the important of parameters, in this procedure assign a=1, b=5 and c=1.

Table 3.4

The initial vertexes of this method

Vertex	[SDS] (mol L^{-1})	Flow rate (mL min^{-1})
1	0.040	0.500
2	0.070	1.000
3	0.100	0.750

3.1.5.4 Validation study

For validation study, calibration curves, repeatability, reproducibility, recovery and limits of detection (LOD) of this method were examined.

a) Calibration curves

Working range of the method was evaluated at nine concentration levels within the range of 1-100 µmol L^{-1} by diluting the standard stock solutions (1000 µmol L^{-1}). These solutions were injected in triplicate and then plotted concentrations of each paraben versus peak areas for calibration curves and linear regressions analysis.

Working standard mixture solutions of parabens were freshly prepared at concentration of 1, 5, 10, 20, 30, 40, 50, 75 and 100 µmol L^{-1} by pipetting of the stock standard solutions of each paraben (1000 µmol L^{-1}) at 10.00, 50.00, 100.00, 200.00, 300.00, 400.00, 500.00, 750.00 and 1000.00 µL, respectively, into 10.0 mL volumetric flask and diluted with mobile phase. These solutions were injected into the optimum conditions.

b) Repeatability

The instrumental system precision was examined by injecting 10 times the same mixed standard parabens solutions at concentration of 10 µmol L^{-1} (100.00 µL of each stock standard solution in 10.00 mL of mobile phase) within one day. The means and the relative standard deviations (%RSD) were evaluated.

c) Reproducibility

Reproducibility was carried out by injecting seven aliquots of three cosmetics (sample number 6, 50 and 60). Seven individual samples were

prepared as described in Section 3.1.3.2 and each solution was injected in one replicate into the system. The relative standard deviations (%RSD, n=7) were investigated.

d) Recovery study

To assess accuracy, placebo sample was freshly prepared as described in Section 3.1.3.2 and spiked with various amounts of methyl paraben, ethyl paraben, propyl paraben and butyl paraben at six concentrations: 5, 10, 20, 40, 60 and 80 μmol L^{-1}.

Moreover, the recovery method was performed by running four samples (sample number 6, 17, 50 and 60) which spiked with various amounts of methyl paraben, ethyl paraben, propyl paraben and butyl paraben at five concentrations: 10, 20, 30, 40 and 50 μmol L^{-1}. The mixtures were analyzed by the proposed method and then calculated the quantity of each paraben recovered in relation to the added amount (%Recovery).

e) Limits of detection (LOD)

This study was carried out to determine the limits of detection (LOD) of the proposed method. They are expressed as a concentration of each paraben at a specified signal-to-noise ratio, usually three (3S/N).

3.1.5.5 Application of the method in cosmetics

The proposed method was also applied for monitoring parabens contents in cosmetic samples. The cosmetic products consisted of two types, which are commercial cosmetics (sample number 1-10) and Thai community cosmetics (sample number 11-64) as listed in Table A1 and A2 (see Appendix A). All samples were freshly prepared as described in Section 3.1.3.2 and analyzed by injecting into the optimum MLC-UV conditions.

3.2 Development of LC-MS/MS method for perchlorate analysis

3.2.1 Instruments

Instruments used in this method, preparation of standard solutions and other reagents were described below. A samples preparation was also presented.

3.2.1.1 LC-MS/MS system

Finnigan SpectraSYSTEM liquid chromatograph (Thermo Finnigan, USA) was used for the analysis featuring binary gradient pump with degaser, dual wavelength UV-visible programmable detector and variable-loop auto sampler with column oven (100 µL loop max.), as shown in Figure B3 (see Appendix B). Zorbax SB-C18, 50 mm length, 4.6 mm i.d. and 5 µm (Agilent Technologies, USA) was used as analytical column (Figure B4, Appendix B). The liquid chromatographic with mass spectrometric systems are given in Table 3.5.

Table 3.5
Instrumentation of LC-MS/MS

Instrument	Model	Company
Liquid chromatograph	SpectraSYSTEM	Thermo Finnigan (USA)
Binary gradient pump	P2000	Thermo Finnigan (USA)
Degaser	SCM1000	Thermo Finnigan (USA)
UV-Visible detector	UV2000	Thermo Finnigan (USA)
Auto sampler with variable loop (100 µL max.)	AS3000	Thermo Finnigan (USA)
Column	Zorbax SB-C18 (50 x 4.6 mm i.d., 5 µm)	Agilent Technologies (USA)
Data processing	Xcalibur	Thermo Finnigan (USA)
Ion-trap mass spectrometer	LCQ Advantage	Thermo Finnigan (USA)

3.2.1.2 Instruments used in the method

Instruments and equipments used in the preparation of reagents, standard solutions and samples are given in Table 3.6.

Table 3.6

Instruments of the preparation of standard solutions and samples

Instrument	Model	Company
Analytical balance	TC-205	Denver Instrument (USA)
Centrifuge	1040	Labquip International Co., Ltd. (England)
Vaccuum pump	A-1000S	Tokyo Rikakikai Co., Ltd. (Japan)
Autopipette (2-20, 20-200 and 100-1000 µL)	-	Eppendorf (Germany)
DI water purification system	ELGASTAT Option3A, 13172G series	Elga (England)
Nylon membrane filter (0.20 µm, 47 mm)	-	Chromex Scientific (United Kingdom)
Nylon syringe filter (0.20 µm, 13 mm)	-	Chromex Scientific (United Kingdom)
OnGuard II Ba (1-cc)	-	Dionex Corporation (USA)

3.2.2 Chemicals

Chemicals and solvents used in this work were listed in Table 3.7. All chemicals and reagents used were of AR grade. All solvents used for chromatographic analysis were HPLC grade. Water was deionized and purified on DI water purification system and used to prepare all solutions. All glasswares used for preparation were cleaned with 10 %w/w nitric acid before used.

Table 3.7

List of chemicals and suppliers

Chemicals	Formula	Suppliers
Sodium perchlorate (99+ %)	$NaClO_4$	Acros (USA)
Oxygen-labeled sodium perchlorate (90+ %, 100 µg mL^{-1} in water)	$NaCl^{18}O_4$	Cambridge Isotope Laboratories, Inc. (USA)
Sodium sulphate	Na_2SO_4	Carlo Erba (Italy)
Sodium chloride	NaCl	Carlo Erba (Italy)
Sodium nitrate	$NaNO_3$	Merck (Germany)
Sodium nitrite	$NaNO_2$	APS Finechem (Australia)
Sodium carbonate	Na_2CO_3	Sigma (USA)
di-Sodium hydrogen orthophosphate	Na_2HPO_4	Ajax Chemicals (Australia)
Potassium bromide	KBr	Carlo Erba (Italy)
Potassium iodate	KIO_3	Carlo Erba (Italy)
Potassium iodide	KI	BHD Laboratory (England)
Magnesium chloride	$MgCl_2$	Ajax Chemicals (Australia)
Barium chloride	$BaCl_2$	Ajax Chemicals (Australia)
Hexamethonium bromide (HMB)	$C_{12}H_{30}Br_2N_2$	Sigma Aldrich (Switzerland)
Hexadecyltrimethylammonium bromide (CTAB)	$C_{19}H_{42}BrN$	Fluka (Switzerland)
Decyltrimethylammonium bromide (DTAB)	$C_{13}H_{30}BrN$	Sigma Aldrich (Switzerland)
Dihexylammonium acetate (DHAA) (0.5 mol L^{-1} in water)	$C_{14}H_{31}NO_2$	Tokyo Chemical Industry Co., Ltd. (Japan)
Methanol	CH_4O	Merck (Germany)
Acetic acid 100 % (glacial)	$C_2H_4O_2$	Merck (Germany)
Nitric acid 65 %	HNO_3	RCI Labscan Ltd. (Thailand)

3.2.3 Environmental samples and sample preparation

In this section, the developed method was applied to determine perchlorate in environmental samples. Samples and sample preparation are described below.

3.2.3.1 Environmental samples

Environmental samples analyzed in the developed method were water and soil in the area of the north of Thailand. All samples were listed in Table A3 and A4 (see Appendix A). Water and soil samples were collected on March, 2011 from three longan fruit gardens in Tambon San Pee Seua, Muang, Chiang Mai provinces, Thailand. For water samples, the first garden used water from irrigation (Water-1) and ground water (Water-2). The second garden used ground water (Water-3). Ground water used in the third garden was filtered before poured water onto the trees (Water-4). All water samples were collected from water surface about 500 mL into precleaned polyethylene bottles. Water-5 and Water-6 were drinking water and tap water, respectively. Soil samples (500 g) from three gardens were taken from the top of soil about 1 meter far from the tree and were collected 2 trees per garden (Soil-1 to Soil-6). Then soils were stored in clean polyethylene bottles. Other soils were collected from three villages in Saraphi (Soil-7 to Soil-9) and one village in Sankampang (Soil-10), Chiang Mai. All samples were stored at 4 ^{o}C until analysis. No preservation was required. According to the literature, perchlorate is stable at room temperature for 50 days or longer. Therefore, the holding time of 28 days was recommended to be consistent with USEPA anion analysis guidelines (Li & George, 2005; Winkler, Minteer, & Willey, 2004).

3.2.3.2 Samples preparation

Water samples: Samples were pretreated by solid phase extraction to eliminate potentially high concentrations of common anions such as sulphate anion prior to analysis, which was accomplished by eluting an aqueous

sample aliquot through Dionex barium OnGuard cartridges (Dionex, USA). Based on the manufacturer-recommended procedures, cartridges were first condition with 10 mL DI water. Next, an aliquot of 5 mL sample was eluted through the cartridges at a speed of ~1 mL min^{-1} (< 2 mL min^{-1}). A 3 mL sample was discarded and sample was collected next 2 mL and was fortified with the internal standard (^{18}O-labeled perchlorate) at a concentration of 50 µg L^{-1} prior to analysis.

Soil samples: Soils were prepared by leaching 1.0000 g of sample with 10.0 mL of DI water and centrifuged at 4000 rpm for 15 minutes. A 5 mL aliquot of the supernatant solution was then prepared for analysis following the aqueous procedure above. Then all samples were filtered through a 0.20 µm nylon syringe filter before injection into LC-MS/MS.

3.2.4 Preparation of standard solutions and other reagents

The preparation procedures of standard solutions and other reagents used in this work are following. All solutions were prepared in DI water and were filtered through a 0.20 µm nylon syringe filter before injected into the LC system. All glasswares used were cleaned with 10 %w/w nitric acid before used.

3.2.4.1 Mobile phase

The LC-MS/MS method was carried out by using hexamethonium bromide (HMB) at concentration of 10 µmol L^{-1} as mobile phase. Mobile phase was prepared by diluting of 10 mmol L^{-1} hexamethonium bromide, as described below.

A 1.8110 g of hexamethonium bromide (MW 362.19) was approximately weight. Then the solid of hexamethonium bromide was dissolved and diluted with DI water into 500.0 mL volumetric flask to give 10 mmol L^{-1} hexamethonium bromide.

Then, 10 µmol L^{-1} hexamethonium bromide used as mobile phase was prepared by pipetting of 0.50 mL of 10 mmol L^{-1} hexamethonium bromide

into 500.0 mL volumetric flask and made up to volume with DI water. This solution was filtered through a 0.20 µm nylon membrane filter before used.

3.2.4.2 Ion-interaction reagents

a) Hexadecyltrimethylammonium bromide, 1 mmol L^{-1}

Hexadecyltrimethylammonium bromide (CTAB, MW 364.45) at concentration of 1 mmol L^{-1} was prepared. A solid CTAB of 0.0364 g was weighed and diluted with DI water into 100.0 mL volumetric flask.

b) Decyltrimethylammonium bromide, 1 mmol L^{-1}

Decyltrimethylammonium bromide (DTAB, MW 280.29) was weighed at 0.0280 g into 100.0 mL volumetric flask by diluting with DI water. This DTAB solution was concentration of 1 mmol L^{-1}.

c) Dihexylammonium acetate, 1 mmol L^{-1}

An aliquot of 0.20 mL of dihexylammonium acetate (DHAA, MW 245.40) at concentration of 0.5 mol L^{-1} was transferred into 100.0 mL volumetric flask and diluted with DI water to give DHAA solution of 1 mmol L^{-1}.

3.2.4.3 Perchlorate standard solution

a) Perchlorate solution, 1000 mg L^{-1}

Stock standard solution of perchlorate at concentration of 1000 mg L^{-1} was prepared by accurately weighing of 0.0616 g of perchlorate. Then DI water was added and diluted into 50.0 mL volumetric flask. For further dilution, this solution was then used as the primary stock solution.

b) Perchlorate solution, 20 mg L^{-1}

20 mg L^{-1} perchlorate was prepared by transferring of the primary stock solution of perchlorate (1000 mg L^{-1}) at exact volume of 1.00 mL into 50.0 mL volumetric flask and made to final volume with DI water.

c) Perchlorate solution, 500 µg L^{-1}

A 25.00 µL aliquot of the primary stock perchlorate solution (1000 mg L^{-1}) was transferred into 50.0 mL volumetric flask and diluted with DI water.

d) Perchlorate solution, 20 µg L^{-1}

Pipetting of 500 µg L^{-1} at a volume of 2.00 mL was diluted with DI water into 50.0 mL volumetric flask to give perchlorate solution of 20 µg L^{-1}.

e) Working perchlorate standard solutions

Working standard solution of perchlorate at concentrations of 4, 10, 20, 30, 40 and 50 µg L^{-1} were freshly prepared by pipetting of 500 µg L^{-1} of perchlorate solution at 0.08, 0.20, 0.40, 0.60, 0.80 and 1.00 mL, respectively, and made up to final volume of 10.0 mL of DI water. These solutions were fortified with the internal standard at a concentration of 50 µg L^{-1} prior to analysis. These solutions were further prepared to construct calibration curves that described in Section 3.2.5.4 (b).

3.2.4.4 Oxygen-labeled sodium perchlorate, 500 µg L^{-1}

Stock oxygen-labeled sodium perchlorate ($Cl^{18}O_4^-$) used as internal standard was prepared at concentration of 500 µg L^{-1}, as followed. A 250.00

µL aliquot of 100 µg mL^{-1} oxygen-labeled sodium perchlorate was transferred into a 50.0 mL volumetric flask and made to volume with DI water.

3.2.4.5 Other anion solutions, 1000 mg L^{-1}

Each anion solution of 1000 mg L^{-1} of sulphate (SO_4^{2-}), phosphate (PO_4^{3-}), chloride (Cl^-), nitrate (NO_3^-), nitrite (NO_2^-), carbonate (CO_3^{2-}), bromide (Br^-), iodate (IO_3^-) and iodide (I^-) were prepared, as described below.

Sulphate (SO_4^{2-}), phosphate (PO_4^{3-}), chloride (Cl^-), nitrate (NO_3^-), nitrite (NO_2^-), carbonate (CO_3^{2-}), bromide (Br^-), iodate (IO_3^-) and iodide (I^-) were accurately weighed at 0.1479, 0.1495, 0.1649, 0.1371, 0.1500, 0.1767, 0.1489, 0.1224 and 0.1308 g, respectively. Then DI water was added and diluted into 100.0 mL volumetric flask to give solution of 1000 mg L^{-1} for each anion.

3.2.4.6 Magnesium chloride solution, 0.5 mol L^{-1}

A 10.1650 g of solid $MgCl_2$ was approximately weighed into 100.0 mL volumetric flask and diluted with DI water. This solution was 0.5 mol L^{-1} $MgCl_2$ (12,155 mg L^{-1} as Mg^{2+}).

3.2.4.7 Barium chloride solution, 0.08 mol L^{-1}

Barium chloride at concentration of 0.08 mol L^{-1} (10,987 mg L^{-1} as Ba^{2+}) was prepared by transferring of 1.9542 g into DI water in 100.0 mL volumetric flask.

3.2.4.8 Sodium carbonate solution, 0.17 mol L^{-1}

Solid Na_2CO_3 was weighed at 1.7700 g and dissolved with DI water into 100.0 mL volumetric flask to give Na_2CO_3 solution of 0.17 mol L^{-1} (10,020 mg L^{-1} as CO_3^{2-}).

3.2.5 Procedures and methods

In this part, liquid chromatography with mass spectrometric detection (LC-MS/MS) was developed incorporating to ion-interaction (ion-pairing) reagent as mobile phase on C18 column for perchlorate analysis. This method was applied for the analysis of perchlorate in environmental samples. Procedures and methods are followed.

3.2.5.1 Investigation of ion-pairing reagent

In this work, the studied cationic ion-pairing reagents were hexadecyltrimethylammonium bromide (CTAB), decyltrimethylammonium bromide (DTAB), dihexylammonium acetate (DHAA) and hexamethonium bromide (HMB). The suitable ion-pairing reagent for determination of perchlorate was selected, as described below.

20 mg L^{-1} of perchlorate (200.00 µL of 1000 mg L^{-1} of stock perchlorate solution in 10.00 mL of methanol), 20 µmol L^{-1} of each ion-pairing reagent (200.00 µL of 1 mmol L^{-1} of each stock ion-pairing reagent solution in 10.00 mL of methanol) and 20 mg L^{-1} of perchlorate mixed with each ion-pairing reagent at concentration of 20 µmol L^{-1} were prepared. These solutions were directly injected into mass spectrometer in a positive mode for selection of the suitable ion-pairing reagent for perchlorate analysis.

3.2.5.2 Optimization of the LC condition

The optimal conditions for separation of perchlorate were studied. The types of ion-pairing formation (pre-column and on-column) in liquid chromatographic system were investigated. The parameters such as compositions of mobile phase (organic modifier, acetic acid and concentration of ion-pairing reagent) and injection volume were studied.

a) Investigation of ion-pairing formation in LC system

After the ion-pairing reagent selected as described in Section 4.2.1, then the types (pre-column and on-column) of ion-pairing formation that introduced into liquid chromatographic system were investigated. Mobile phase was mobile phase A: 10 % methanol and mobile phase B: 90 % acidic aqueous (0.1 %v/v acetic acid in DI water). Other LC/MS conditions are showed in Table 3.8. The studied LC systems were consisted of three systems (System 1, 2 and 3), as given in Table 3.9. System 1-(a) was not used ion-pairing reagent and system 1-(b) was pre-column ion-pairing formation. System 2-(a) and 2-(b) were on-column ion-pairing formation. System 3-(a) and 3-(b) were both pre-column and on-column ion-pairing formation.

Table 3.8
LC/MS conditions

Instruments	Parameters	Conditions
Liquid chromatograph	Mobile phase	MeOH : 0.1 % CH_3COOH (10 : 90)
	Column	Zorbax SB-C18 (50 x 4.6 mm i.d., 5 µm)
	Flow rate	0.40 mL min^{-1}
	Injection volume	20 µL
Ion-trap mass spectrometer	Acquisition mode	Positive ESI
	Sheath gas (L h^{-1})	40
	Auxiliary gas (L h^{-1})	10
	Spray voltage (kV)	4.50
	Spray current (µA)	0.39
	Capillary temperature (^oC)	300
	Capillary voltage (V)	46
	Tube lens offset (V)	25

In the first system, 1-(b), mobile phase did not contained ion-pairing reagent but ion-pairing reagent mixed with perchlorate was injected into LC system. This system was in term ‘pre-column ion-pairing formation’. In the second system, ion-pairing reagent was introduced into LC system by mixing with mobile phase which called ‘on-column ion-pairing formation’. The third system was both pre-column and on-column ion-pairing formation. System 2-(a) and 2-(b), ion-pairing reagent was mixed in mobile phase line B and A, respectively, and perchlorate was directly injected into LC system without ion-pairing reagent. System 3-(a) and 3-(b), ion-pairing reagent was mixed in mobile phase line B and A, respectively, and perchlorate mixed with ion-pairing reagent was injected into LC system. Perchlorate at concentrations of 1, 5, 10, 20 and 30 mg L^{-1} were prepared by pipetting of 1000 mg L^{-1} of perchlorate solution at 0.01, 0.05, 0.10, 0.20 and 0.30 mL, respectively, into 10.0 mL volumetric flask. These solutions were injected into all system. Concentration of ion-pairing reagent studied was 20 µmol L^{-1}. The suitable system for perchlorate analysis was selected.

Table 3.9

The study of ion-pairing formation in LC system

Ion-pairing formation	System	Mobile phase A (10 %)		Mobile phase B (90 %)		Injection	
		MeOH	20 µM HMB	0.1 % acetic â	20 µM HMB	ClO_4^-	20 µM HMB
-	1-(a)	√	-	√	-	√	-
Pre-column	1-(b)	√	-	√	-	√	√
On-column	2-(a)	√	-	√	√	√	-
	2-(b)	√	√	√	-	√	-
Pre-column	3-(a)	√	-	√	√	√	√
and on-column	3-(b)	√	√	√	-	√	√

b) Injection volume

Liquid chromatograph (Thermo Finnigan, USA) that used in this work consisted of variable-loop injection (100 µL loop max.). Injection volume was also studied and varied at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µL. Injection volume that obtained the highest sensitivity was selected. The LC conditions were given in Table 3.10. Perchlorate at concentration of 0.005, 0.02 and 1 mg L^{-1} were prepared and were fortified with the internal standard at a concentration of 50 µg L^{-1} for the analysis.

Table 3.10
LC-MS/MS conditions

Instruments	Parameters	Conditions
Liquid chromatograph	Mobile phase	MeOH : 20 µM HMB with 0.1 % CH_3COOH (10 : 90)
	Column	Zorbax SB-C18 (50 x 4.6 mm i.d., 5 µm)
	Flow rate	0.40 mL min^{-1}
	Injection volume	10-100 µL
Ion-trap mass spectrometer	Acquisition mode	Positive ESI
	Sheath gas (L h^{-1})	40
	Auxiliary gas (L h^{-1})	10
	Spray voltage (kV)	4.50
	Spray current (µA)	0.39
	Capillary temperature (^oC)	300
	Capillary voltage (V)	10
	Tube lens offset (V)	5
	Scanning mode	Full scan
	Collision energy (%)	30

c) Compositions of mobile phase

The compositions of mobile phase such as organic modifier, acetic acid and concentration of ion-pairing reagent were studied, as described below.

- Effect of organic modifier

Organic modifier used in this method was methanol. Effect of organic modifier in mobile phase were studied at 0, 5, 10, 15 and 20 %v/v of methanol (mobile phase A) and mobile phase B was fixed with 18 µmol L^{-1} hexamethonium bromide with 0.1 % acetic acid in DI water. Injection volume was 100 µL and other conditions were given in Table 3.10. Perchlorate, chloride, sulphate and phosphate at concentration of 0.5, 50, 50 and 50 mg L^{-1} were prepared and were fortified with the internal standard at a concentration of 50 µg L^{-1}. These solutions were injected into the LC system.

- Effect of acid

In this method, acid added in mobile phase was acetic acid. Acid contents in mobile phase were varied at 0, 0.05 and 0.10 %v/v. Acid contents must not higher than 0.10 %v/v because pH of mobile phase was less than 2 (limit of column was pH 2-8). Perchlorate at concentration of 0.05, 0.1 and 0.2 mg L^{-1} were prepared and were fortified with the internal standard at a concentration of 50 µg L^{-1} for the analysis. The LC conditions were given in Table 3.10 which used methanol : 20 µM hexamethonium bromide (10 : 90) as mobile phase and 100 µL loop as injection volume.

- Concentration of ion-pairing reagent

The concentrations of ion-pairing reagent in mobile phase were varied at 5, 10, 20, 30, 40 and 80 µmol L^{-1}. The concentration of ion-

pairing reagent that obtained the highest sensitivity was selected. Perchlorate at concentration of 10, 30 and 50 µg L^{-1} were prepared and were fortified with the internal standard at a concentration of 50 µg L^{-1}. These solutions were injected into the LC system as given in Table 3.11.

Table 3.11
LC-MS/MS conditions

Instruments	Parameters	Conditions
Liquid chromatograph	Mobile phase	MeOH : HMB in DI water (10 : 90)
	Column	Zorbax SB-C18 (50 x 4.6 mm i.d., 5 µm)
	Flow rate	0.40 mL min^{-1}
	Injection volume	100 µL
Ion-trap mass spectrometer	Acquisition mode	Positive ESI
	Sheath gas (L h^{-1})	40
	Auxiliary gas (L h^{-1})	10
	Spray voltage (kV)	4.50
	Spray current (µA)	0.39
	Capillary temperature (^{o}C)	300
	Capillary voltage (V)	40
	Tube lens offset (V)	30
	Scanning mode	Full scan
	Collision energy (%)	30

3.2.5.3 Optimization of the MS/MS condition

The MS/MS conditions which were collision energy and scanning mode were studied.

a) Collision energy

The collision induced dissociation step for MS/MS was optimized. The collision energies were varied from 26.0-35.0 % in order to obtain the maximum response of the fragment ions. Perchlorate of 0.1 mg L^{-1} was prepared for the analysis and was injected into the LC system as given in Table 3.11.

b) Scanning mode in MS/MS

Scanning mode in ion-trap mass spectrometer (Xcalibur software) for MS/MS detection are the selected ion monitoring (SIM) and full scan mode. The type of scanning mode that obtained the highest sensitivity was selected for the analysis. The selected ion monitoring (SIM) was monitored at mass of *m/z* 301, 242, 187 and 128. The full scan mode was monitored at mass range of *m/z* 80-315. Perchlorate at concentration of 10, 30 and 50 µg L^{-1} were prepared and were fortified with the internal standard at a concentration of 50 µg L^{-1}. These solutions were injected into the LC system as given in Table 3.11.

3.2.5.4 Validation study

For validation study, linearity range, repeatability, reproducibility, recovery, limit of detection (LOD) and interferences study were examined.

a) Linearity range

Twenty concentrations of perchlorate were prepared in the range of 0.5-50,000 µg L^{-1} and were fortified with the internal standard at a concentration of 50 µg L^{-1}. Linearity range of this method was studied.

b) Calibration curve (Working range)

The calibration curve of the proposed method was evaluated at six concentration levels within the range of 4-50 µg L^{-1} by diluting the standard stock solution that described in Section 3.2.4.3 (e). These were fortified with the internal standard at a concentration of 50 µg L^{-1} and were injected in triplicate. Then calibration curve was constructed for linear regression analysis and this calibration curve was used for determination of perchlorate in environmental samples.

c) Repeatability

Repeatability of the determination of perchlorate was studied for the instrumental system precision by injecting 10 times the same sample solutions (Soil-1 and Water-1) that added perchlorate at concentration of 10 µg L^{-1} and 50 µg L^{-1} for soil and water samples, respectively, within one day. The sample preparation was described in Section 3.2.3.2 and was fortified with the internal standard at a concentration of 50 µg L^{-1} prior to analysis. The means and the relative standard deviations (%RSD) of ten replicate analyses were evaluated.

d) Reproducibility

Reproducibility was studied by injecting ten aliquots of samples. Ten individual soil sample (Soil-1) that added perchlorate at concentration of 10 µg L^{-1} and ten individual water sample (Water-1) that added perchlorate at concentration of 50 µg L^{-1} were prepared as described in Section 3.2.3.2. All solutions were fortified with the internal standard at a concentration of 50 µg L^{-1}. Each solution was injected in one replicate into the LC system. The %RSD (n=10) were evaluated.

e) Recovery study

For accuracy study, drinking water (Water-5) and tap water (Water-6) were prepared as described in Section 3.2.3.2 and spiked with perchlorate

at concentration of 50 µg L^{-1} and were fortified with the internal standard at a concentration of 50 µg L^{-1}. The recoveries were examined without using solid phase extraction.

In addition, samples (Soil-1 and Water-1) were prepared as described in Section 3.2.3.2 and spiked with amount of perchlorate at concentration of 50 µg L^{-1}. These solutions were fortified with the internal standard at a concentration of 50 µg L^{-1}. The recoveries that treated with solid phase extraction and without solid phase extraction were compared. Then the recoveries were calculated.

f) Limit of detection (LOD)

The limit of detection (LOD) of the proposed method was examined. LOD was performed as the lowest concentration that still obtained the same ion fragments and the same relative intensity of fragments (±20 % if relative intensity of base peak > 50 %) (EU, 2002).

g) Interferences study

Interferences which studied in this method were sulphate (SO_4^{2-}), phosphate (PO_4^{3-}), chloride (Cl^-), nitrate (NO_3^-), nitrite (NO_2^-), carbonate (CO_3^{2-}), bromide (Br^-), iodate (IO_3^-) and iodide (I^-), as described below.

- Effect of Cl^-, NO_3^-, NO_2^-, CO_3^{2-}, Br^-, IO_3^- and I^-

Perchlorate of 0.5 mg L^{-1} was mixed with each anion at concentration of 50 mg L^{-1}. All solutions were injected into the LC system (Table 3.11) for interferences study without using solid phase extraction.

- The tolerant limits of SO_4^{2-} and PO_4^{3-}

Sulphate and phosphate were the major common anions and the spectral interferences (*m/z* 99). Therefore, the tolerant limits of

sulphate and phosphate that interfered perchlorate analysis were studied without using solid phase extraction, as described following. Perchlorate at concentration of 10 µg L^{-1} which mixed with each sulphate and phosphate were prepared as given in Table 3.12. The LC system was given in Table 3.11.

Moreover, the tolerant limit of sulphate using solid phase extraction was examined. Perchlorate at concentration of 30 µg L^{-1} mixed with each sulphate at concentrations of 0, 10, 50, 100 and 150 mg L^{-1} were prepared in 10.0 mL volumetric flask. The cartridges were first condition with 10 mL DI water. Next, all solutions were eluted through the cartridges at a speed of ~1 mL min^{-1} (< 2 mL min^{-1}). A first 3 mL of solution was discarded and was collected next 2 mL for injection into LC system.

Table 3.12

The study of the tolerant limits of sulphate and phosphate

Bottles	$[ClO_4^-]$ (µg L^{-1})	$[SO_4^{2-}]$ or $[PO_4^{3-}]$ (µg L^{-1})
1	10	0
2	10	10
3	10	50
4	10	100
5	10	300
6	10	500
7	10	1,000
8	10	5,000
9	10	10,000
10	10	20,000
11	10	50,000
12	10	100,000

3.2.5.5 Application of the method in environmental samples

Environmental samples which analyzed in the proposed method were soil and water samples. Samples preparation was investigated by using solid phase extraction (SPE) for sulphate removal prior to the analysis. Then all samples were analyzed.

a) Samples preparation

In this method, solid phase extractions (OnGuard-Ba) were used for preparation of the samples. Dry and wet elutions of the cartridges were studied and the suitable elution was then chosen. Perchlorate at concentration of 10 µg L^{-1} was prepared in 25.0 mL volumetric flask. First, Cartridges were condition with 10 mL DI water. Next, a solution of 10 µg L^{-1} perchlorate was eluted through the cartridges at a speed of ~1 mL min^{-1} (< 2 mL min^{-1}). A first 3 mL of solution was collected and next solution was collected each 2 mL. Dry elution means the solution in the cartridges must dry before the next solution eluting. Wet elution always had the solution in the cartridges. Each fraction collected was injected into the LC system (Table 3.11).

b) Samples determination

The developed method was applied to determine perchlorate in environmental samples such as soil and water. All samples were listed in Table A3 (see Appendix A). The samples preparation was described in Section 3.2.3.2. The sample solutions were fortified with the internal standard at a concentration of 50 µg L^{-1} and were analyzed by injecting into the optimum conditions. Perchlorate content in samples was calculated from the calibration curve in Section 3.2.5.4 (b).