Chapter 4

Results and discussion

In this chapter, results and discussion consist of two sections. Section 4.1 describes the development of MLC-UV method for parabens analysis in cosmetics. Section 4.2, the development of LC-MS/MS for perchlorate analysis in environmental samples is described.

4.1 Development of MLC method for parabens analysis

4.1.1 Preliminary study

4.1.1.1 Suitable detection wavelength

Maximum absorption wavelengths (λ_{max}) of all parabens were studied by measuring 20 µmol L^{-1} of each methyl paraben, ethyl paraben, propyl paraben and butyl paraben in 0.046 mol L^{-1} SDS using UV-visible spectrophotometer. The results showed that maximum absorption wavelengths (λ_{max}) of all parabens were 254 nm. Therefore, the suitable detection wavelength of all parabens was selected at 254 nm. UV spectrum of methyl paraben was shown in Figure 4.1.

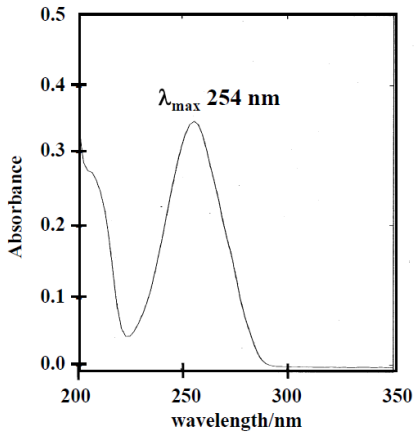


Figure 4.1 UV spectrum of methyl paraben

4.1.1.2 Suitable micellar mobile phase

Micellar mobile phase was investigated the types of surfactant between anionic surfactant and cationic surfactant. The studied anionic surfactant was sodium dodecyl sulfate (SDS) and cationic surfactant was hexadecyltrimethyl-ammonium bromide (CTAB) incorporating to guard column Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 µm) as analytical column.

This study consists of two systems. In the first system, the condition consisted of 0.075 mol L^{-1} SDS as mobile phase at flow rate of 0.75 mL min^{-1} and UV detection at 254 nm. In the second system, 0.025 mol L^{-1} CTAB was used as mobile phase and other conditions were as same as the first system. Chromatograms of methyl paraben, ethyl paraben, propyl paraben and butyl paraben at concentration of 20 µmol L^{-1} obtained at two different micellar mobile phase are illustrated in Figure 4.2 (a) and 4.2 (b), respectively.

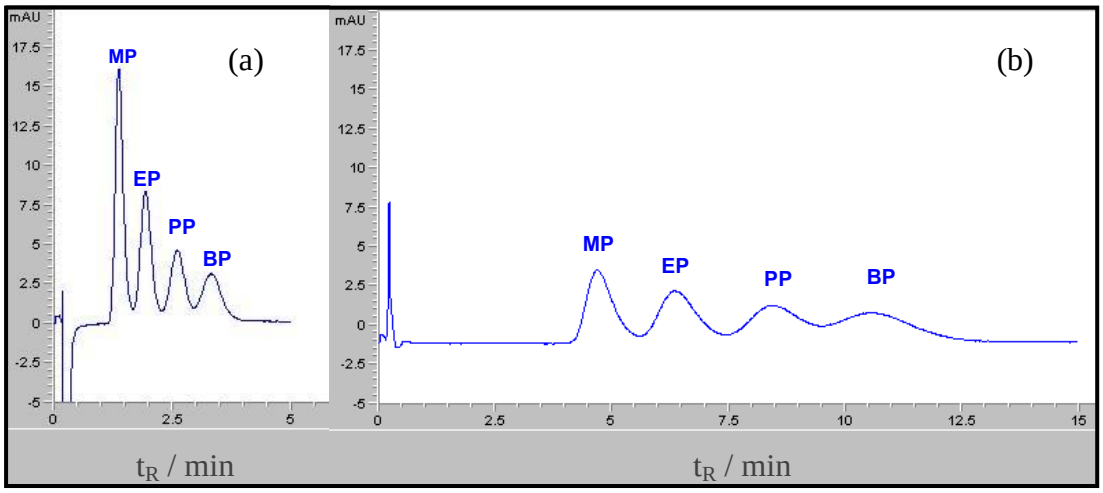


Figure 4.2 Chromatograms of 20 µmol L^{-1} parabens when used (a) 0.075 mol L^{-1} SDS and (b) 0.025 mol L^{-1} CTAB as mobile phase at flow rate of 0.75 mL min^{-1} and UV detection at 254 nm

The elution orders of parabens in both different micellar mobile phases were methyl paraben, ethyl paraben, propyl paraben and butyl paraben,

respectively, because of hydrophobic interaction of parabens which increase following carbon chain of functional group of parabens at the C-4 position. However, the elution orders of parabens were not depend on hydrophilic group of SDS and CTAB which are anionic and cationic surfactant, respectively. In this method, the concentrations of SDS and CTAB were higher than 9 and 19 times of its critical micellar concentration, respectively, which was on micelle concept or in term micellar liquid chromatographic method.

SDS can be improved the separation, obtaining the higher sensitivity and faster separation than CTAB. Moreover, concentration of CTAB was not increased for increasing retention time because parabens cannot be separated. Therefore, SDS was selected as the mobile phase composition for this method. Retention time (t_R) and peak area of all parabens when used two different micellar mobile phase are given in Table C1 (see Appendix C).

4.1.2 Chromatographic behavior

The retention behaviors of methyl paraben, ethyl paraben, propyl paraben and butyl paraben were studied in micellar mobile phase at various concentrations of SDS, 0.025-0.150 mol L^{-1}. The capacity factors (k) of parabens were calculated as shown in Table C2 (see Appendix C). 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 also calculated as shown in Table C3 (see Appendix C). It was found that the reciprocal value of capacity factor of each paraben was a linear function of concentration of surfactant as micelle foam in mobile phase, as equations 4.1, 4.2, 4.3 and 4.4 for methyl paraben, ethyl paraben, propyl paraben and butyl paraben, respectively, and the relation coefficient (R^2) > 0.99.

All equations were corresponding to chromatographic behavior of solutes in micelle system with common analytical column C8 and C18 that reviewed by Arunyanart and Cline-Love (Arunyanart & Cline-Love, 1984), as shown in equation 1.3 (see Chapter 1, Section 1.1.1.3). Where k is capacity factor, [M] is concentration of surfactant as micelle foam in mobile phase, and [M] is equal to the difference between total concentration of SDS and critical micelle concentration.

$$\frac{1}{k} = 1.139[M] + 0.063 \quad (R^2 = 0.9966) \qquad \text{.....(4.1)}$$

$$\frac{1}{k} = 0.968[M] + 0.030 \quad (R^2 = 0.9958) \qquad \text{.....(4.2)}$$

$$\frac{1}{k} = 0.759[M] + 0.015 \quad (R^2 = 0.9957) \qquad \text{.....(4.3)}$$

$$\frac{1}{k} = 0.590[M] + 0.010 \quad (R^2 = 0.9961) \qquad \text{.....(4.4)}$$

The results showed that the use of a short guard column Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 µm) as analytical column obtained the possibility for parabens separation in micellar liquid chromatography. The relationship between 1/k and [M] are given in Figure 4.3.

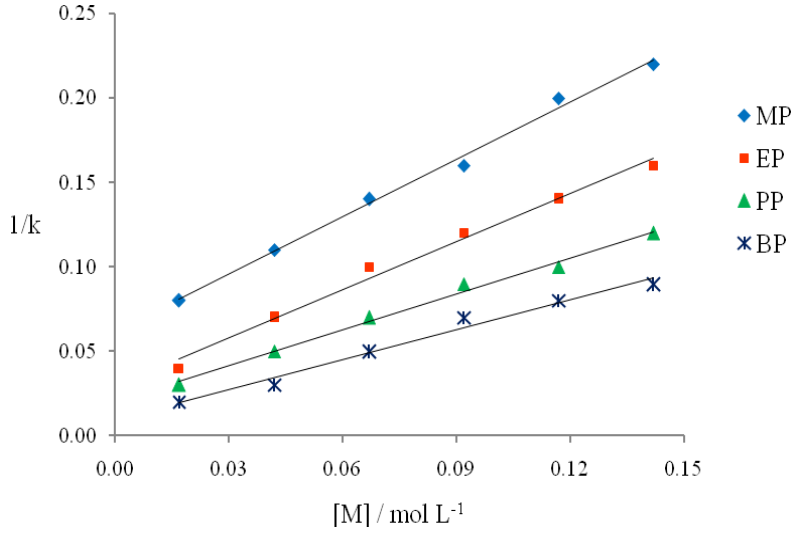


Figure 4.3 Relation of 1/k and [M]

4.1.3 Optimization of the MLC condition

The simplex optimization method was used for optimization of micellar chromatographic conditions for parabens analysis. The variables of this method were the flow rate and the concentration of micellar mobile phase. Therefore,

the numbers of variables (n) in this method were two and the numbers of trials (n+1) were three. The chromatographic response functions (CRF) were calculated, as described in equation 3.1 (see Chapter 3, Section 3.1.5.3). The calculations of CRF of all vertexes are illustrated in Table C4 (see Appendix C). In order to optimize the conditions for obtaining the maximum response or the responses cannot be improved further, the relationship between chromatographic response and vertex number were plotted and given in Figure 4.4 and Table C5 (see Appendix C). The results showed that chromatographic response functions of vertex number of 16-21 were little variation and cannot be improved further with standard deviation = 1.32 (or %RSD = 13) that the advantage was robustness of the method. The vertex number of 17 obtained the highest CRF which was the maximum response, so these conditions were selected.

The optimum conditions, by simplex optimization, for simultaneous determination of four parabens were 0.046 mol L^{-1} sodium dodecyl sulfate and a flow rate of 0.612 mL min^{-1}, as summarized in Table 4.1. The results showed that these conditions were found to achieve the complete separation within 7 minutes as shown in Figure 4.5.

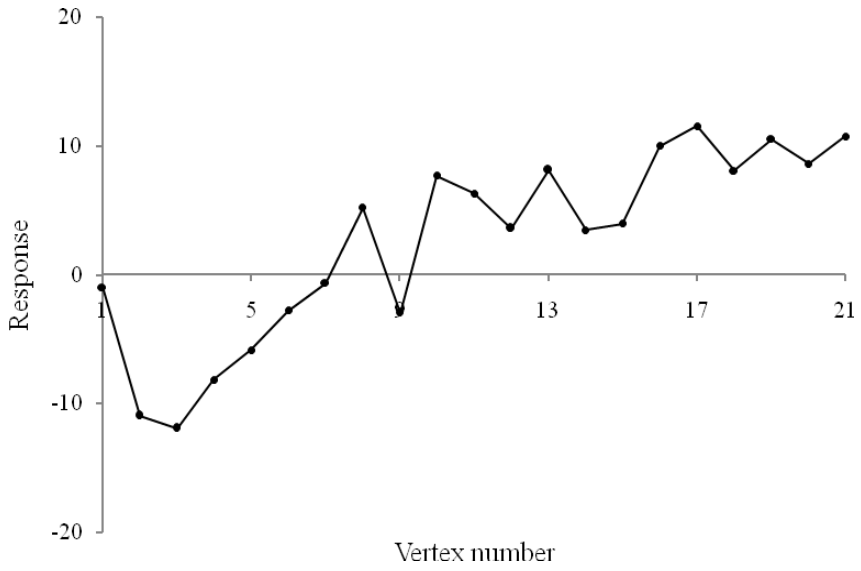


Figure 4.4 Response vs. vertex number for variable-size simplex self-test results

Table 4.1

The optimal conditions of this method

Parameters	Optimal Condition
Column	Zorbax SB-C18 (12.5 x 4.6 mm i.d., 5 µm)
Mobile phase	0.046 mol L^{-1} SDS
Flow rate	0.612 mL min^{-1}
Diode array detector	254 nm
Injection volume	20 µL

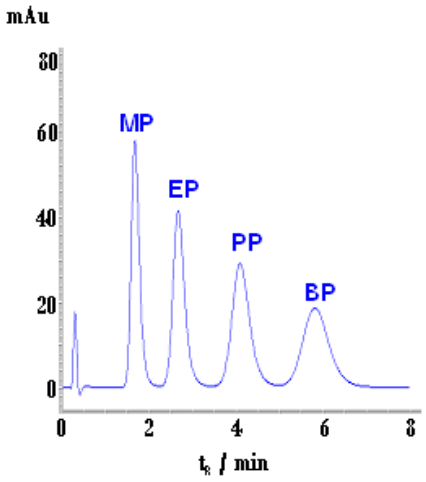


Figure 4.5 Chromatogram of the standard parabens mixture at concentration of 40 µmol L^{-1}

4.1.4 Validation study

4.1.4.1 Calibration curves

Working range of the method was examined by performing three replicate injections at nine standard concentrations: 1, 5, 10, 20, 30, 40, 50, 75 and 100 µmol L^{-1}. The calibration curves were plotted from peak area versus concentration as shown in Figure 4.6 (a), (b), (c) and (d) for methyl paraben, ethyl paraben, propyl paraben and butyl paraben, respectively (Table C6, Appendix C). The

results of the equations and the correlation coefficients (R^2) were reported in Table 4.2. The calibration curves showed good linearity over the concentration range and the correlation coefficients of four parabens were higher than 0.9990.

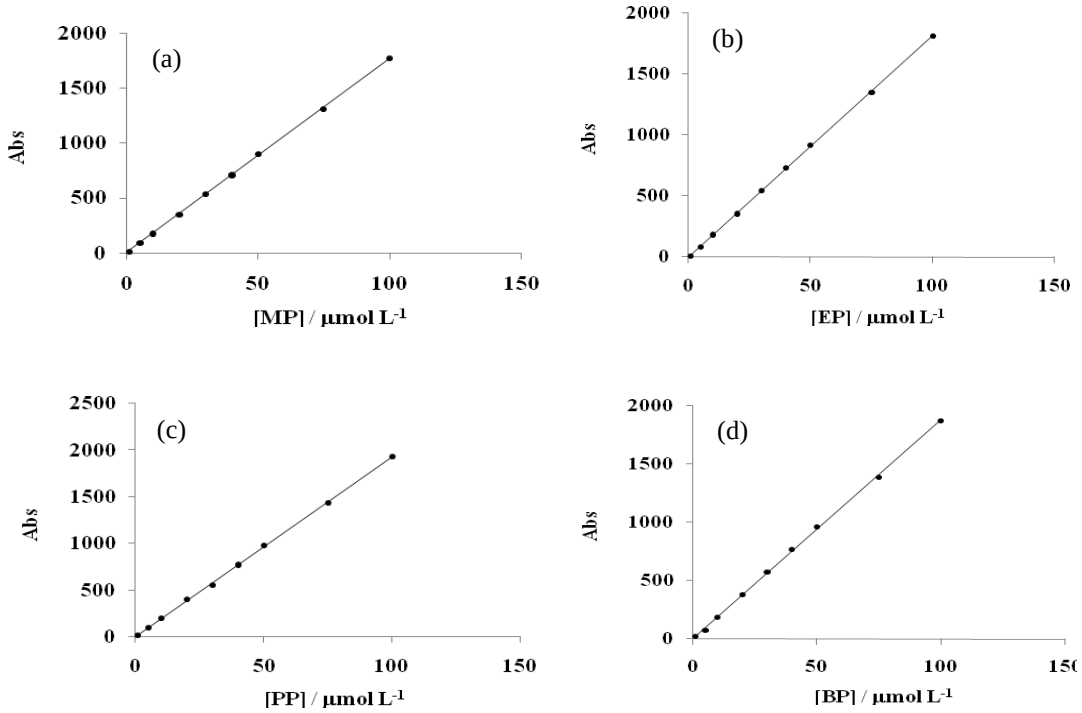


Figure 4.6 Calibration curves of (a) methyl paraben, (b) ethyl paraben, (c) propyl paraben and (d) butyl paraben at concentration in the range of 1-100 µmol L^{-1}

Table 4.2

Retention time (t_R), resolution factor (R_S) and calibration equations

Analytes	t_R (min)	R_S	Linearity			
			Range (µmol L^{-1})	Slope	Intercept	R^2
MP	1.543	-	1-100	17.6230	2.5001	0.9998
EP	2.698	2.87	1-100	18.1360	0.3114	0.9998
PP	4.040	2.83	1-100	19.1960	2.1231	0.9997
BP	6.004	2.15	1-100	18.6750	1.2670	0.9993

4.1.4.2 Repeatability

To determine the precision of instrumental system, parabens were analyzed ten times using the same standard mixture solution (10 µmol L^{-1}). The results were summarized in Table C7 (see Appendix C). From the results, the RSD values of retention time and peak area response of parabens were in the range of 0.51-3.76 and 1.01-3.01 %, respectively, indicating good repeatability of the assay system.

4.1.4.3 Reproducibility

Reproducibility of injecting seven aliquots of three cosmetics which were body lotion (sample no. 6), facial cream (sample no. 50) and body lotion (sample no. 60) were studied. Seven aliquots of each sample were analyzed one injection per aliquot for the determination of the precision of the method including sample preparation and instrumental performance. In all instances, the RSD values of the amount of parabens in sample solutions were 2.21-2.41, 1.24-1.35, 1.78-2.65 % for sample number 6, 50 and 60, respectively as summarized in Table C8 (see Appendix C). The values of RSD obtained for the amount of parabens showed the satisfactory precision of the method (< 3 %).

4.1.4.4 Recovery study

The recovery studies were carried out by spiking six known amounts of methyl paraben, ethyl paraben, propyl paraben and butyl paraben in placebo shampoo (range from 5-80 µmol L^{-1}). As shown in Table C9 (see Appendix C), the recoveries of placebo at each level were within 95.3 to 102.9 %. Moreover, the recovery studies of four samples (sample number 6, 17, 50 and 60) were studied. The results obtained for the accuracy study from four samples, by spiking five known amounts of parabens (range from 10-50 µmol L^{-1}), are presented in Table C10 (see Appendix C). For body lotion (sample no. 6), shower gel (sample no. 17), facial cream (sample no. 50) and body lotion (sample no. 60), the recoveries of parabens ranged from 92.4 to 109.2, 96.9 to 103.5, 97.5 to 104.9 and 97.6 to 103.5 %,

respectively. Therefore, recovery values demonstrated that the method was sufficiently accurate within the desired range.

4.1.4.5 Limits of detection (LOD)

In this method, the detection limits were the amount of compound that would still give a signal three times greater than the noise of the baseline (3S/N), by injecting progressively low concentration. The limits of detection were 0.040, 0.050, 0.075 and 0.100 µmol L^{-1} for methyl paraben, ethyl paraben, propyl paraben and butyl paraben, respectively. The chromatogram of the limit of detection, which was an estimation of 3S/N, was given in Figure 4.7.

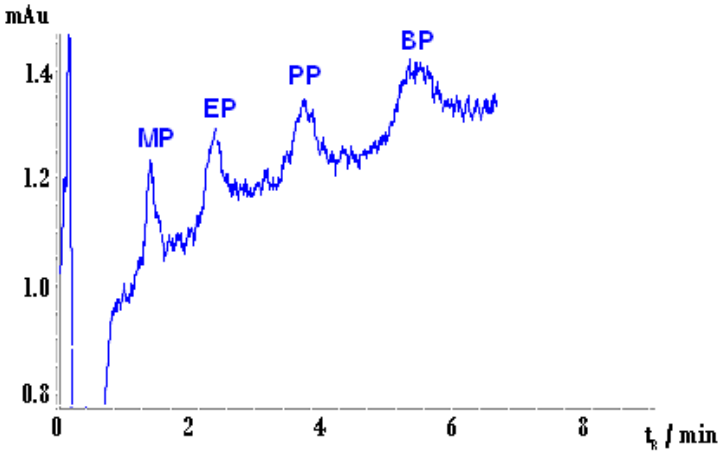


Figure 4.7 Chromatogram of methyl paraben, ethyl paraben, propyl paraben and butyl paraben at concentration of 0.040, 0.050, 0.075 and 0.100 µmol L^{-1}, respectively

4.1.5 Application of the method in cosmetics

The MLC-UV method was applied for determining methyl paraben, ethyl paraben, propyl paraben and butyl paraben presented in commercial cosmetics (sample number 1-10) and Thai community cosmetics (sample number 11-64). Figure 4.8 showed some cosmetics analyzed.

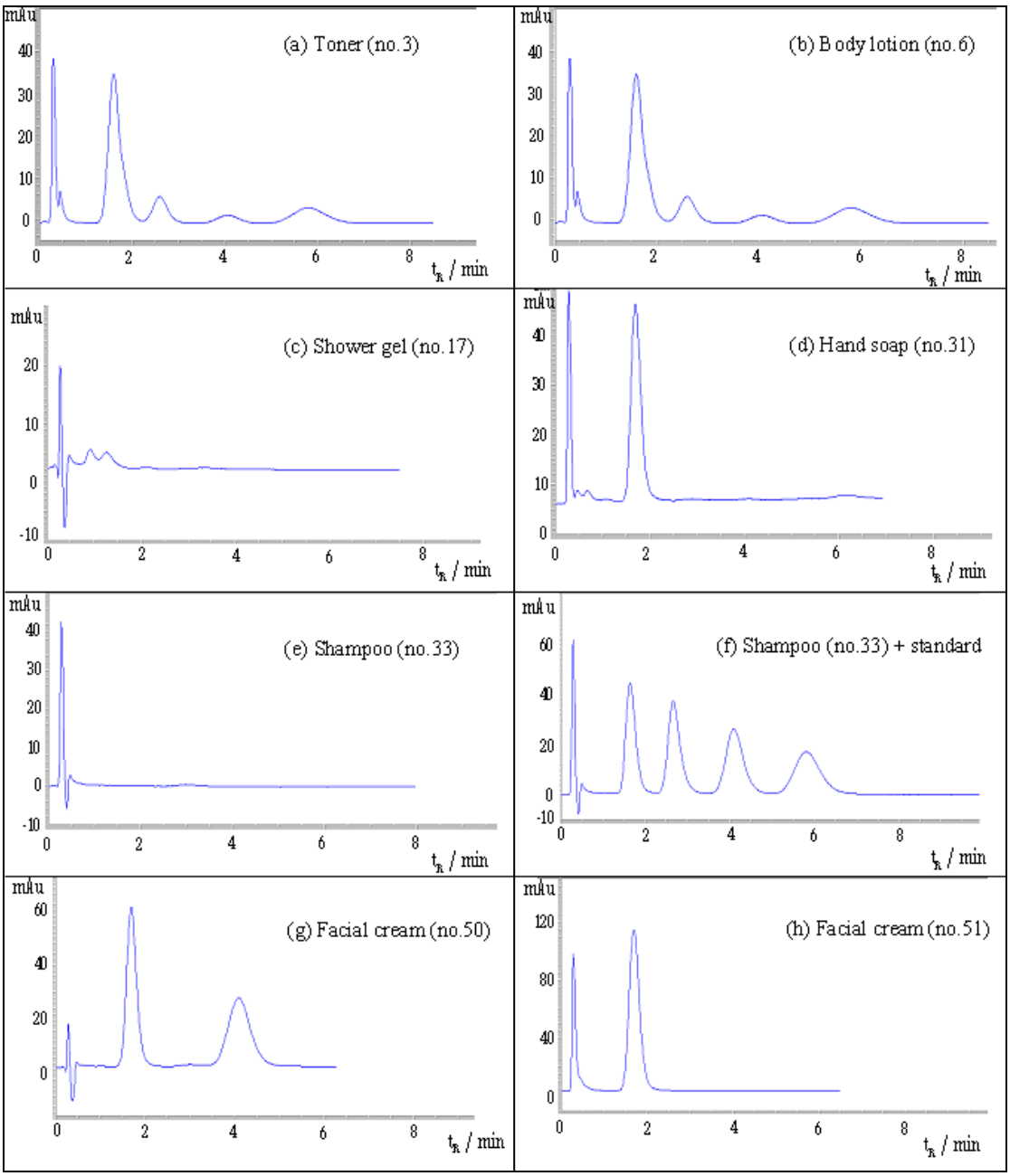


Figure 4.8 Chromatograms of some samples (a) sample no.3, (b) sample no.6, (c) sample no.17, (d) sample no.31, (e) sample no.33 (no parabens), (f) sample no.33 added standard parabens at 40 mg L^{-1}, (g) sample no.50 and (h) sample no.51

In Figure 4.8, commercial cosmetics (a) toner and (b) body lotion were found methyl paraben, ethyl paraben, propyl paraben and butyl paraben. Sample no. 17, 31, 50 and 51 were Thai community cosmetics. Sample no. 33 was placebo

shampoo which was not added parabens preservatives as shown in Figure 4.9 (e). Figure 4.9 (f) was sample no.33 added standard mixture parabens at concentration of 40 mg L^{-1} for accuracy study of the method. Other cosmetic samples analyzed were summarized in Table 4.3.

Table 4.3

Parabens contents in commercial cosmetics (sample number 1-10) and Thai community cosmetics (sample number 11-64)

No.	Type	MP/mg Kg^{-1}		EP/mg Kg^{-1}		PP/mg Kg^{-1}		BP/mg Kg^{-1}		%w/w[a]
		AV	SD	AV	SD	AV	SD	AV	SD	
1	Facial foam	475.3	7.4	681.6	17.5	386.0	4.8	1219.8	31.2	0.22
2	Cleansing lotion	2263.3	62.6	-	-.	63.2	0.1	-	-	0.21
3	Toner	818.4	24.1	140.6	4.6	96.5	1.2	254.8	2.1	0.11
4	Facial cream	604.6	15.4	-	-	792.5	17.7	-	-	0.12
5	Hand and nail lotion	717.5	16.1	-	-	591.7	15.5	-	-	0.11
6	Body lotion	745.0	13.9	-	-	872.4	9.2	-	-	0.13
7	Body scrub	1734.8	1.6	-	-	1774.7	3.2	-	-	0.29
8	Body lotion	2320.4	5.3	-	-	1029.9	4.9	-	-	0.29
9	Body lotion	1401.5	9.4	-	-	775.0	1.7	-	-	0.19
10	Body lotion	1371.9	27.0	394.0	9.4	434.9	8.6	-	-	0.19
11	Soap	-	-	-	-	-	-	-	-	-
12	Soap	-	-	-	-	-	-	-	-	-
13	Soap	-	-	-	-	-	-	-	-	-
14	Soap	-	-	-	-	-	-	-	-	-
15	Soap	-	-	-	-	-	-	-	-	-
16	Shower gel	166.0	5.1	146.2	4.1	106.8	2.7	279.5	5.6	0.06
17	Shower gel	-	-	-	-	-	-	-	-	-

Table 4.3 (Continued)

No.	Type	MP/mg Kg^{-1}		EP/mg Kg^{-1}		PP/mg Kg^{-1}		BP/mg Kg^{-1}		%w/w[a]
		AV	SD	AV	SD	AV	SD	AV	SD	
18	Shower gel	-	-	-	-	-	-	237.5	3.4	0.02
19	Shower gel	-	-	-	-	-	-	233.0	1.4	0.02
20	Shower gel	-	-	-	-	-	-	-	-	-
21	Shower gel	-	-	-	-	-	-	-	-	-
22	Shower gel	-	-	-	-	-	-	-	-	-
23	Shower gel	-	-	-	-	-	-	-	-	-
24	Shower gel	-	-	-	-	-	-	-	-	-
25	Shower gel	-	-	-	-	-	-	-	-	-
26	Shower gel	-	-	-	-	-	-	-	-	-
27	Shower gel	-	-	-	-	-	-	-	-	-
28	Facial foam	-	-	-	-	-	-	-	-	-
29	Facial foam	-	-	-	-	73.6	2.3	-	-	0.01
30	Facial foam	-	-	-	-	-	-	-	-	-
31	Hand soap	89.0	1.7	-	-	-	-	-	-	0.01
32	Shampoo	339.4	5.8	-	-	136.1	3.2	-	-	0.04
33	Shampoo	-	-	-	-	-	-	-	-	-
34	Shampoo	-	-	-	-	-	-	-	-	-
35	Shampoo	-	-	-	-	-	-	-	-	-
36	Shampoo	-	-	-	-	-	-	-	-	-
37	Shampoo	-	-	-	-	-	-	-	-	-
38	Shampoo	-	-	-	-	-	-	-	-	-
39	Shampoo	-	-	-	-	-	-	-	-	-
40	Shampoo	-	-	-	-	-	-	-	-	-
41	Shampoo	114.3	1.4	-	-	114.4	4.5	-	-	0.02
42	Shampoo	-	-	-	-	-	-	-	-	-
43	Shampoo	-	-	-	-	-	-	-	-	-
44	Shampoo	-	-	-	-	-	-	-	-	-

Table 4.3 (Continued)

No.	Type	MP/mg Kg^{-1}		EP/mg Kg^{-1}		PP/mg Kg^{-1}		BP/mg Kg^{-1}		%w/w[a]
		AV	SD	AV	SD	AV	SD	AV	SD	
45	Conditioner	-	-	-	-	-	-	-	-	-
46	Conditioner	213.7	11.9	-	-	41.0	1.1	-	-	0.02
47	Conditioner	-	-	-	-	-	-	-	-	-
48	Conditioner	-	-	-	-	-	-	-	-	-
49	Facial cream	952.4	5.4	-	-	1016.4	5.1	-	-	0.16
50	Facial cream	1034.7	3.4	-	-	1162.4	6.6	-	-	0.18
51	Facial cream	4311.5	6.8	-	-	-	-	-	-	0.39
52	Facial cream	-	-	-	-	-	-	-	-	-
53	Facial cream	1305.9	33.9	299.9	4.2	174.2	2.4	505.2	17.1	0.19
54	Facial cream	915.1	2.4	-	-	817.5	3.6	-	-	0.15
55	Facial cream	-	-	-	-	-	-	-	-	-
56	Facial cream	-	-	-	-	-	-	-	-	-
57	Facial mask	-	-	-	-	-	-	-	-	-
58	Body scrub	-	-	-	-	-	-	-	-	-
59	Body scrub	2875.8	2.6	-	-	160.3	2.2	-	-	0.27
60	Body lotion	175.4	2.8	48.9	0.8	29.4	1.4	66.1	1.3	0.03
61	Body lotion	4198.9	5.1	-	-	299.9	3.5	-	-	0.40
62	Body lotion	-	-	-	-	-	-	-	-	-

Table 4.3 (Continued)

No.	Type	MP/mg Kg^{-1}		EP/mg Kg^{-1}		PP/mg Kg^{-1}		BP/mg Kg^{-1}		%w/w[a]
		AV	SD	AV	SD	AV	SD	AV	SD	
63	Body lotion	-	-	-	-	-	-	-	-	-
64	Armpit cream	3911.6	1.9	-	-	4751.7	3.3	-	-	0.72

[a] parabens contents in the finished product (%w/w) expressed as *p*-hydroxybenzoic acid (MW=138.12)

AV: average; SD: standard deviation (n=3)

(-) means cannot be detected (The limits of detection of MP, EP, PP, and BP were 0.040, 0.050, 0.075 and 0.100 µmol L^{-1}, respectively.

The developed method was applied to the real commercial cosmetic samples. The results showed that 10 samples contained parabens. The most samples contained methyl paraben and propyl paraben (7 samples). Sample number 1 and 3 contained four parabens and sample number 10 contained methyl paraben, ethyl paraben and propyl paraben. The parabens contents in commercial cosmetics are shown in Figure 4.9. Sample number 7 contained the highest parabens contents which was 0.29 %w/w in acid form (*p*-hydroxybenzoic acid).

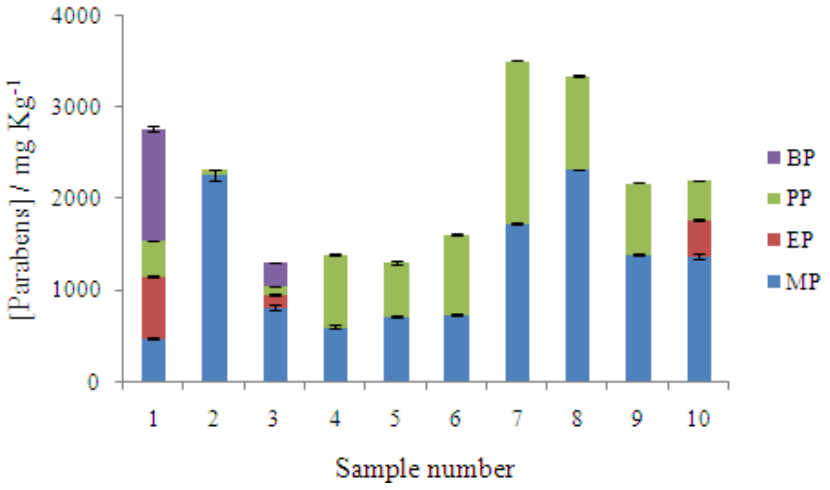


Figure 4.9 Parabens contents in commercial cosmetics

Fifty four Thai community cosmetics (sample number 11-64) were analyzed. The 37 community cosmetics were not detected all parabens but 17 samples were found. The most samples found mixed parabens (12 samples) and 5 samples found single paraben. Methyl paraben and propyl paraben were the most commonly used added together in cosmetics (9 samples). Sample number 16, 53 and 60 found four parabens. Sample number 64 contained the highest parabens contents which was 0.72 %w/w in acid form (*p*-hydroxybenzoic acid). The parabens contents in community cosmetics are shown in Figure 4.10.

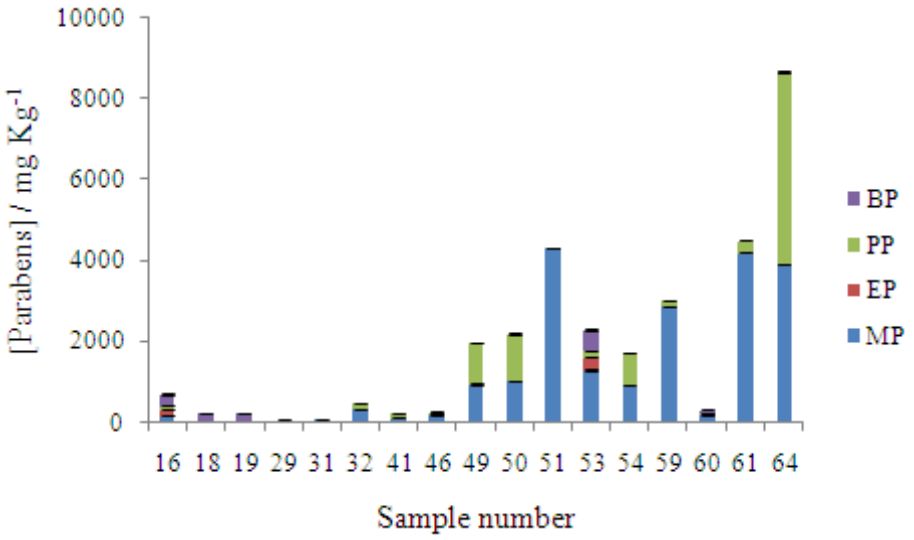


Figure 4.10 Parabens contents in Thai community cosmetics

The results showed that the parabens contents in commercial cosmetics and Thai community cosmetics were within the EU requirement (0.40 %w/w for single paraben and 0.80 %w/w for mixed parabens in acid form) as shown in Figure 4.11.

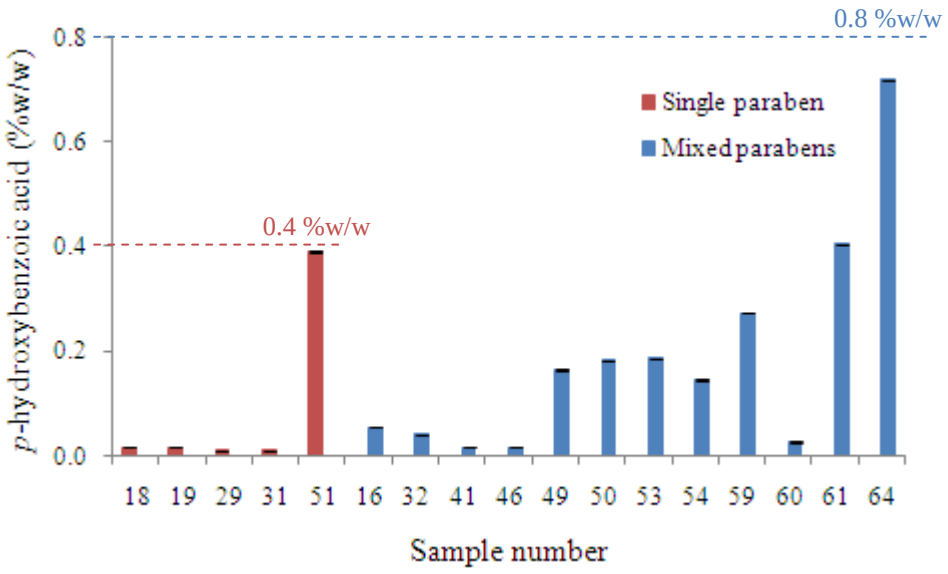


Figure 4.11 Parabens contents in Thai community cosmetics

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

4.2.1 Investigation of ion-pairing reagent

In this part, reverse phase ion interaction (ion pair) chromatography incorporating to C18 column was developed for determination of perchlorate in environmental samples. Because of anion analysis that weakly retained in reverse phase column, an ion-interaction (ion-pairing) reagent that has opposite charge was therefore added for ionic separation on reverse phase column. Moreover, this system was given one advantage that was converted a low mass analyte (a low mass perchlorate, *m/z* 99) to a higher-mass measurand that formed an adduct with a reagent of appreciable mass to avoid chemical noise region in mass spectrometric detection. Therefore, cationic ion-pairing reagent was used for ion-pair formation with perchlorate to form the positive charge that was detected by ion-trap mass spectrometer in a positive mode.

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 positive charge ion-pairing reagent for determination of perchlorate was chosen for the chromatographic separation and mass spectrometric detection. The procedure was described in Section 3.2.5.1. The results showed in Table 4.4.

The results showed that mass spectrums of CTAB, DTAB and DHAA mixed with perchlorate were as same as mass spectrums of CTAB, DTAB and DHAA, as shown in Figure 4.12-4.14. Therefore, CTAB, DTAB and DHAA were not formed a positive adduct with perchlorate. On the other hands, HMB (D^{2+}) can be formed with perchlorate (ClO_4^-) to give an adduct ($DClO_4^+$) at *m/z* 301 and 303, as shown in Figure 4.15 (b). Because of chlorine isotopic ratio (^{35}Cl and ^{37}Cl), isotopic ratio of $D^{\mathbf{35}}\mathbf{Cl}O_4^+$ (*m/z* 301) and $D^{\mathbf{37}}\mathbf{Cl}O_4^+$ (*m/z* 303) were therefore 3:1 (Figure 4.15 (b)). For MS/MS detection, product ions (daughter ions) of $DClO_4^+$ were three at *m/z* 128, 187 and 242 (base peak). Moreover, fragments of *m/z* 128 and 187 obtained nearly relative abundance, as shown in Figure 4.15 (c).

Table 4.4

The suitable ion-pairing reagent studied for perchlorate analysis

Bottles	Perchlorate	Ion-pairing reagent[a] (20 µmol L^{-1})	Expected mass[b]	Detected mass[c]	Ion-pair formation
1	-	CTAB	284.33	284.4	
2	-	DTAB	200.24	200.3	
3	-	DHAA	185.21	186.2	
4	-	HMB	281.16	101.2, 281.2	
5	5 mg L^{-1}	-	98.95	101.3	
6	5 mg L^{-1}	CTAB	383.28	284.4	x
7	5 mg L^{-1}	DTAB	299.19	200.2	x
8	5 mg L^{-1}	DHAA	284.16	186.2	x
9	5 mg L^{-1}	HMB	301.19	301.2, 303.2	√

[a] CTAB: hexadecyltrimethylammonium bromide; DTAB: decyltrimethylammonium bromide; DHAA: dihexylammonium acetate; HMB: hexamethonium bromide

[b] monoisotopic mass; [c] positive mode detection

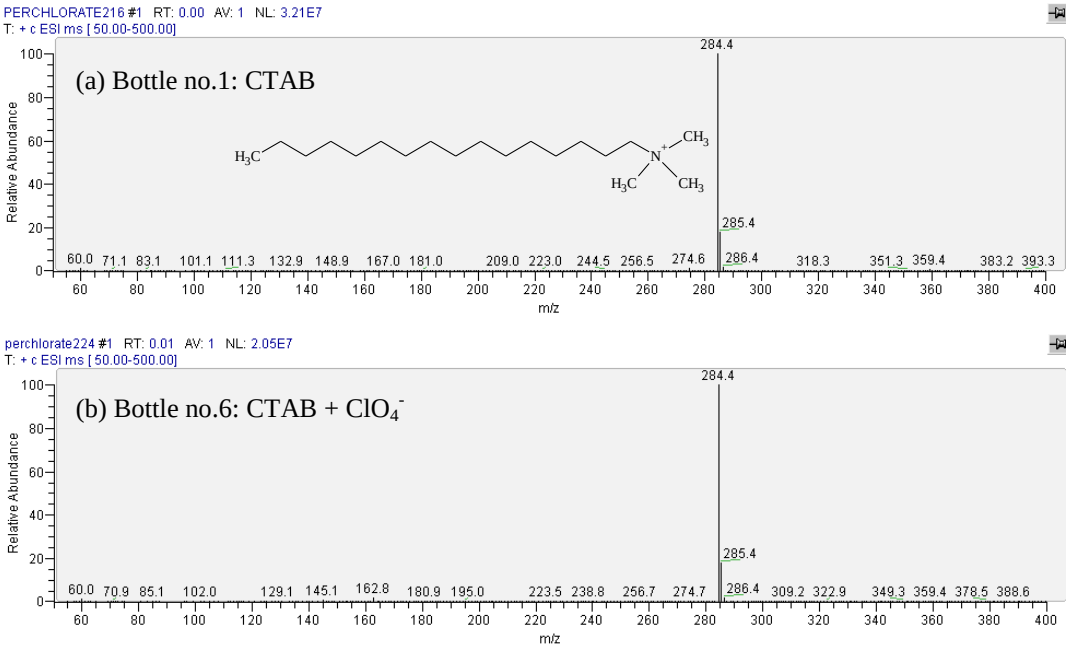


Figure 4.12 Mass spectrums of (a) CTAB and (b) CTAB mixed with ClO_4^-

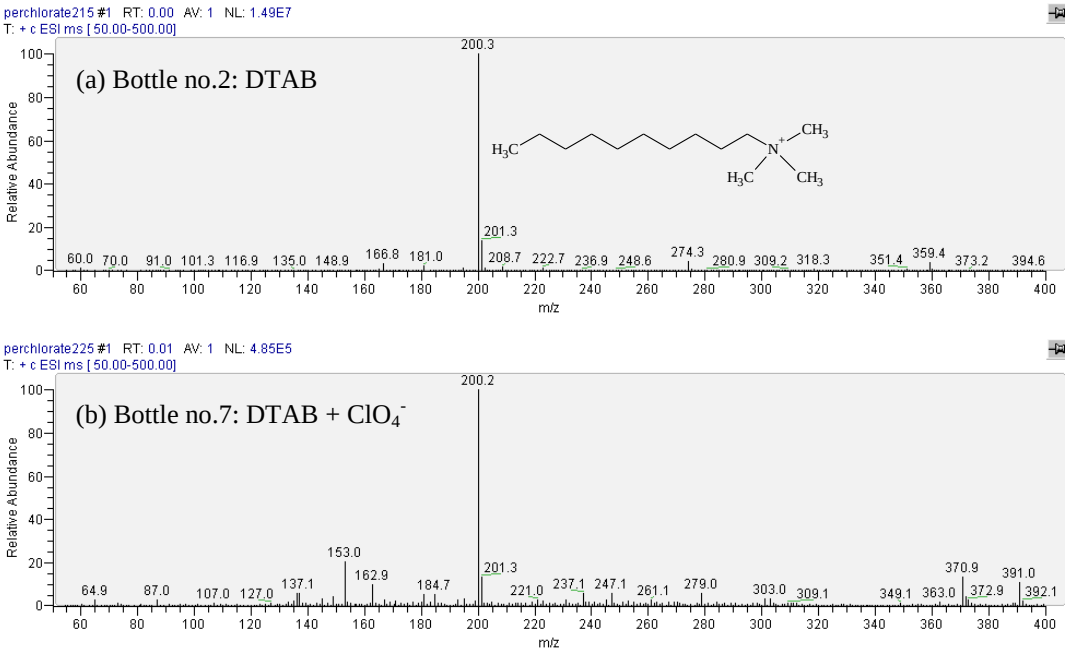


Figure 4.13 Mass spectrums of (a) DTAB and (b) DTAB mixed with ClO_4^-

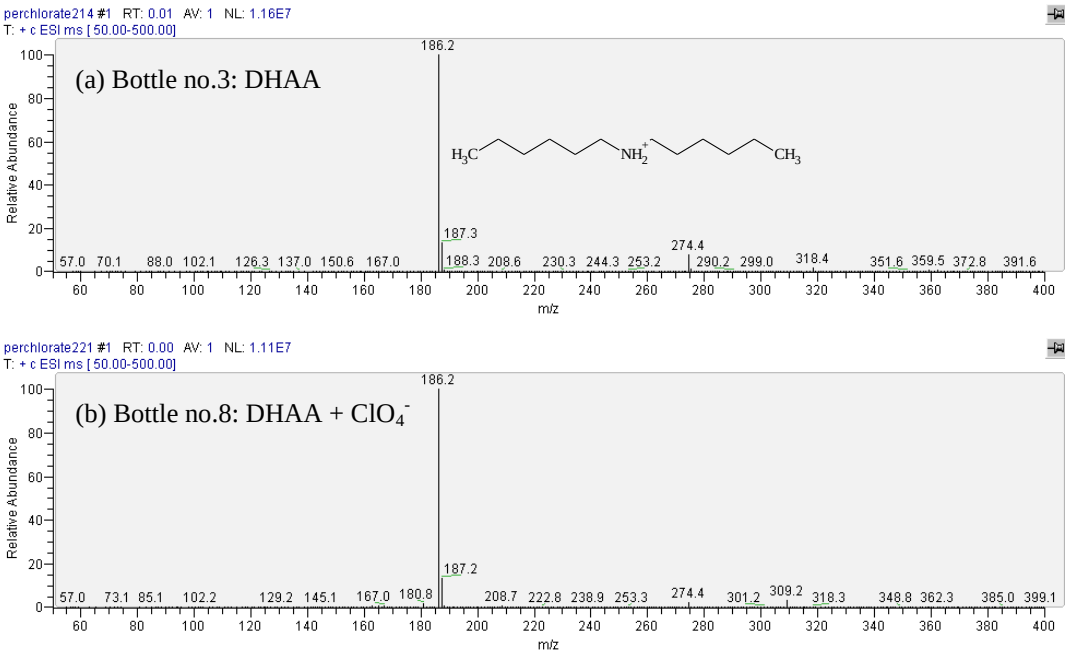
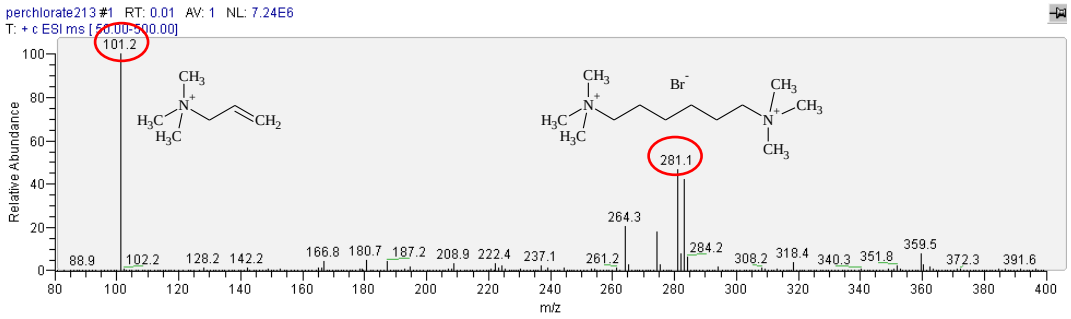


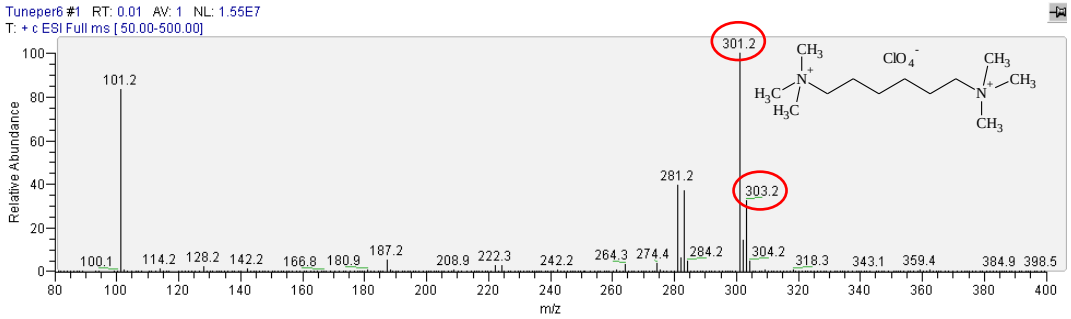
Figure 4.14 Mass spectrums of (a) DHAA and (b) DHAA mixed with ClO_4^-

As presented above, DTAB and DHAA were not detected an adduct with perchlorate in positive mode mass spectrometry. From literature survey, Magnuson reported ion-pair extraction method for perchlorate analysis in drinking water using negative electrospray ionization mass spectrometry (ESI-MS) that used DTAB as extractive ion-pairing reagent and dichloromethane as extraction solvent (Magnuson, Urbansky, & Kelty, 2000). Chen presented the used of DHAA as ion-pairing reagent and 1-octanol as extraction solvent for determination of perchlorate in river using negative electrospray ionization mass spectrometry (Chen, Chen, & Ding, 2009). Due to the limit of instrument that cannot detected in negative mode MS, DTAB and DHAA were therefore cannot used. CTAB cannot be formed an adduct with perchlorate because CTAB is too long carbon chain length (Magnuson, et al., 2000). In this method, HMB was therefore selected for ion-pair formation with perchlorate which had molecular mass as shown in Figure 4.15.

(a) Bottle no.4: HMB (MS spectrum)



(b) Bottle no.9: HMB + ClO_4^- (MS spectrum)



(c) Bottle no.9: HMB + ClO_4^- (MS/MS spectrum)

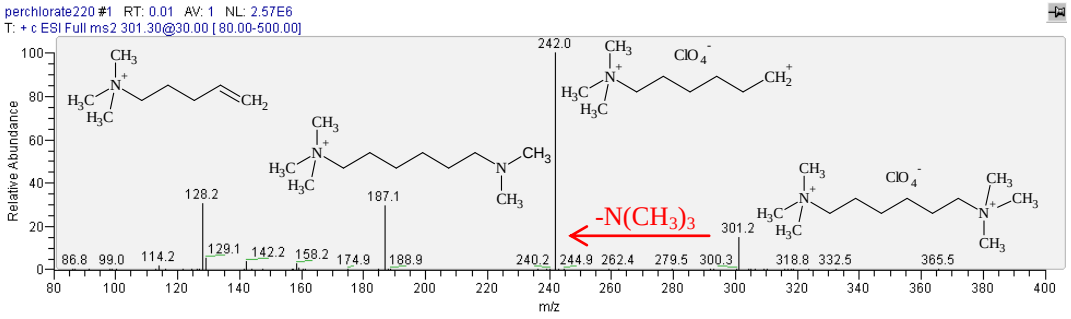


Figure 4.15 MS spectrums of (a) HMB, (b) HMB formed with ClO_4^- and MS/MS spectrum of (c) HMB formed with ClO_4^-

In this work, a confirmatory and quantitative LC-MS/MS method was developed for perchlorate analysis. Chlorine isotopic ratio and oxygen-labeled sodium perchlorate ($NaCl^{18}O_4$) as internal standard were used for the improvement of specification and sensitivity. The MS spectrum and MS/MS spectrum of HMB formed with $Cl^{18}O_4^-$ as internal standard ($DCl^{18}O_4^+$) were given in Figure 4.16 (a) and Figure 4.16 (b), respectively. Chlorine isotopic ratio of $D^{\mathbf{35}}\mathbf{Cl}^{18}O_4^+$ (*m/z* 309) and $D^{\mathbf{37}}\mathbf{Cl}^{18}O_4^+$ (*m/z* 311) were three fold (Figure 4.16 (c)) as same as isotopic ratio of $DClO_4^+$. Figure

4.16 (b) showed product ions (daughter ions) of $DCl^{18}O_4^+$ which were *m/z* 128, 187 and 250. Base peak was *m/z* 250. Product ions at *m/z* 128 and 187 had nearly relative abundance.

In addition, the criteria of confirmatory method of perchlorate presented in environmental samples were the suspect compound that elute at the same retention time of perchlorate standard by comparison of its mass spectrum with the mss spectrum of standard. The ion fragment with the greatest abundance was selected as the quantifying ion which is used for calculating the concentration. In this method, the quantifying ion was *m/z* 242 (base peak) and the qualifying ions were *m/z* 242, 187 and 128.

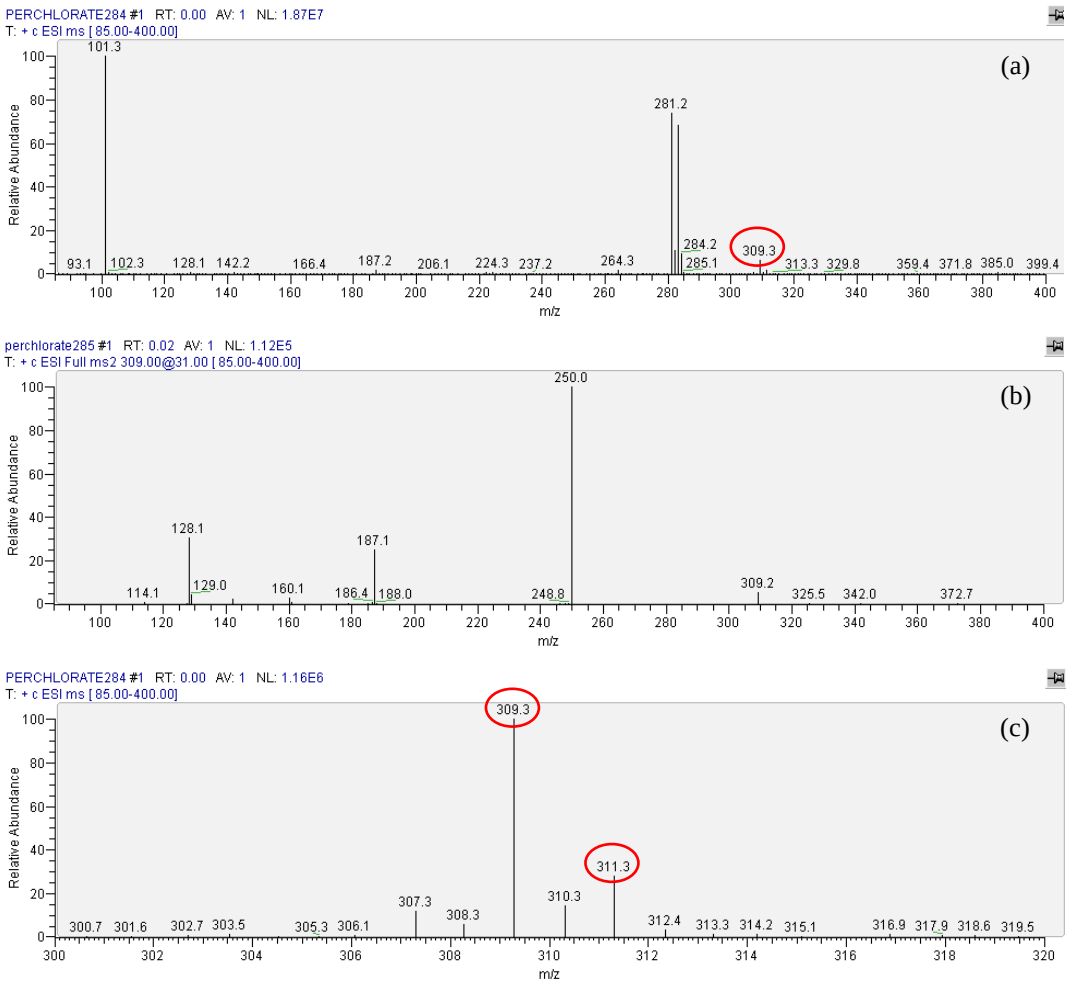


Figure 4.16 MS spectrum of (a) and (c) HMB formed with $Cl^{18}O_4^-$ and MS/MS spectrum of (b) HMB formed with $Cl^{18}O_4^-$

4.2.2 Optimization of the LC condition

4.2.2.1 Investigation of ion-pairing formation in LC system

The types of ion-pairing formation (pre-column and on-column) in liquid chromatographic system were investigated. The procedures were described in Section 3.2.5.2 (a) and Table 4.5.

Table 4.5
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)	√	√	√	-	√	√

The results showed that the direct analysis of perchlorate without ion-pairing reagent obtained low sensitivity because of low mass of perchlorate (*m/z* 99) and chemical noise region in mass spectrometric detection (Figure 4.17, system 1-(a)). On the other hands, on-column ion-pairing formation (system 2) and both pre-column and on-column ion-pairing formation (system 3) obtained good peak sharp and higher sensitivity than pre-column ion-pairing formation (system 1-(b)), as shown in Figure 4.17. In system 2 and 3, it was found that ion-pairing reagent that introduced into LC system obtained the good peak shape and high sensitivity when mixed with mobile phase B (Figure 4.17, system 2-(a) and 3-(a)) because of the difference final concentration of ion-pairing reagent in the

column (the mixing ratio of mobile phase A and B was 10 and 90 %, respectively). The ion-paring concentration in system (a) was therefore higher than in system (b). Thus, system 2-(a) and 3-(a) were considered by injecting perchlorate at concentration within the range of 0.1-20 mg L^{-1} and linearity ranges of both systems were then examined.

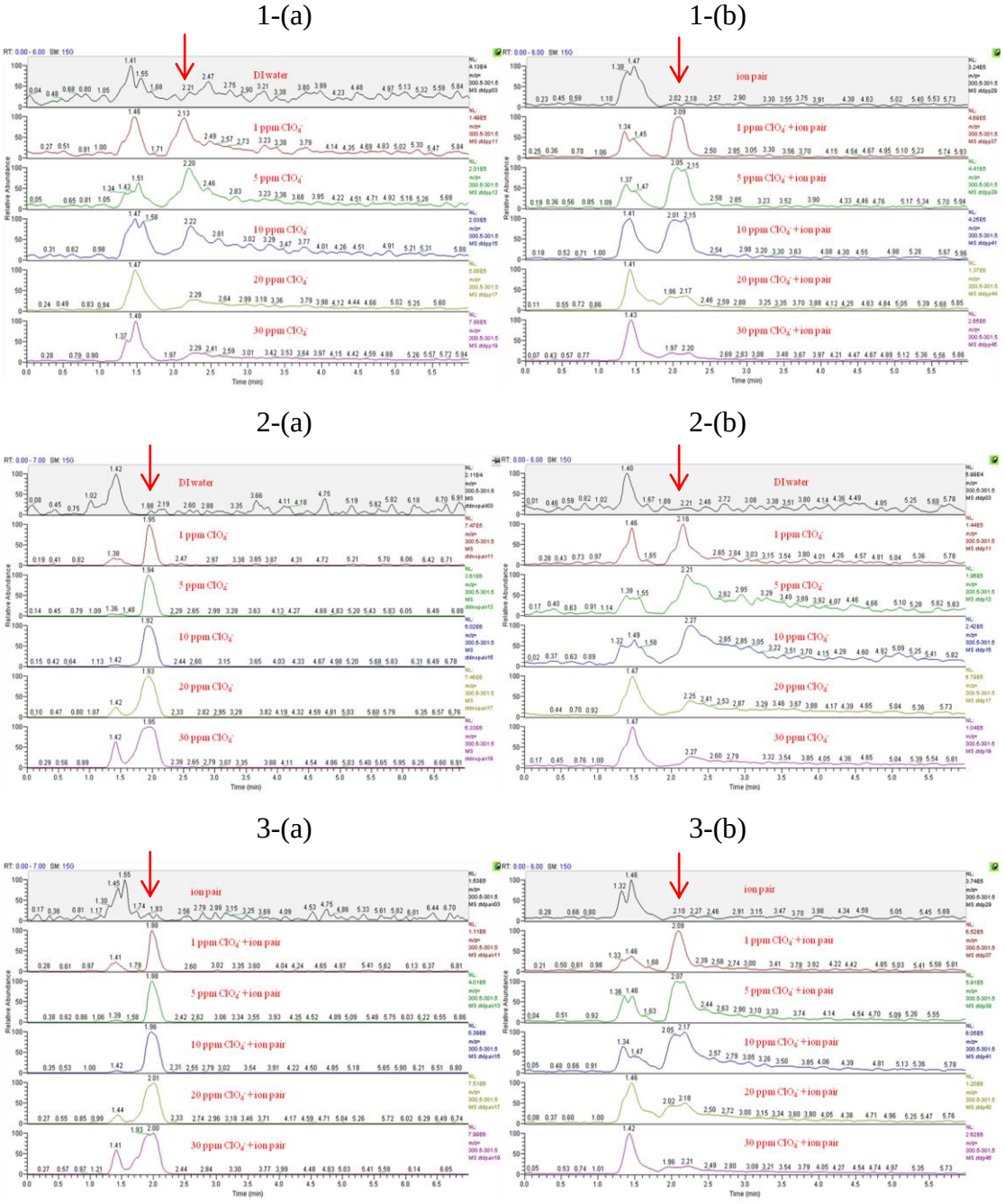


Figure 4.17 LC/MS chromatograms of ion-pairing formation in LC system

Figure 4.18 showed chromatograms of perchlorate at concentration of 0.1-20 mg L^{-1}. Linearity ranges of system 2-(a) and 3-(a) were 0.1-3 mg L^{-1}, as shown in Table 4.6. The results showed that system 2-(a) and system 3-(a) obtained the same sensitivity. Therefore, system 2-(a) was selected because of directly injection of perchlorate. This condition was ion-pairing reagent mixed with mobile phase line B (0.1 %v/v acetic acid in DI) and mobile phase A was MeOH.

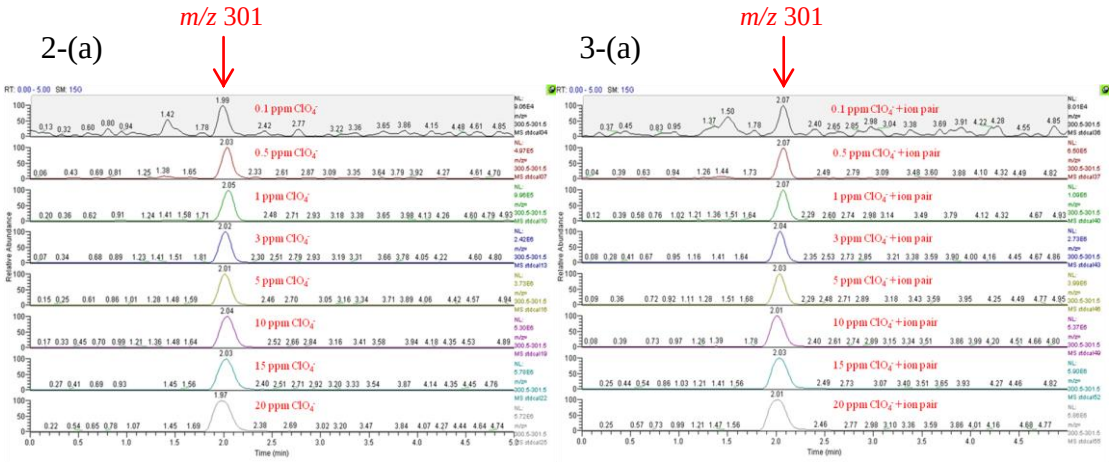


Figure 4.18 LC/MS chromatograms of ion-pairing formation in LC system 2-(a) (left) and 3-(a) (right)

Table 4.6

Linearity ranges of system 2-(a) and 3-(a)

System	Linearity			
	Range (mg L^{-1})	Slope (x 10^3)	Intercept (x 10^3)	R^2
2-(a)	0.1-3	6932.10	349.64	0.9952
3-(a)	0.1-3	7013.79	438.06	0.9958

4.2.2.2 Injection volume

The studied injection volumes were 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µL. Peak area ratio of perchlorate and internal standard at

concentration of 0.005, 0.02 and 1 mg L^{-1} of each injection volume were given in Table C11 (see Appendix C). It was found that injection volume at 100 µL obtained the highest sensitivity without overload peak at trace perchlorate analysis, as presented in Figure 4.19 and 4.20. Calibration equations of perchlorate at various injection volumes were shown in Table C12 (see Appendix C). On the other hands, injection volumes at 10-40 µL cannot be analyzed perchlorate at low concentration. Therefore, 100 µL loop injection was selected for this method.

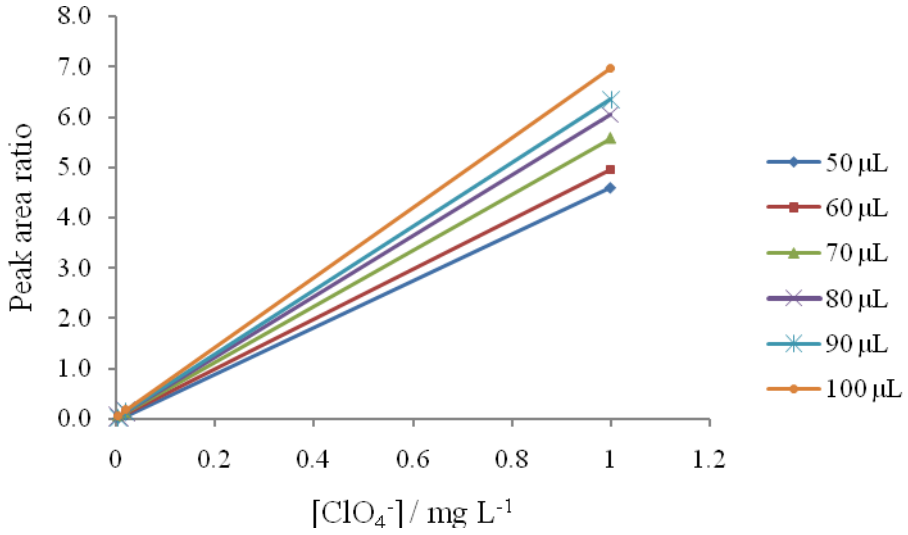


Figure 4.19 Calibration curves of perchlorate at various injection volumes

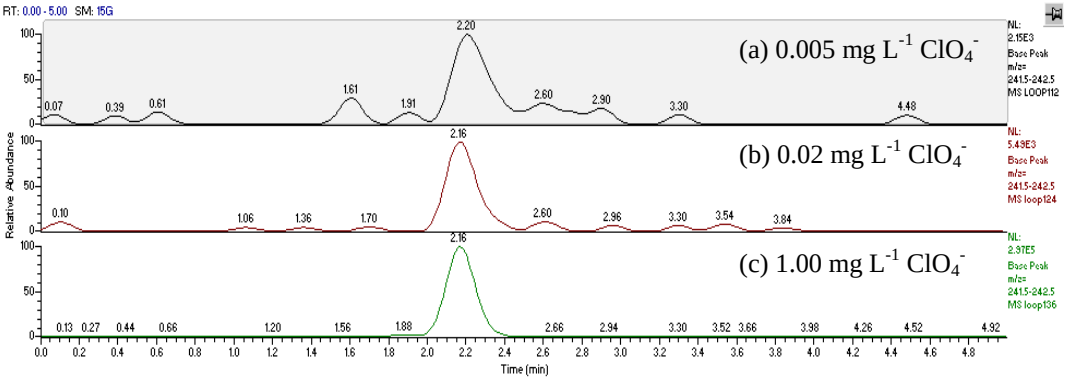


Figure 4.20 LC-MS/MS chromatograms of perchlorate at injection volume 100 µL

4.2.2.3 Compositions of mobile phase

The compositions of mobile phase were investigated. The parameters such as effect of organic modifier, effect of acid and concentration of ion-pairing reagent were studied.

a) Effect of organic modifier

Electrospray ionization mass spectrometry (ESI-MS) is not good for high water percentages in eluent but it requires volatile mobile phase components. On the other hands, the stationary phase coverage by the ion-pairing reagent decreases with increasing organic modifier in the eluent. In spite of this, the presence of at least small amounts of organic modifier in the mobile phase is crucial to enhance the separation of the reverse phase chromatographic method (Cecchi, 2010).

Therefore, organic modifier used in this method was added a little which was good for both ESI-MS and ion-pairing formation. Organic modifier used in this method was methanol which the advantages were proton generation and easy desolvation (volatile compound) in ESI. Moreover, organic modifier can be affected on retention time of analyte. Other anions such as chloride, sulphate and phosphate were therefore selected for the study of elution on C18 column that resulted from organic modifier. Methanol contents in mobile phase were varied at 0, 5, 10, 15 and 20 %v/v. The results showed that retention time of all anions decreased when organic modifier increased. Perchlorate, chloride, sulphate and phosphate were quite separated at 0 %v/v methanol (Figure 4.21) but it obtained low sensitivity (Figure 4.22). Because of trace analysis, organic modifier at 10 %v/v methanol that obtained the highest sensitivity was therefore selected (Figure 4.22). Figure 4.23 showed chromatograms of ClO_4^-, Cl^-, SO_4^{2-} and PO_4^{3-} using 10 %v/v methanol. All data are given in Table C13 (see Appendix C). Clearly, both analyte retention and the ion-pairing reagent adsorption isotherm were weakened in organic modifier-rich eluents.

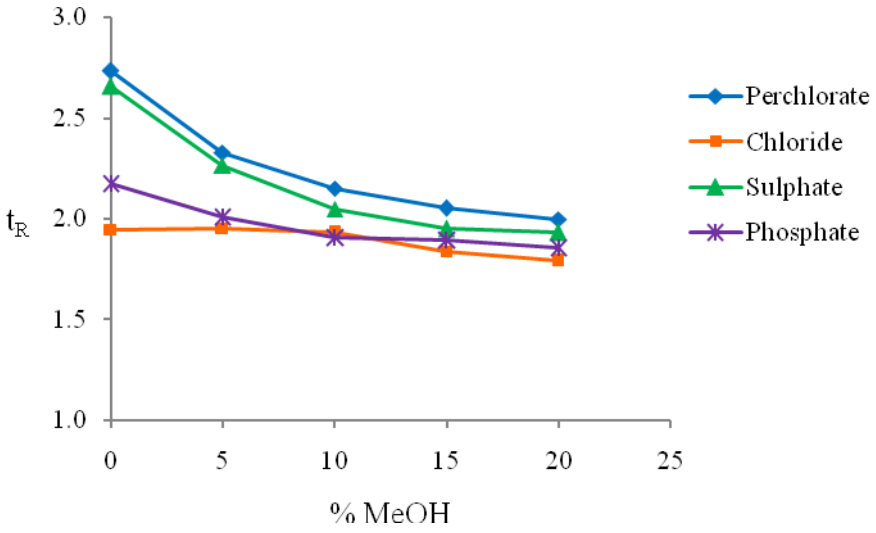


Figure 4.21 Retention times of ClO_4^-, Cl^-, SO_4^{2-} and PO_4^{3-} at various methanol contents

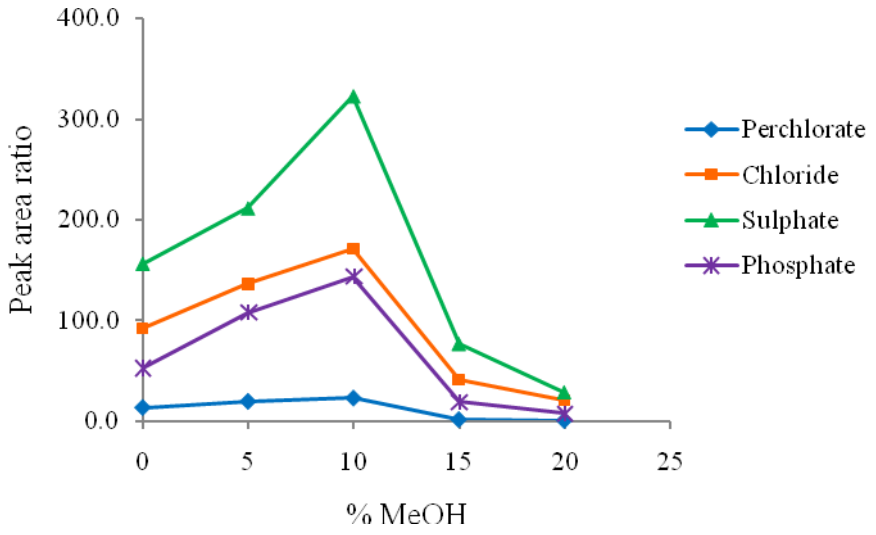


Figure 4.22 Peak area ratios of ClO_4^-, Cl^-, SO_4^{2-} and PO_4^{3-} at various methanol contents

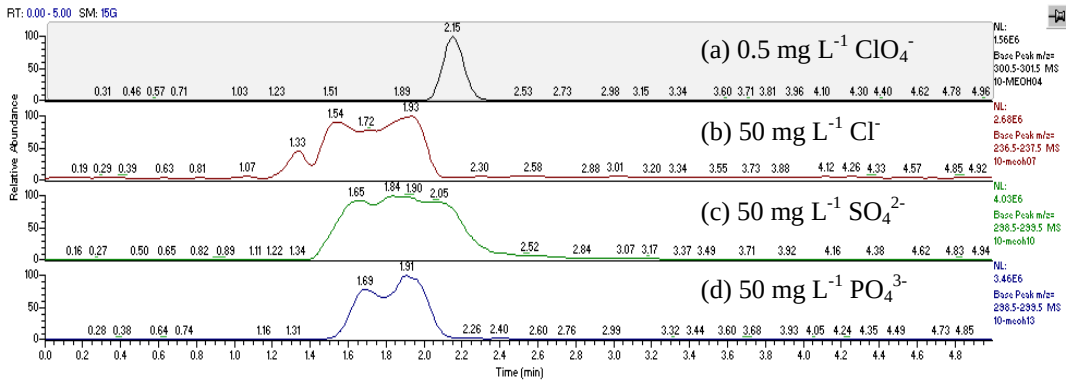


Figure 4.23 LC-MS chromatograms of (a) ClO_4^-, (b) Cl^-, (c) SO_4^{2-} and (d) PO_4^{3-} at 10 %v/v methanol

b) Effect of acid

The advantage of the use of acid content in mobile phase was proton generation in ESI-MS. From literature survey, ion pair chromatography-mass spectrometry (RPIP-ESI-MS) that used acid content in mobile phase has been reported (Kojima, Inagaki, Tomita, Watanabe, & Uchida, 2010). LC-ESI-MS/MS methods for perchlorate analysis were also presented the used of acid such as acetic acid and formic acid as mobile phase (Li & George, 2005, 2006; Snyder, Pleus, Vanderford, & Holady, 2006; Snyder, Vanderford, & Rexing, 2005).

Acid used in this method was acetic acid at various contents of 0, 0.05 and 0.10 %v/v. The results showed that acid in mobile phase was not effect on the retention time of the analyte but it was effect on the sensitivity (peak area of analyte decreased), as shown in Table C14 (see Appendix C). When acid content in mobile phase increased, sensitivity of the analysis decreased, as shown in Figure 4.24 and Table C15 (see Appendix C). Therefore, this method was not used acetic acid in mobile phase (0 %v/v) for the analysis.

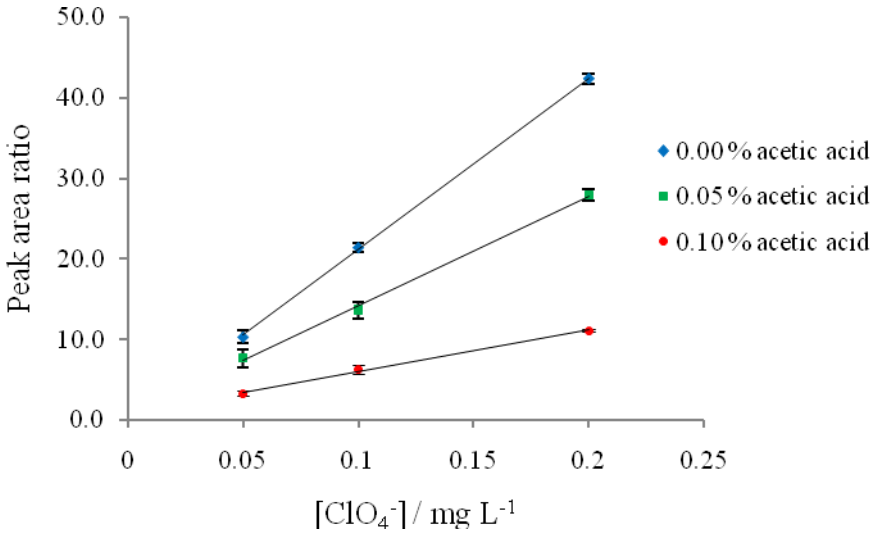


Figure 4.24 Calibration curves of perchlorate at various acetic acid contents

c) Concentration of ion-pairing reagent

The concentration of ion-pairing reagent in mobile phase was the important parameter. The high concentration of ion-pairing reagent caused ion suppression in ESI. The low concentration of ion-pairing reagent was not enough for ion-pairing formation with perchlorate. In this method, the concentrations of ion-pairing reagent in mobile phase were varied at concentration of 5, 10, 20, 30, 40 and 80 µmol L^{-1}. It was found that hexamethonium bromide at concentration of 10 µmol L^{-1} obtained the highest sensitivity. The higher concentration of ion-pairing reagent obtained lower sensitivity because of ion suppression in ESI. The results were given in Figure 4.25 (Table C16) and Figure 4.26 (Table C17, Appendix C). Finally, hexamethonium bromide at concentration of 10 µmol L^{-1} used as mobile phase was selected for the proposed method.

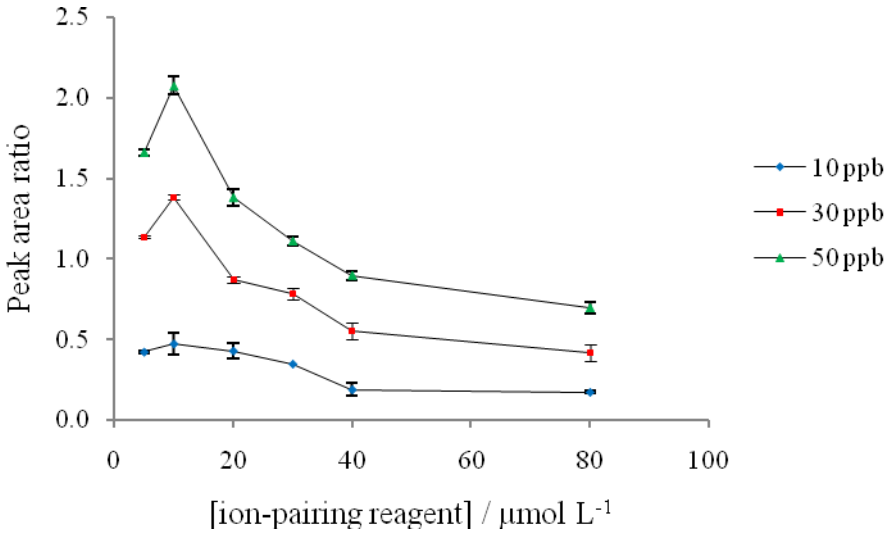


Figure 4.25 Peak area ratios of perchlorate at various concentrations of ion-pairing reagent in mobile phase

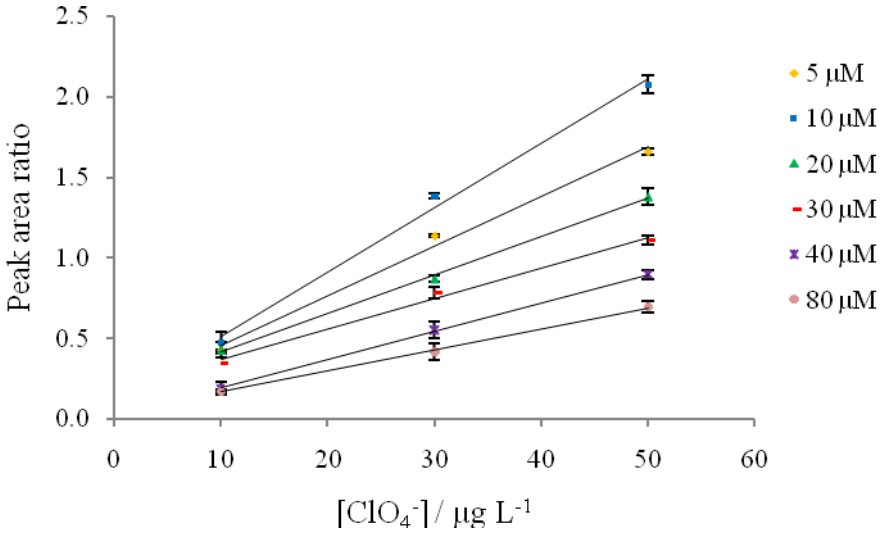


Figure 4.26 Calibration curves of perchlorate at various concentrations of ion-pairing reagent in mobile phase

4.2.3 Optimization of the MS/MS condition

4.2.3.1 Collision energy

The collision energies were varied from 26.0-35.0 %. The results showed that the suitable collision energy that obtained the highest yield of daughter ions and minimized parent ion was 30.0 %, as given in Figure 4.28 and Table C20 (see Appendix C). This collision energy also obtained about 10 % relative abundance of parent ion (*m/z* 301), as shown in Figure 4.29.

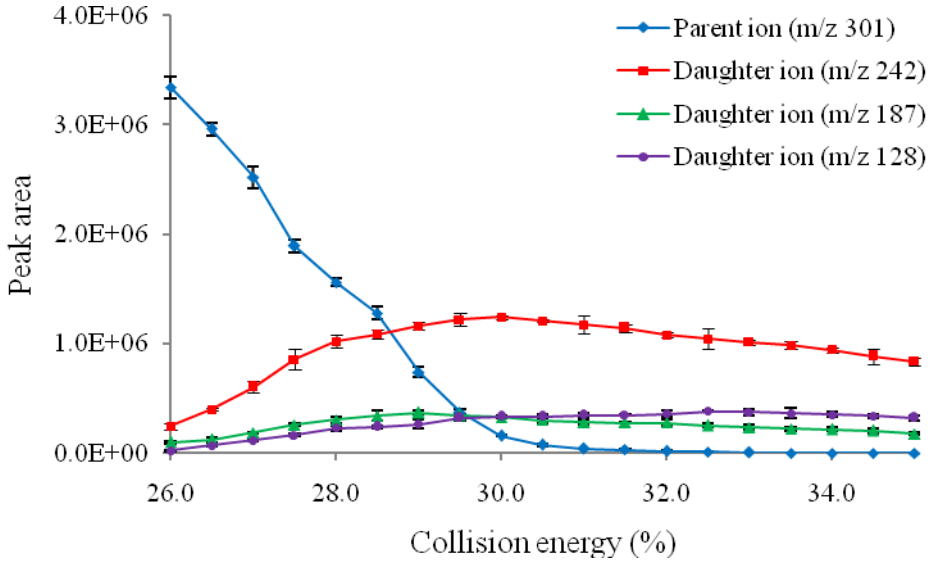


Figure 4.28 Ion abundance curve obtained by plotting initial parent ion and fragment daughter ions abundances (expressed as the peak area) as a function of the collision energy

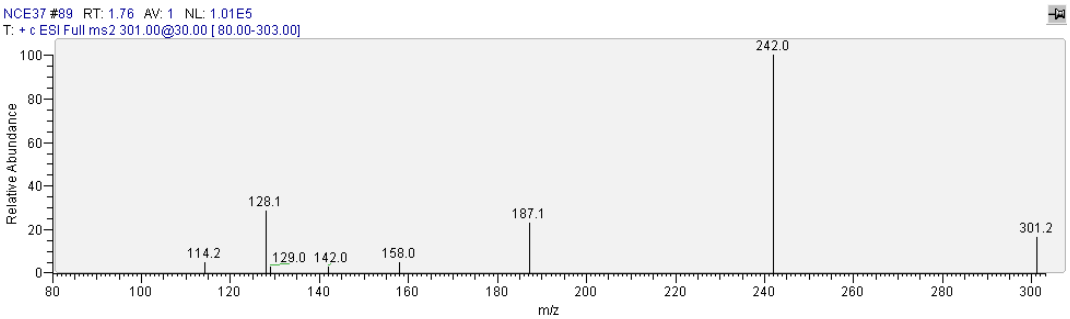


Figure 4.29 MS/MS spectrum of HMB formed with ClO_4^-

4.2.3.2 Scanning mode in MS/MS

Scanning mode in ion-trap mass spectrometer (Xcalibur software) are the selected ion monitoring (SIM) and full scan mode. In the SIM scan type, the ions in the ion source are stored in the mass analyzer. Then, ions of interest for one or more mass-to-charge ratios are isolated and are scanned out of the mass analyzer to produce a SIM mass spectrum. With the full scan type, the mass analyzer is scanned from the first mass to the last mass without interruption in a given scan time.

Scanning mode in mass spectrometric detection was investigated by injecting perchlorate at concentration of 10, 30 and 50 µg L^{-1} with the internal standard at a concentration of 50 µg L^{-1}. Then the calibration curves of the extract ion chromatogram of *m/z* 242 were plotted and were shown in Figure 4.27, Table C18 and Table C19 (see Appendix C). It was found that full scan mode obtained higher sensitivity than SIM mode. Therefore, full scan mode was chosen for the analysis. The optimal conditions of the LC system for perchlorate analysis were summarized in Table 4.7.

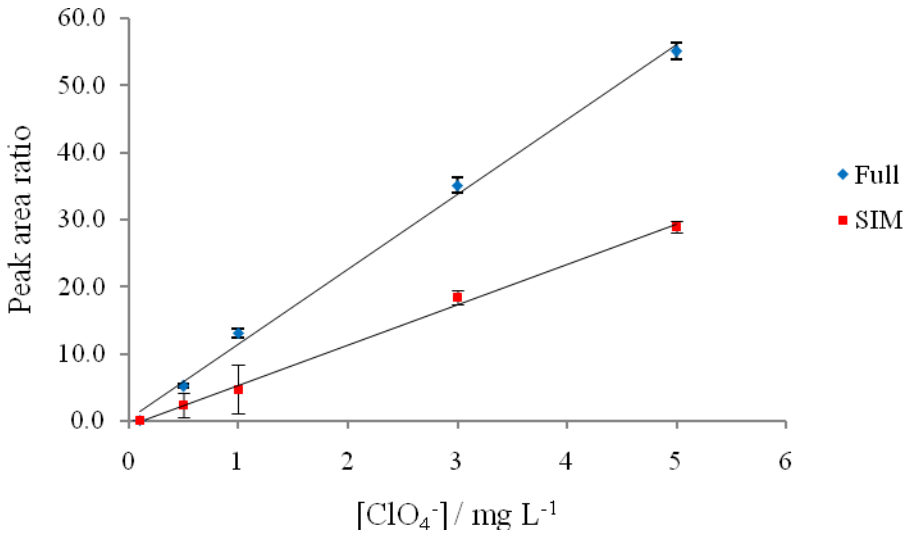


Figure 4.27 Calibration curves of perchlorate between Full and SIM mode in ion-trap mass spectrometer (Xcalibur software)

Table 4.7

The optimal conditions of LC-MS/MS method

Instruments	Parameters	Conditions
Liquid chromatograph	Mobile phase	MeOH : 10 µmol L^{-1} 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)	44
	Tube lens offset (V)	15
	Scanning mode	Full scan
	Collision energy (%)	30.0
	Mass range (*m/z*)	80.0-315.0
	Isolation width (*m/z*)	1.0
	Parent ion (*m/z*)	301
	Daughter ions (*m/z*)	242*, 128, 187

* Quantifying ion (base peak) was *m/z* 242.
Qualifying ions were *m/z* 242, 187 and 128.

4.2.4 Validation study

Linearity range, repeatability, reproducibility, recovery, limit of detection (LOD) and interferences study were examined for validation of the method.

4.2.4.1 Linearity range

Peak area ratios of twenty concentrations of perchlorate (in the range of 0.5-50,000 µg L^{-1}) and internal standard were plotted versus concentrations of perchlorate. The results showed in Figure 4.30 and Table C21 (see Appendix C). It was summarized that linearity range of the method was 4-1000 µg L^{-1} of perchlorate. The correlation coefficient (R^2) was 0.9998, as shown in Figure 4.31.

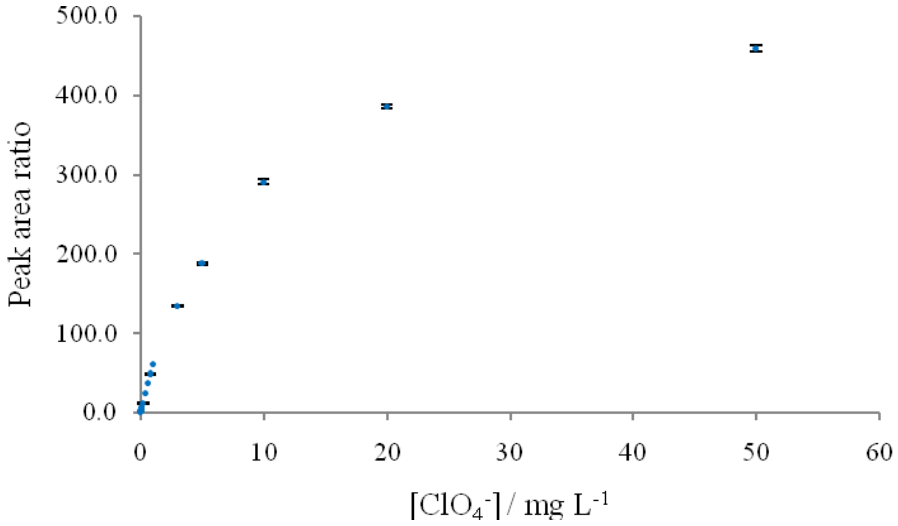


Figure 4.30 Linearity range study

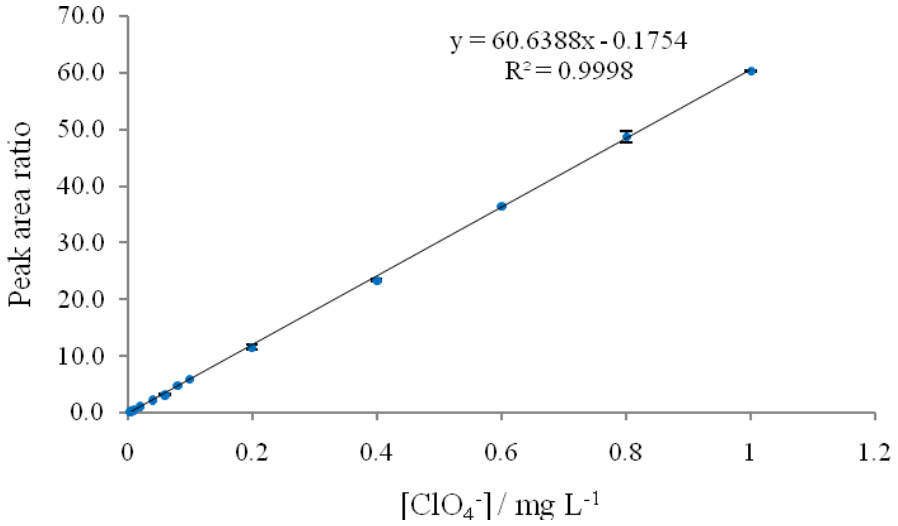


Figure 4.31 Linearity range

4.2.4.2 Calibration curve (Working range)

The calibration curve of six concentrations of perchlorate (4-50 µg L^{-1}) that used for samples determination was constructed. Working range was shown in Figure 4.32 and Table C22 (see Appendix C).

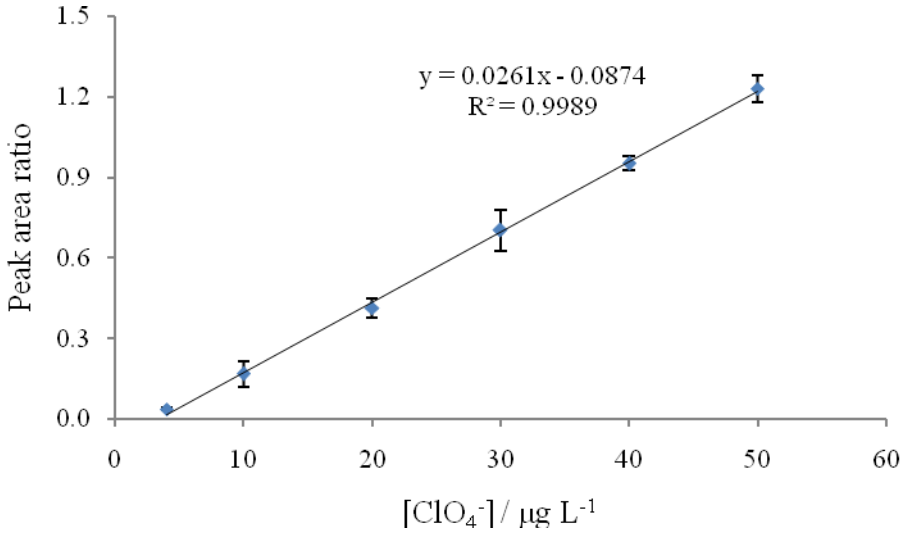


Figure 4.32 Working range

4.2.4.3 Repeatability

The results show that the RSD values of perchlorate content in Soil-1 and Water-1 were 9.80 and 15.54 %, respectively, as given in Table C23 (see Appendix C). This proposed method indicated good repeatability that agreed with the EPA method 331.0 (%RSD must be ≤ 20%, n=7) (U.S. Environmental Protection Agency, 2005).

4.2.4.4 Reproducibility

Reproducibility of injecting ten aliquots of each sample (Soil-1 and Water-1) were analyzed one replicate per aliquot for the determination of the

precision of the method including sample preparation and instrumental performance. It was found that the %RSD of perchlorate content in sample solutions were 15.57 and 19.77 % for Soil-1 and Water-1, respectively, which showed good reproducibility of this method (Table C24, Appendix C). These agreed with the EPA method 331.0 (%RSD must be ≤ 20%, n=7) (U.S. Environmental Protection Agency, 2005).

4.2.4.5 Recovery study

The recoveries were studied by spiking of samples with perchlorate at concentration of 50 µg L^{-1}. The recoveries of drinking water (Water-5) and tap water (Water-6) were 86.59 and 80.10 %, respectively, without using SPE (Table C25, Appendix C). These recoveries agreed with the EPA method 331.0 because of less interferences (mean recovery 80-120 % of the true value) (U.S. Environmental Protection Agency, 2005).

The recoveries of Soil-1 were 12.68 and 35.91 % when treated without and with SPE prior to analysis, respectively. The recoveries of Water-1 were 19.99 and 36.08 % when treated without and with SPE prior to analysis, respectively. All data were given in Table C25 (see Appendix C). The results showed that the use of solid phase extractions (OnGuard-Ba) can be improved the recovery because of sulphate removal that caused ion suppression. However, other interferences might be disturbed the mass spectrometric detection.

4.2.4.6 Limit of detection (LOD)

The detection limit of this method was 2 µg L^{-1} (a regulatory limit for perchlorate of 4 µg L^{-1} in water), as given in Figure 4.33 (a). At this concentration, it still obtained the same ratio of relative abundance of daughter ions, as given in Figure 4.33 (b) for confirmatory and quantitative of the method.

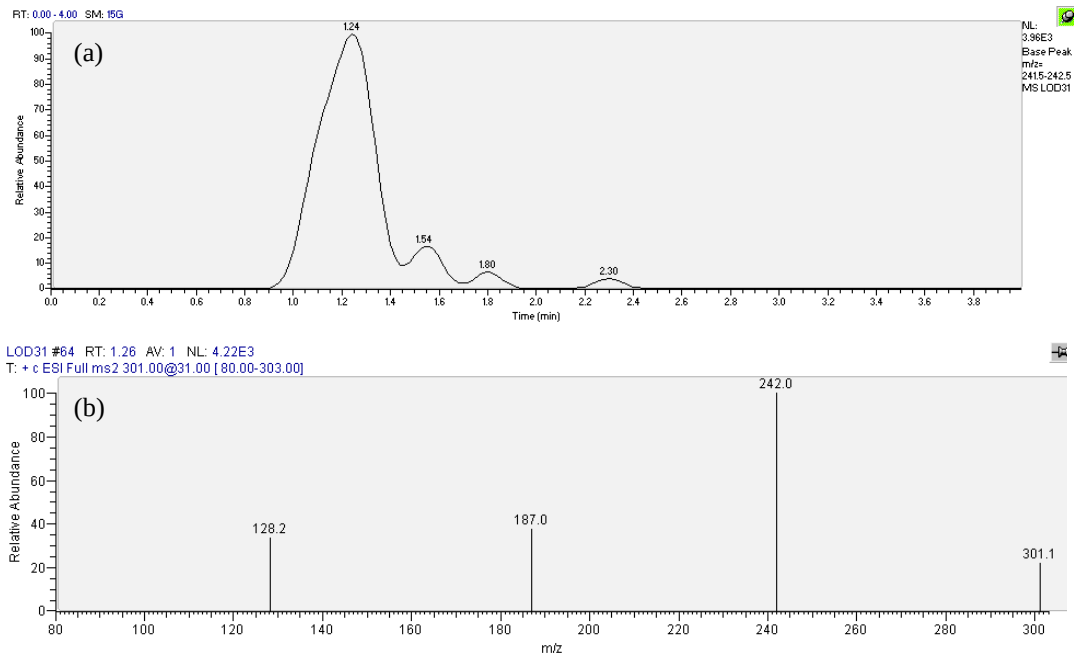


Figure 4.33 (a) Chromatogram and (b) MS/MS spectrum of perchlorate at concentration of 2 µg L^{-1}

4.2.4.7 Interferences study

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 common anions that can be found in environmental samples and caused ion suppression in ESI for determination of perchlorate in the environments. The regulatory limits of these anions in environmental samples were given in Table 4.8.

From preliminary results, interferences can be classified in two groups which were not interfered and interfered. Interferences that were not interfered were non-detectable interferences (NO_2^-, CO_3^{2-} and Br^-) and detectable interferences (Cl^-, NO_3^-, IO_3^- and I^-). Detectable interferences that interfered were SO_4^{2-} and PO_4^{3-}. In this method, interferences studies were therefore examined.

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

For interferences study without using solid phase extraction, 0.5 mg L^{-1} of perchlorate that mixed with each anion at concentration of 50

mg L^{-1} was prepared. The results showed that all anions at concentration of 50 mg L^{-1} (100 times) were not interfered perchlorate analysis in environmental samples that peak area of perchlorate still obtained deviation less than 3SD (standard deviation of replicated injection of standard perchlorate solution), as shown in Figure 4.34.

Table 4.8

Monoisotopic mass, average mass and regulatory limits of anions in water samples

Anions	Monoisotopic mass[b]	Average mass[b]	EU (mg L^{-1})[c]
SO_4^{2-}	95.95172	96.06360	250
PO_4^{3-}, [a]	94.95342	94.97136	Not mentioned
Cl^-	34.96885	35.45270	250
NO^{3-}	61.98781	62.00494	50
NO_2^-	45.99290	46.00553	0.50
IO_3^-	174.88940	174.90267	Not mentioned
I^-	126.90466	126.90447	Not mentioned
CO_3^{2-}	59.98474	60.00919	Not mentioned
Br^-	78.91839	79.90400	0.01

[a] The EPA water quality criteria state that phosphate should maintain at 0.01-0.03 mg L^{-1} in surface water (NC State University, 1976).

[b] (Rozenski, 1999); [c] (EU, 1998)

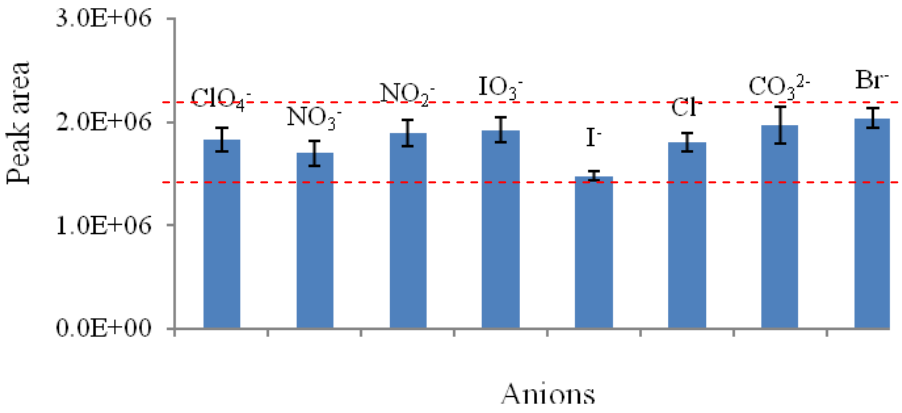


Figure 4.34 Interferences study

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

Sulphate and phosphate were major common anions that interfered perchlorate separation and detection. In 2005, Li presented $H^{34}S^{16}O_4^-$, $H^{32}S^{18}O^{16}O_3^-$ and $H_2P^{18}O^{16}O_3^-$ that could cause the spectral interferences (*m/z* 99) (Li & George, 2005), as shown in Table 4.9. Figure 4.35 showed the spectral interferences of isotopic hydrogen sulphate and phosphate obtaining the same mass as perchlorate that required the high resolution mass spectrometer for separation. For this work, the concentration level of sulphate and phosphate anions contributing the alteration of signal size at 3SD without using solid phase extraction was considered. In this experiment, various concentrations of sulphate and phosphate were added to a constant concentration of perchlorate standard (10 µg L^{-1}), as described in Table 3.12 (Section 3.2.5.4 (g)). It was found that the tolerant limits of both phosphate and sulphate were 0.5 mg L^{-1}, as shown in Figure 4.36 (Table C26) and 4.37 (Table C27, Appendix C), respectively. The regulatory limits of sulphate and phosphate were 250 and 0.03 mg L^{-1} in natural water, respectively. Therefore, the tolerant limit of sulphate at 0.5 mg L^{-1} was not sufficient for application to environmental analysis. Environmental samples containing sulphate was necessary to remove by using solid phase extraction for accurate results that described in next section.

Table 4.9

Mass of perchlorate, isotopic sulphate and isotopic phosphate

Anions	Mass of anions	Expected mass of ion-pairing formation[b]
ClO_4^-	98.94850[a]	301.18938
HSO_4^{2-}	96.95172[a]	299.19260
$H_2PO_4^{3-}$	96.95342[a]	299.19430
$H^{34}S^{16}O_4^-$	98.95955	301.20043
$H^{32}S^{18}O^{16}O_3^-$	98.96463	301.20551
$H_2P^{18}O^{16}O_3^-$	98.97415	301.21503

[a] monoisotopic mass (Rozenski, 1999); [b] monoisotopic mass of HMB: 202.24088

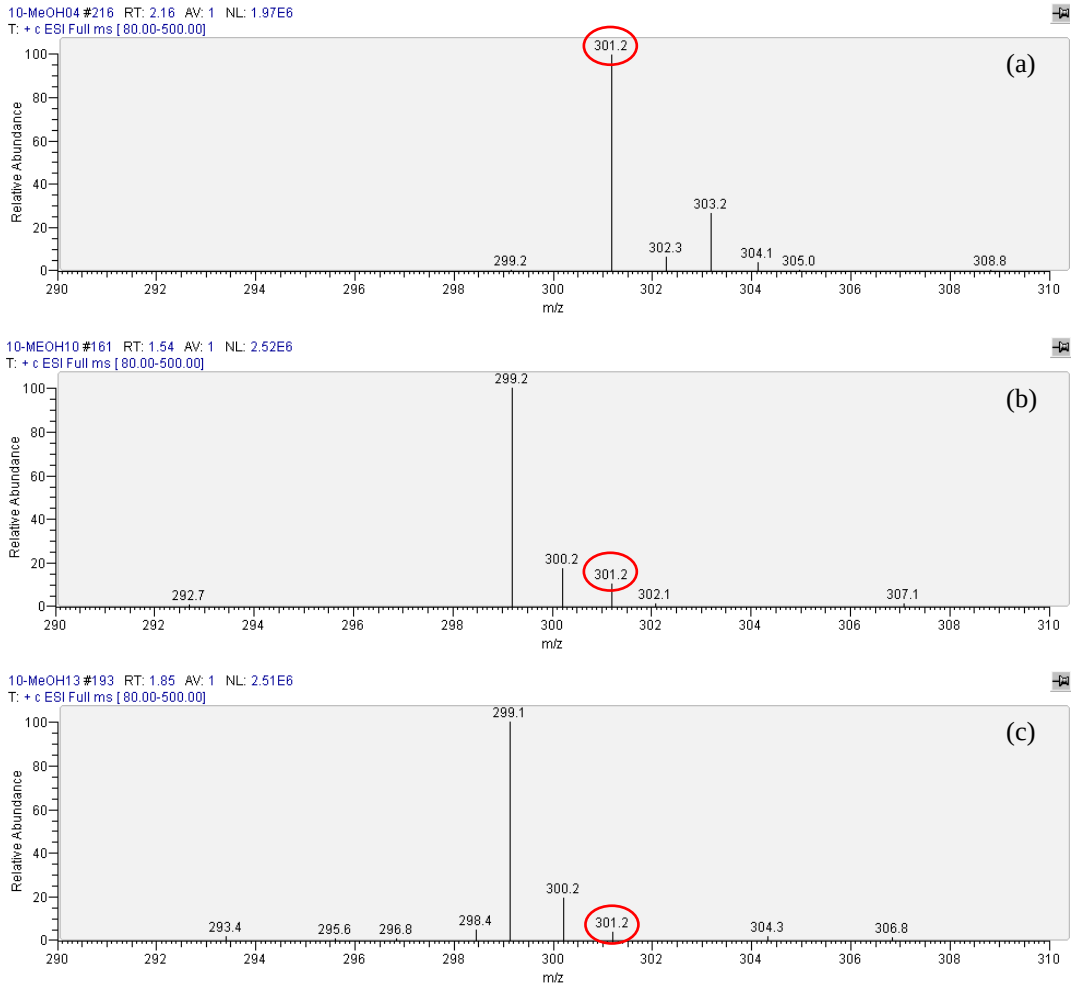


Figure 4.35 MS spectrums of ion-pair formation of (a) ClO_4^-, (b) SO_4^{2-} and (c) PO_4^{3-}

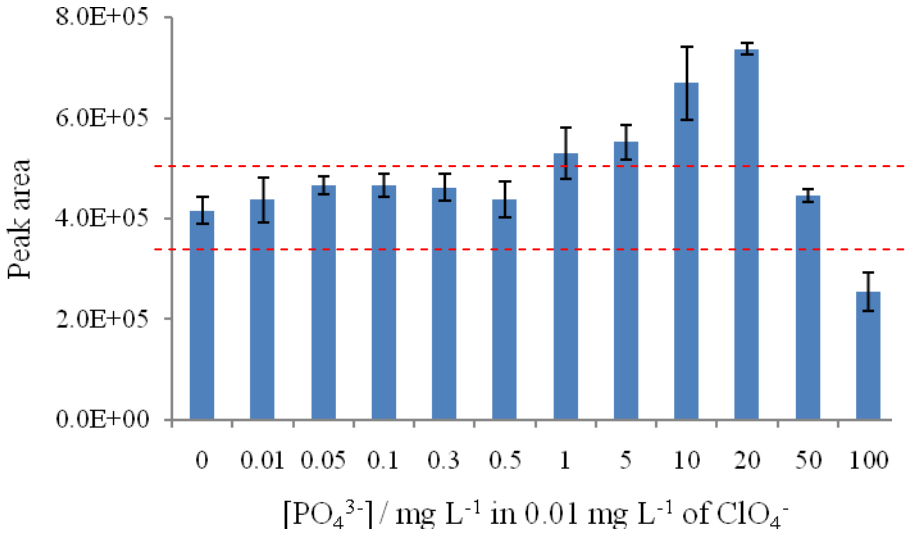


Figure 4.36 Tolerant limit of phosphate

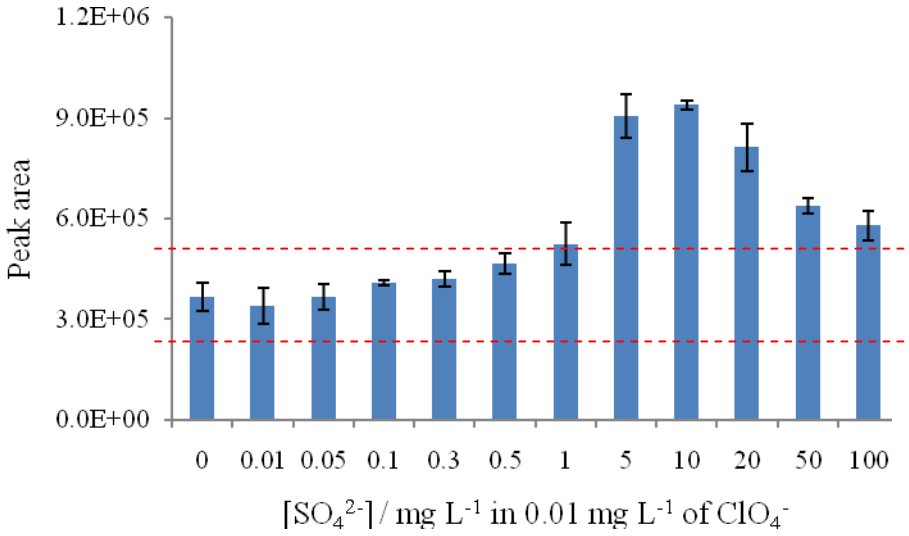


Figure 4.37 Tolerant limit of sulphate

Figure 4.37 showed the results obtaining from the experiments that were carried out using LC-MS/MS. The results clearly demonstrated that sulphate can be interfered the analysis by increasing the signal reading. With use of solid phase extraction (OnGuard-Ba) for sample clean-up, the interference can be more tolerated to approximately 100 mg SO_4^{2-} L^{-1}, as shown in Figure 4.38 and Table C28 (see Appendix C).

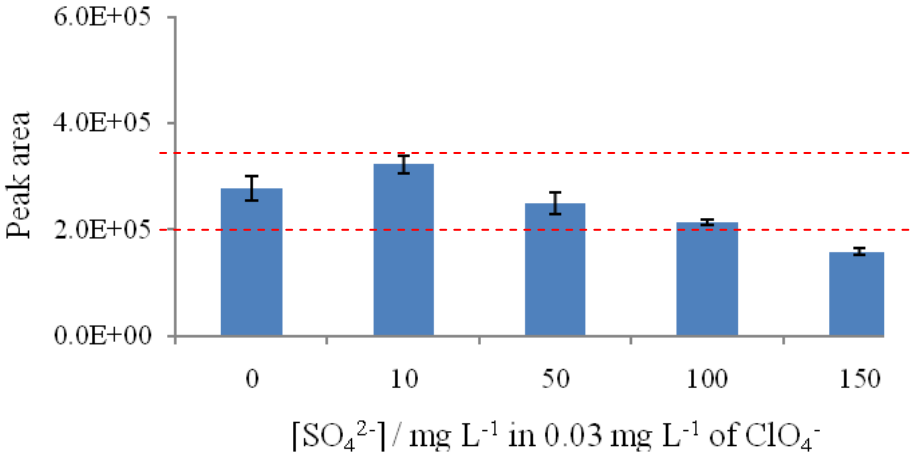


Figure 4.38 Tolerant limit of sulphate using SPE

4.2.5 Application of the method in environmental samples

4.2.5.1 Samples preparation

In this method, solid phase extractions (OnGuard-Ba) were used for sulphate removal prior to the analysis. Dry and wet elutions of the cartridges were investigated by eluting perchlorate, as described in Section 3.2.5.5 (a). Figure 4.39 and 4.40 showed the results of dry and wet elution of the cartridges, respectively. Because the solutions were obstructed from bubbles of dry elution, it was found that dry elution obtained lower peak area of perchlorate when compared with peak area of non-treatment perchlorate which was not passed the cartridges, as shown in Figure 4.39 (Table C29, Appendix C). On the other hands, wet elution obtained well results and was chosen, as shown in Figure 4.40 (Table C29, Appendix C). A first 3 mL of solution (F1-3) was discarded because of dilution and next 2 mL of solution (F4-5) was collected for the analysis.

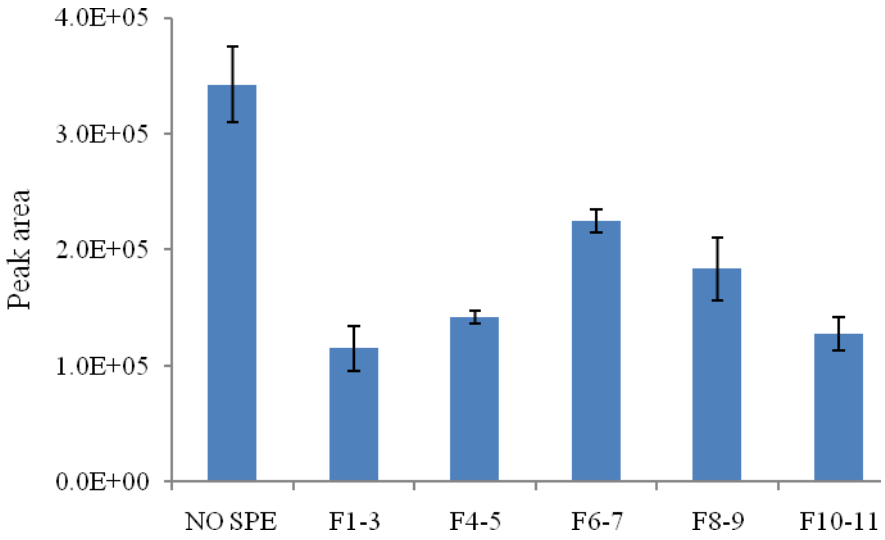


Figure 4.39 Dry elution of the cartridges

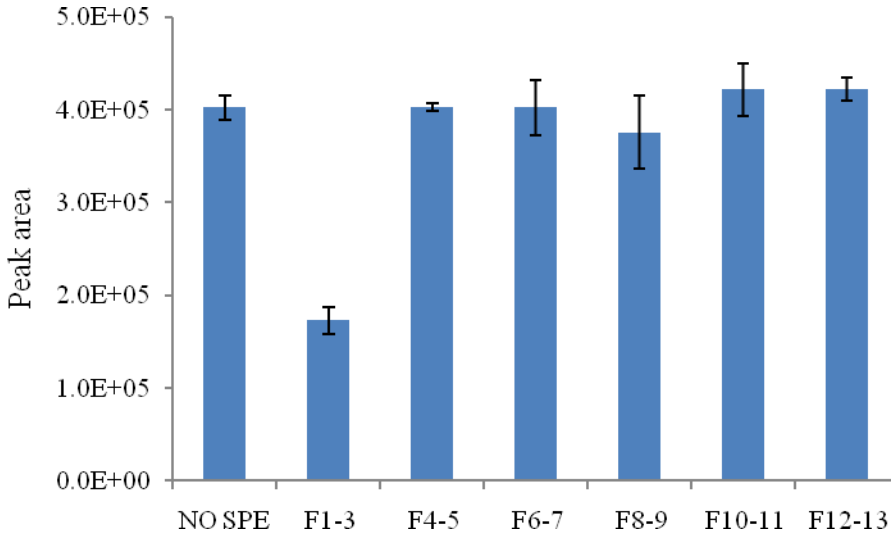


Figure 4.40 Wet elution of the cartridges

4.2.5.2 Samples determination

The proposed method was applied for perchlorate analysis in soils (Soil-1 to Soil-10) and water samples (Water-1 to Water-6). The samples preparation was described in Section 3.2.3.2. It was found that perchlorate was not detected in all samples, as shown in Figure 4.41 and Figure 4.42 for water and soil samples, respectively. (t_R of perchlorate standard was 1.22 minutes)

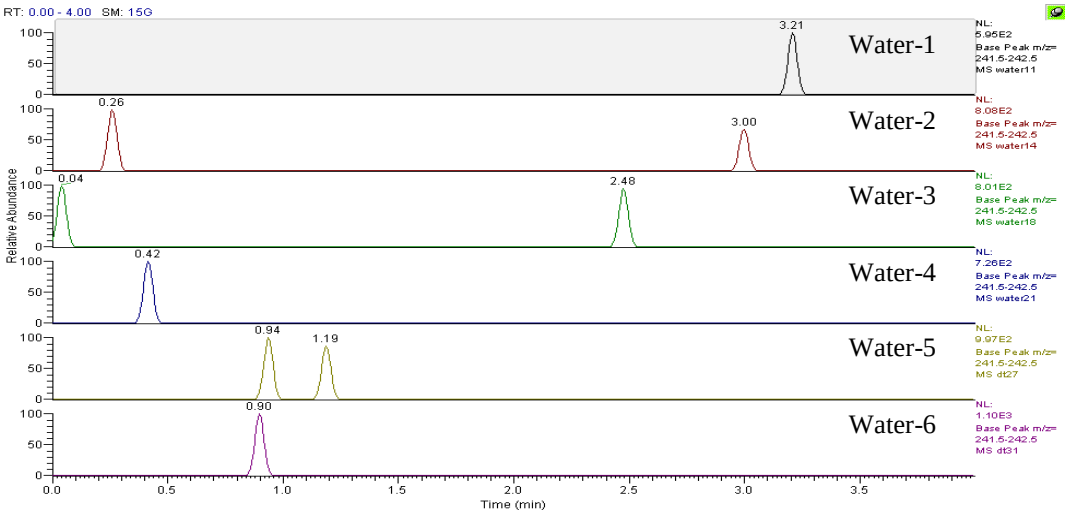


Figure 4.41 Water samples (Water-1 to Water-6)

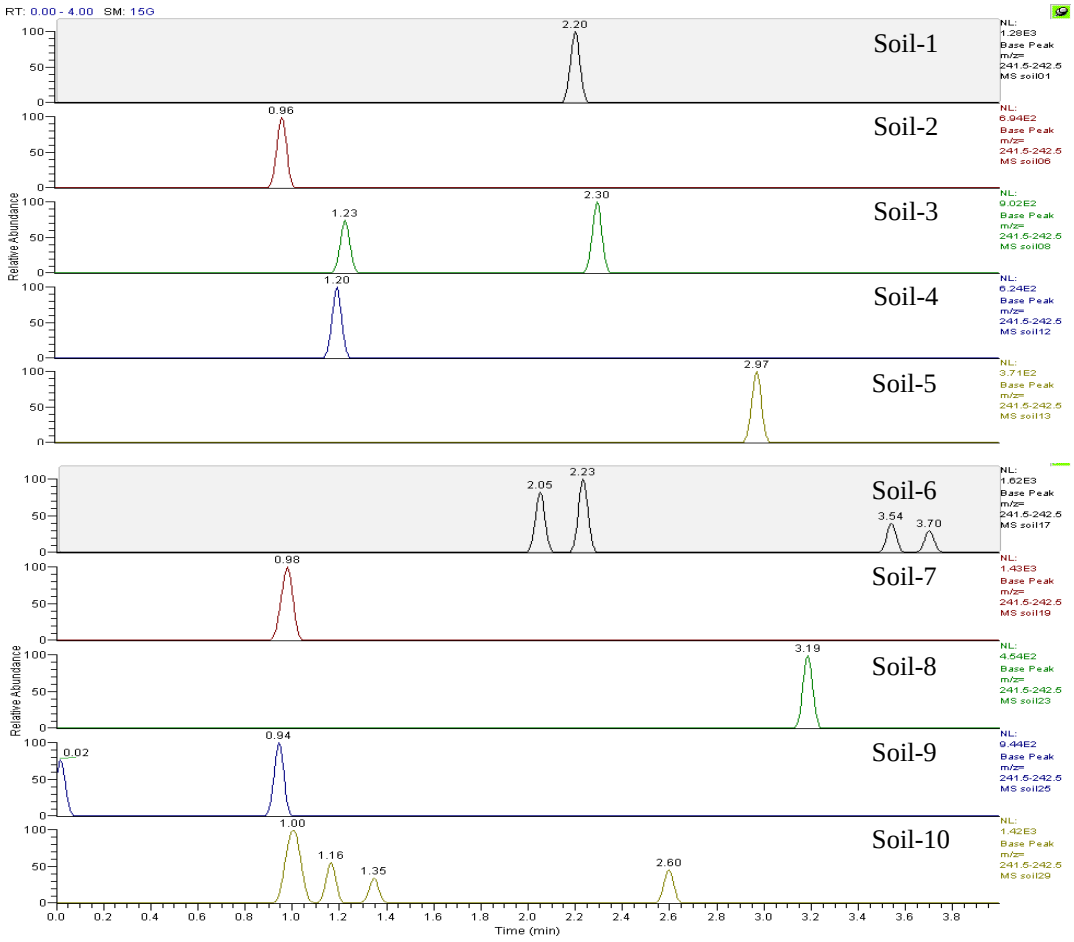


Figure 4.42 Soil samples (Soil-1 to Soil-10)