CHAPTER IV

RESULTS AND DISCUSSION

The optimization of high performance liquid chromatography-mass spectrometer

A previous ESI-MS studies of corticosteroids have reported different forms for precursor ions: $[M+H]^+$ [58, 59] and $[M+HCOO]^-$ [60, 61, 62, 63]. In this study, full scans under positive and negative modes for each compound were selected the most abundant mass-to-charge ratio (*m/z*). Optimum separation of hydroquinone, retinoic acid and the six corticosteroids was achieved on a 150 mm x 3.0 mm I.D., 3 µm Hypersil BDS C8 column with guard column (10 mm x 3 mm I.D., 3 µm) using a step gradient elution with mobile phase of 0.1% formic acid in water and acetonitrile at a flow rate of 0.5-0.55 mL/min (Table 6). The column temperature was maintained at 25°C. For mass spectrometric conditions initially both positive and negative ionization modes were tested for all compounds. The mass spectrometer was operated in positive ESI mode with selected ion monitoring (SIM) for the analytes under investigation, since hydroquinone produced a stronger signal to noise ratio/ sensitivity than the negative ion mode. Because of the polar nature of the analytes, ESI is the source of choice for their MS detection. The marker was the $[M+H]^+$ ion. All the analytes were identified and one characteristic ion was selected for each analyte in order to determine it in SIM mode (Table 12 to Table 13).

Table 12 Identification of all the analytes by HPLC-MS in positive ion mode

Analyte	Retention time (min)	Molecular weight	Ion selected in SIM mode, *m/z*
Hydroquinone	2.8	110.11	111.1
Retinoic acid	60.3	300.44	301.2
Betamethasone	25.1	392.47	393.1
Betamethasone17-valerate	47.0	476.59	477.2
Dexamethasone	26.8	392.47	393.1
Hydrocortisone	33.8	404.5	405.2
Prednisolone	15.9	360.45	361.2
Triamcinolone acetonide	33.1	434.50	435.2

In this study, the mobile phase composition for reversed phase HPLC separations of the analytes was selected for compatibility with electrospray mass spectrometry. Essentially, nonvolatile buffers reagents were excluded to prevent precipitation in the ion source. The mobile phase here presented is suitable for HPLC-MS analysis, because we replaced them by volatile formic acid.

Nine different fragmentation voltages (70-150V) were applied in order to obtain ion a quantifying ion of the protonated molecular ion. Appendix A, Table 34, was compared effect of the different fragmentation voltages under investigation. The results show that these values of ion intensity depended on the fragmentation. The most intense fragmentation voltage for the found precursor ions was chosen for each analyte (Table 7). In this study, the results indicated that the increase a voltage improves the signal intensity. However, the signal intensity of $[M+H]^+$ decreased at higher linearity of fragmentration voltage.

Figure 7 shows the mass spectra of all analytes obtained under the analytical conditions. The protonated molecular ion $[M+H]^+$ of all compounds were observed as base peak at *m/z* 111.1, 301.2, 361.2, 393.1, 405.2, 435.2 and 477.2. More specifically, at 100V ion at *m/z* 111.1 was selected for hydroquinone; *m/z* 361.2 was selected for prednisolone and *m/z* 393.1 was selected for betamethasone and dexamethasone. At 110V ion at *m/z* 301.2 was selected for retinoic acid and *m/z* 477.2 was selected for

betamethasone 17-valerate. At 120V ion at *m/z* 405.2 was selected for hydrocortisone acetate and *m/z* 435.2 was selected for triamcinolone acetonide. Figure 8 shows the HPLC-ESI-MS SIM mass chromatograms of standard mixture for hydroquinone (12 μg/mL), retinoic acid (0.41 μg/mL), betamethasone (3.23 μg/mL), betamethasone 17-valerate (0.81 μg/mL), dexamethasone (3.26 μg/mL), hydrocortisone acetate (1.63 μg/mL), prednisolone and triamcinolone acetonide (1.68 μg/mL).

The separation of the mixture is quite difficult, because the main peak is an isomer in the mixture. The other major challenge is the ability of the method to distinguish between betamethasone and dexamethasone as these two analytes are epimers and only differ from each other orientation of the methyl group of the C16 position. In our conditions, the step is crucial to distinguish between the isomer on the basic of the retention time. The exceptional stability and selectivity of Hypersil BDS C8 can be used to accomplish a difficult separation between betamethasone and dexamethasone.

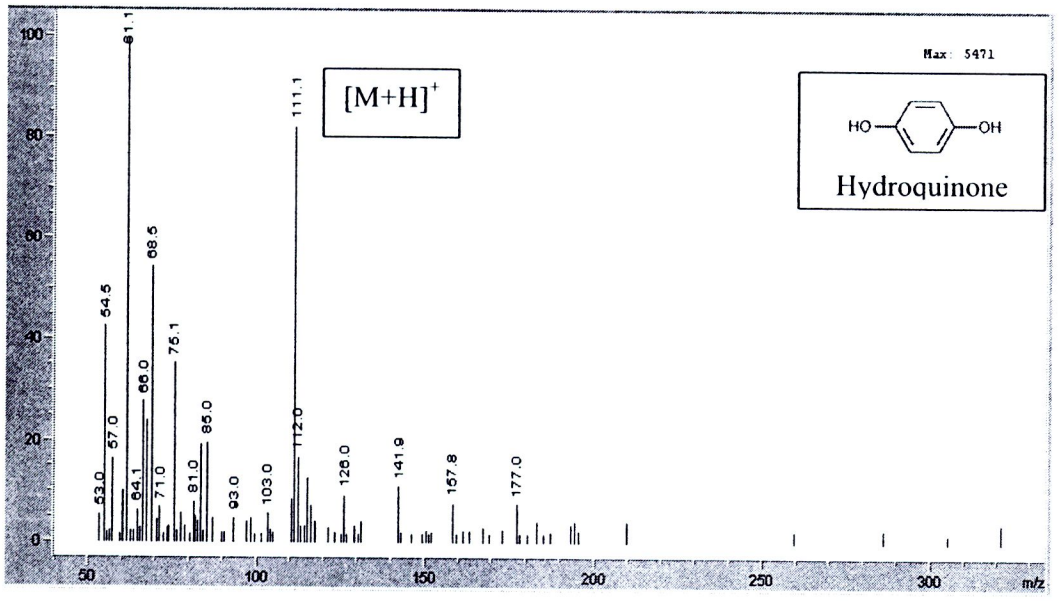
The characteristic $[M+H]^+$ fragment ions were observed for betamethasone and dexamethasone (*m/z* 393), corresponding to the protonated molecules. Further minor ions, at *m/z* 373, correspond to loss of HF for both analytes [64, 65]. Both of them have the same fragment under the optimized HPLC-MS condition. However, our results are separated by retention times. HPLC-MS/MS are also reported fragmentation of dexamethasone (*m/z* 393). The main fragment is the result of the breaking up of the C-F bond with release of a HF molecule [66]. Luo, et al reported the simultaneous separation of betamethasone and dexamethasone in equine on Hypercarb column by LC-MS/MS. Retention times for both compounds are 3.7 and 4.7 min, and the results are presented same precursor ion of them (*m/z* 393). Both betamethasone and dexamethasone also produce the same fragment ions (*m/z* 279, 237,147 and 171). However, the intensity of the major fragment ion *m/z* 237 for dexamethasone is higher than betamethasone, whereas *m/z* 279 for betamethasone is higher than dexamethasone. Therefore, they used ion ratios and retention times to distinguish between both analytes [47]. Although, MS-MS conditions for detection of corticosteroids have same precursor ion (*m/z* 393) and same product ions (*m/z* 147, 335, 355 and 373). Other authors [41] use the different relative abundances,

betamethasone and dexamethasone, of the ions *m/z* 355 and 373 for the two isomers, when MS-MS product ions are considered.

We found that equilibrium times for stable retention was rather long (data not shown). For system suitability, the resolution greater than 1.5 corresponds to baseline separation. The column efficiency is not less than 1500 theoretical plates of all analytes. Percent RSD of peak area of three injections is less than 5.

McDonald, et al. [63] have been reported that by reducing the formic acid concentration from 0.3 to 0.1%, a two-fold increase in sensitivity could be achieved again giving higher sensitivity at low concentration. Separation was achieved on a Hypercarb 100 mm x 2.1 mm I.D., 5 μm column with isocratic mobile phase, acetonitrile and 0.1% formic acid in water (9:1), at a flow rate of 0.6 mL/min. Results are shown giving higher sensitivity and a good separation of betamethasone and dexamethasone with a total LC-MS/MS run time of 6 minutes. A Hypercarb (100 mm x 2.1 mm I.D., 3 μm) was tested and worked very well in separation of betamethasone and dexamethasone. However, the other corticosteroids did not chromatograph very well, so this column was not utilisable for a multi-class method. Moreover, we could not detect hydroquinone.

(A)



(B)

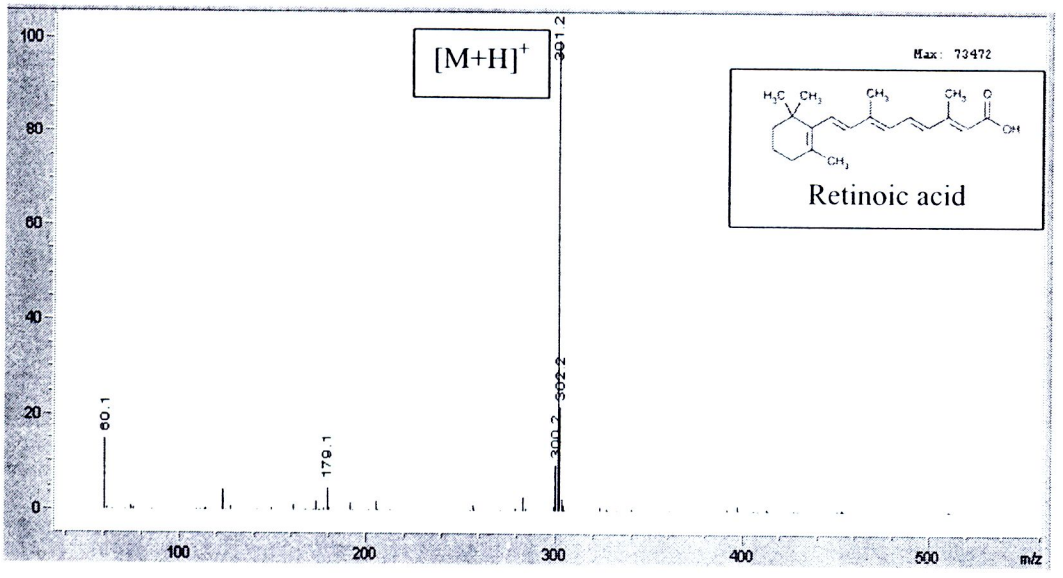
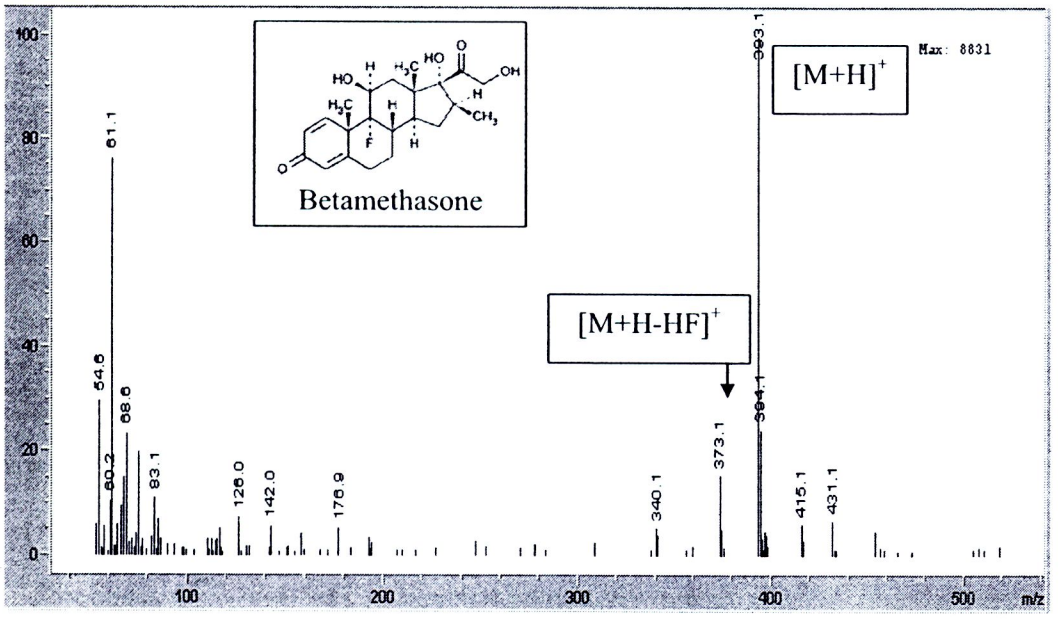


Figure 7 Mass spectra acquired by electrospray source in positive-ion mode of each standard solution for hydroquinone (A), retinoic acid (B), betamethasone (C), dexamethasone (D), betamethasone 17-valerate (E), hydrocortisone acetate (F), prednisolone (G) and triamcinolone acetonide (H)

(C)



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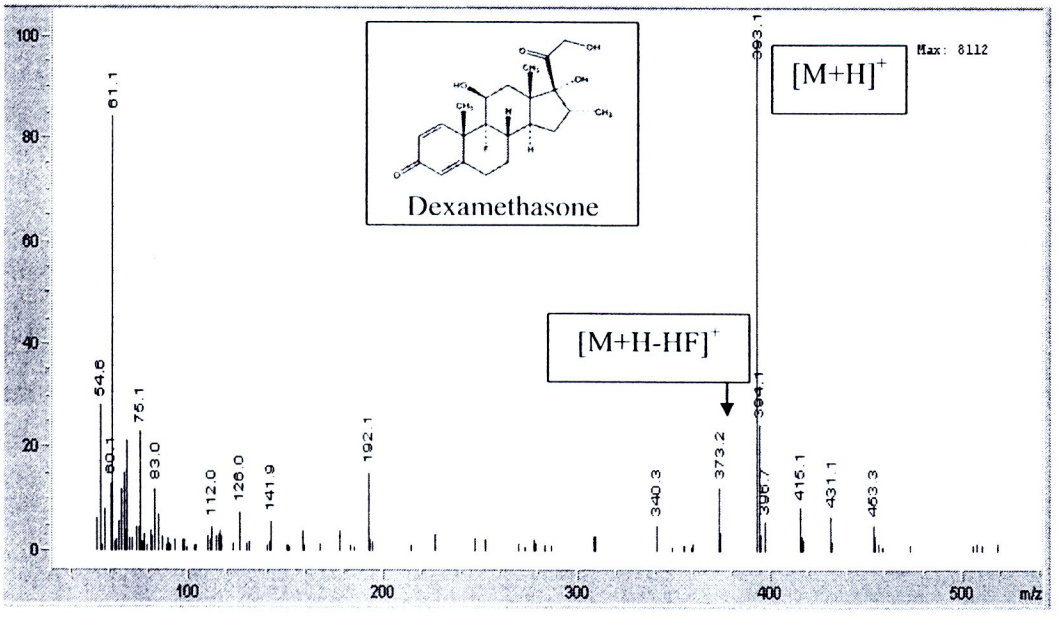
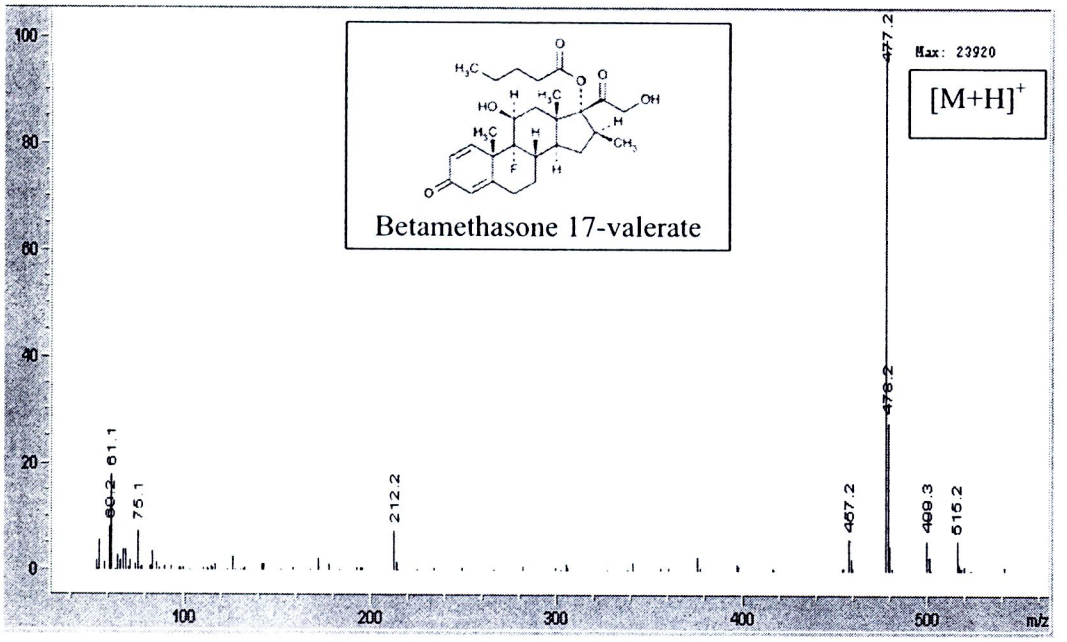


Figure 7 (Cont.)

(E)



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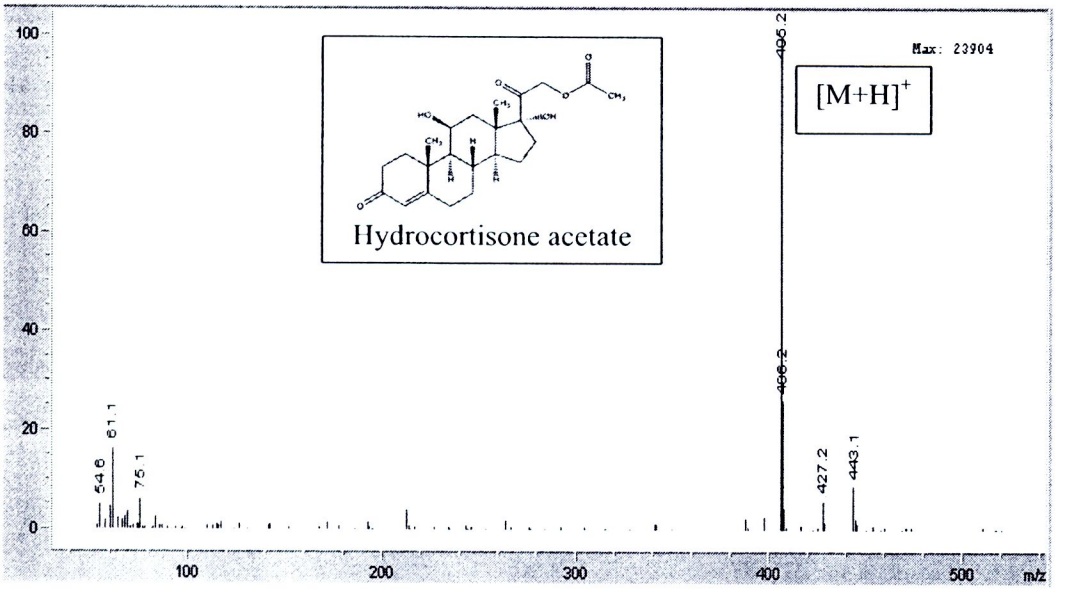

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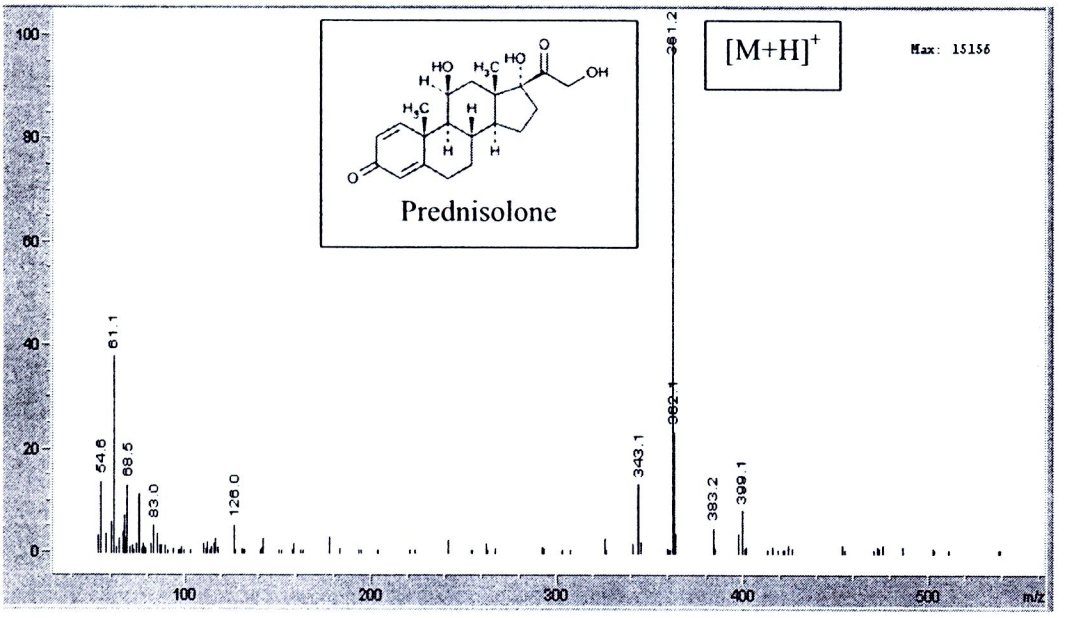
(G)



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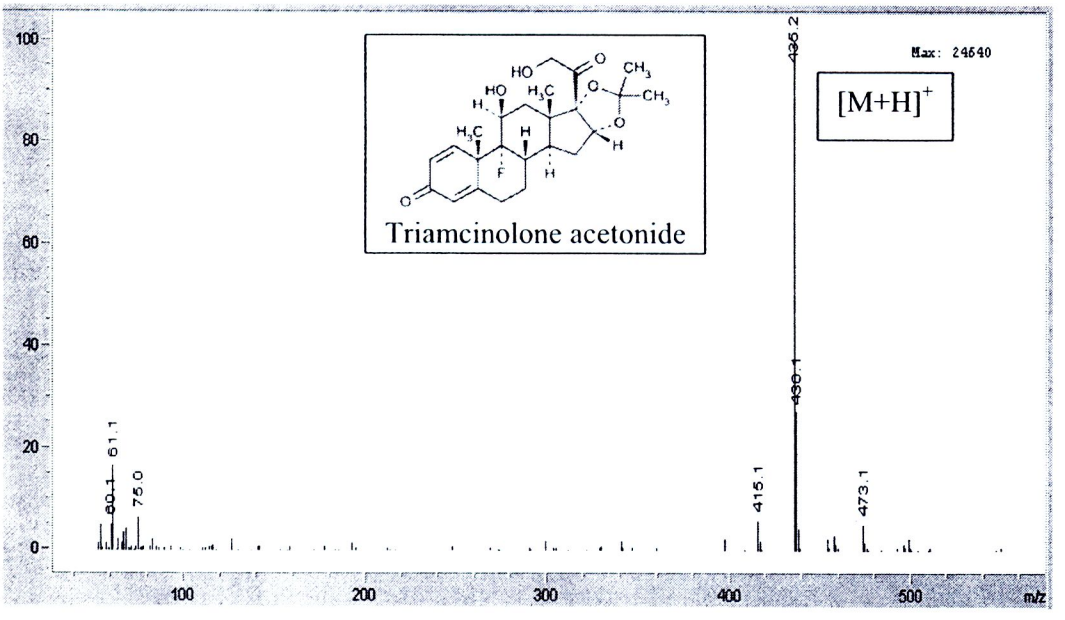


Figure 7 (Cont.)

Table 13 Fragmentation obtained from HPLC-MS spectra of all the analytes

Analyte	Fragment	Possible structure	Associated loss
HQ	111	$C_6H_6O_2$	-
RA	301	$C_{20}H_{28}O_2$	-
BM	393	$C_{22}H_{29}FO_5$	-
	373	$C_{22}H_{28}O_5$	HF
BMV	477	$C_{27}H_{37}FO_6$	-
DM	393	$C_{22}H_{29}FO_5$	-
	373	$C_{22}H_{28}O_5$	HF
HCA	405	$C_{23}H_{32}O_6$	-
PRL	361	$C_{21}H_{28}O_5$	-
TA	435	$C_{24}H_{31}FO_6$	-

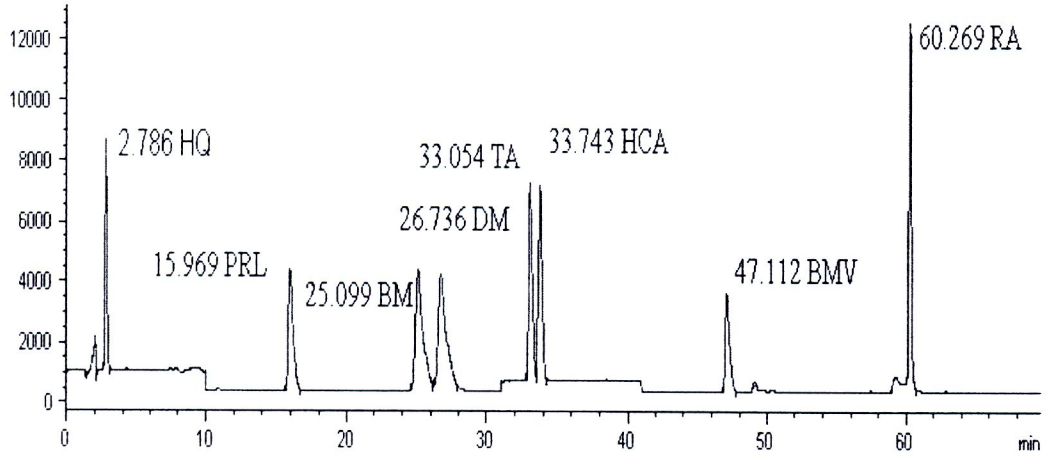


Figure 8 HPLC-ESI-MS TIC chromatogram (SIM mode) of standard mixture for hydroquinone (12 µg/mL), retinoic acid (0.41 µg/mL), betamethasone (3.23 µg/mL), betamethasone 17-valerate (0.81 µg/mL), dexamethasone (3.26 µg/mL), hydrocortisone acetate (1.63 µg/mL), prednisolone and triamcinolone acetonide (1.68 µg/mL)

Method validation

The method was validated under the EURACHEM GUIDE (1998). The parameters measured in the validation process included specificity/selecttivity, linearity and range, recovery, precision, limit of detection, limit of quantitation and measurement uncertainty, as is described in the following sections.

1. Specificity/Selectivity

A typical chromatogram from standard solutions, matrix blank and spiked matrix blank with hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide are shown in Figure 9. For the specificity, no co-eluting interfering compounds were observed in the chromatogram of the matrix blank. Chromatograms demonstrate that the compounds of interest could be detected separately from matrix components. The separated hydroquinone, retinoic acid and the corticosteroids were measured without interference, indicating that the method is selective for eight analytes.

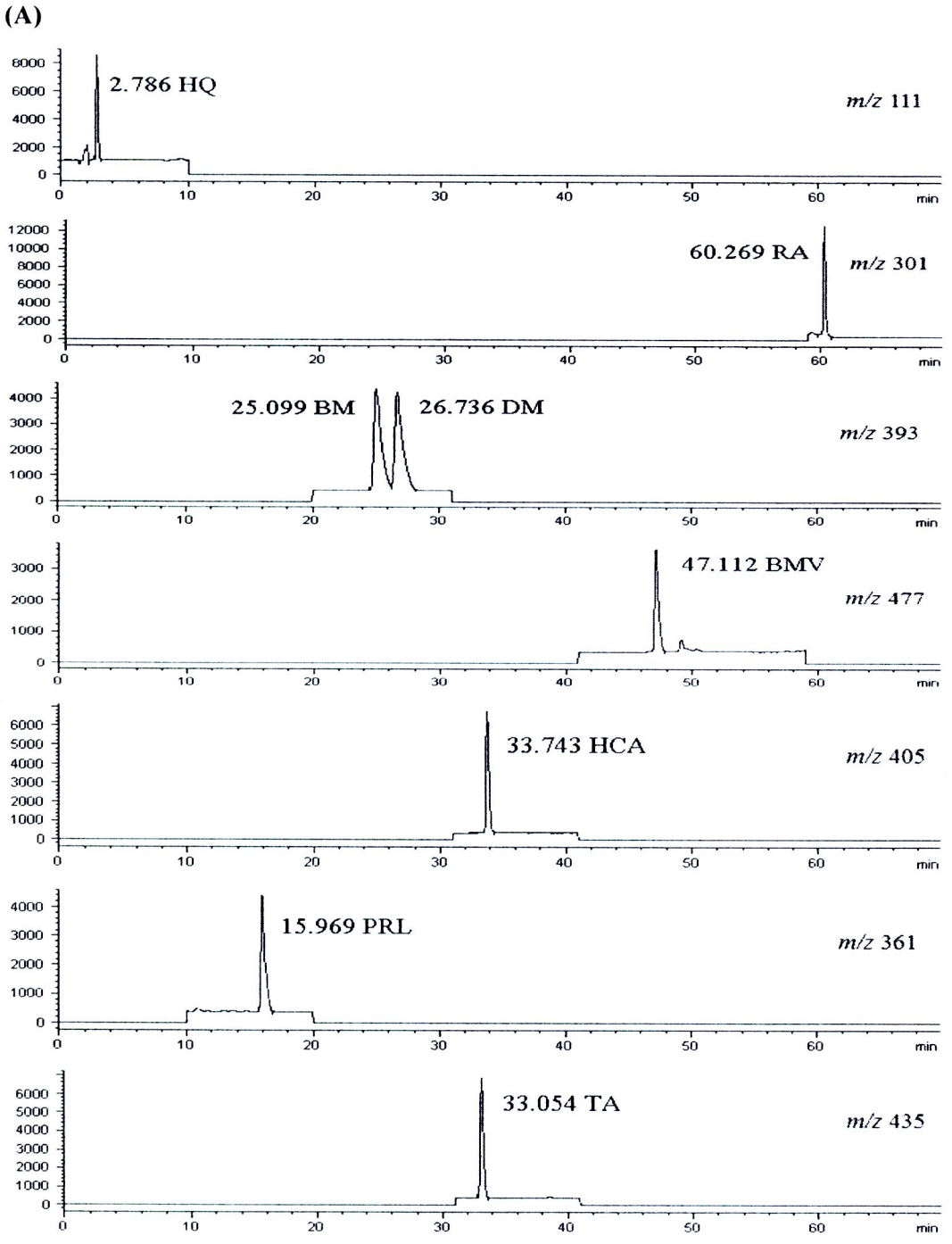


Figure 9 HPLC-ESI-MS EIC chromatograms of standard solution (A), matrix blank (B) and spiked matrix blank (C) with hydroquinone, retinoic acid, betamethasone, dexamethasone, betamethasone 17-valerate, hydrocortisone acetate, prednisolone, and triamcinolone acetonide

(B)

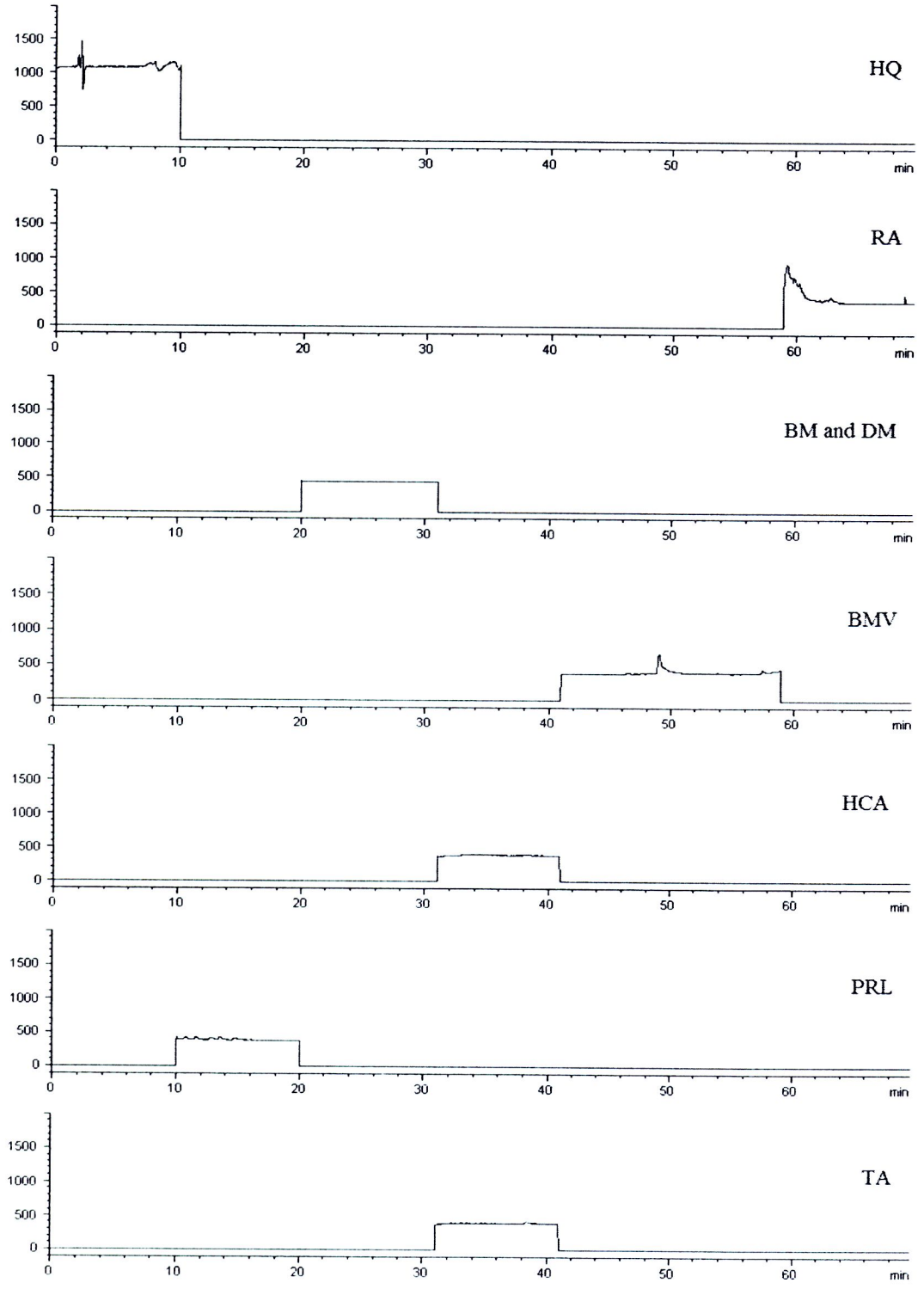


Figure 9 (Cont.)

(C)

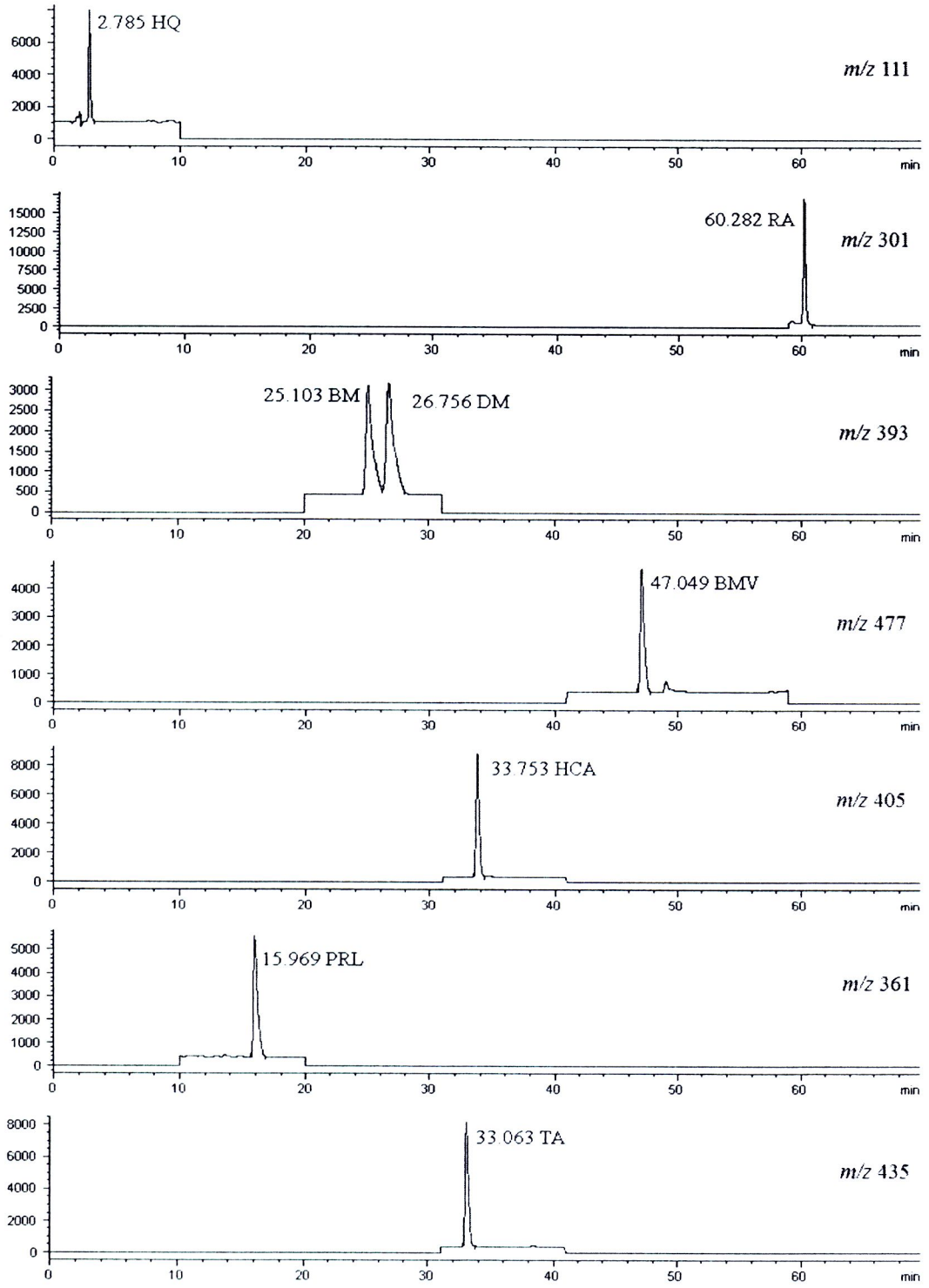


Figure 9 (Cont.)

2. Linearity and range

2.1 System linearity

Standard curves were fitted by linear regression equation $y = ax + b$, where y represents the peak areas, a and b the contents, x is the concentration of the compounds. The seven point calibration curve was established in the concentration ranges from 1.5-60 μg/mL for hydroquinone, from 0.05-2 μg/mL for retinoic acid, from 0.4-16.3 μg/mL for betamethasone, from 0.1-4.2 μg/mL for betamethasone 17-valerate, from 0.4-16.7 μg/mL for dexamethasone, from 0.2-8.3 μg/mL for hydrocortisone acetate, from 0.2-8.1 μg/mL for prednisolone and from 0.2-8.2 μg/mL for triamcinolone acetonide. The calculated concentrations and the peak areas all of the analytes displayed linear relationship over the selected concentration range with consistent slopes and correlation coefficients (r) greater than 0.995 throughout the validation runs. The results of the system linearity, with all analytes, evaluation are summarized in Figure 10 to Figure 17 and Table 14. Table 35 to Table 42 (Appendix B) are also shown raw data of the system linearity. Linearity can be tested informally by examination of a plot of residuals produced by linear regression of the responses on the concentrations in an appropriate calibration set. For residual plots, the x-axis is the concentration value of x, and the y-axis is the residual of x, and the results are shown in Figure 18 to Figure 25. The residuals were acceptable insofar as they are normally distributed with an approximate mean of zero. Most of data points are randomly scattered about the mean. This random pattern indicates that a linear model provides a decent fit to the data.

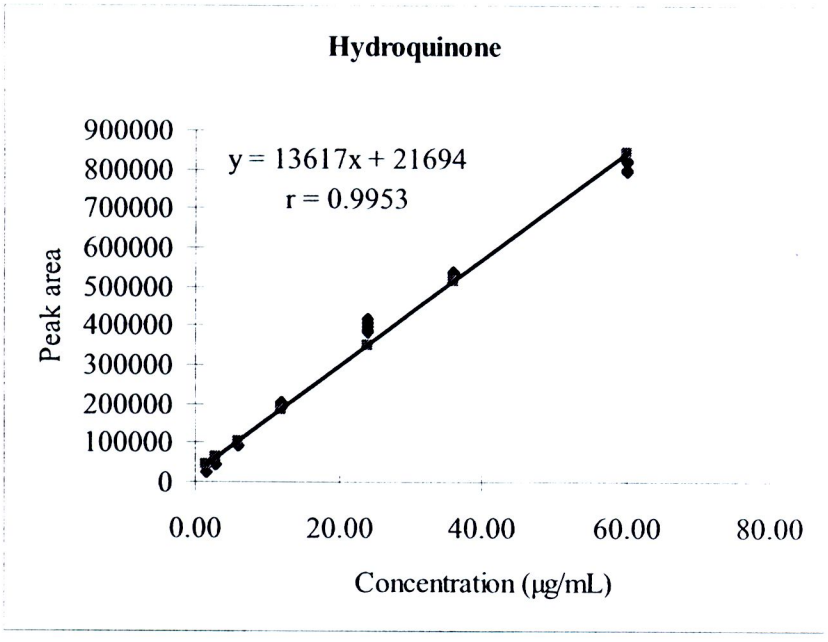


Figure 10 System linearity of hydroquinone

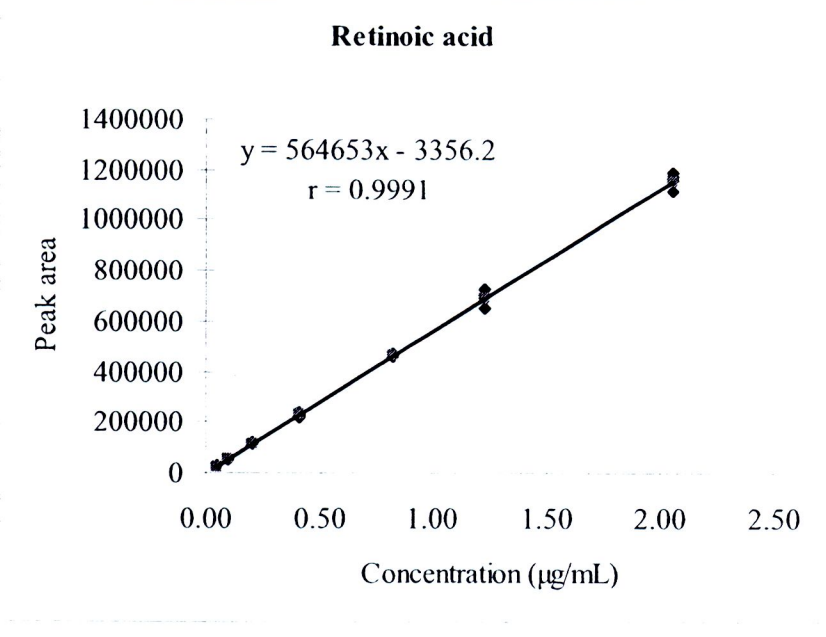


Figure 11 System linearity of retinoic acid

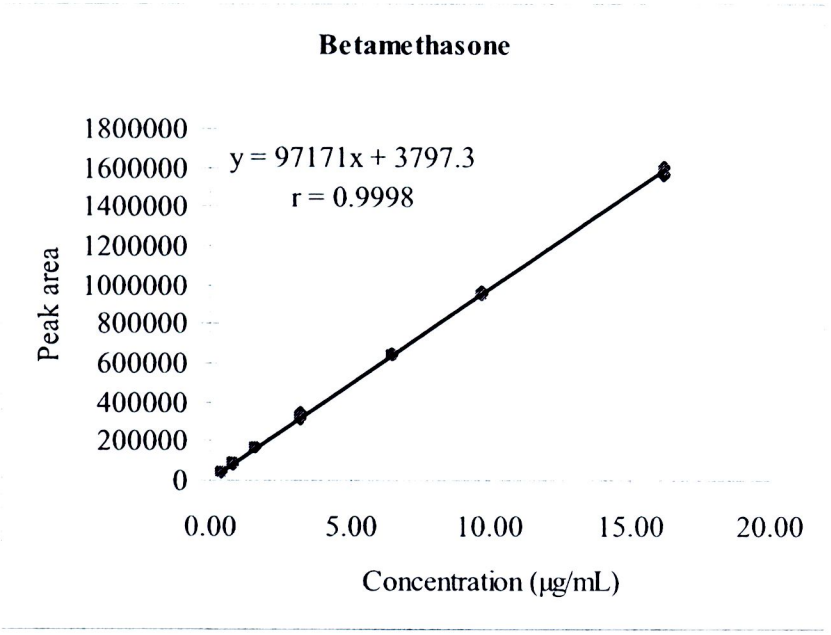


Figure 12 System linearity of betamethasone

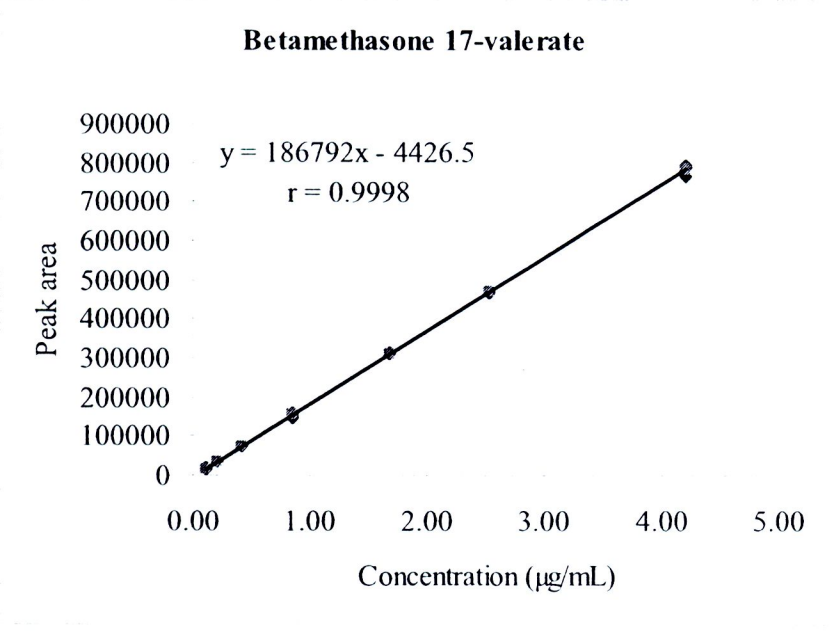


Figure 13 System linearity of betamethasone 17-valerate

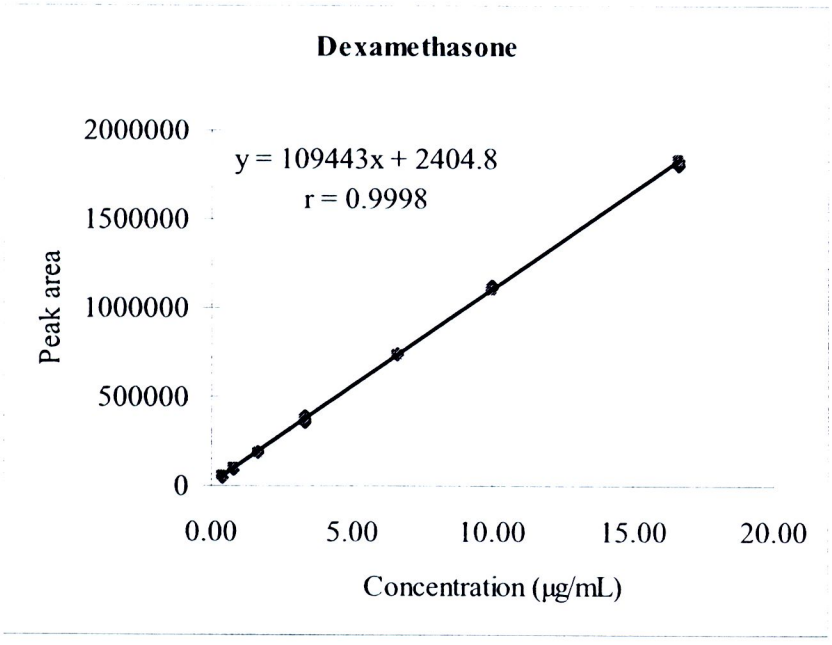


Figure 14 System linearity of dexamethasone

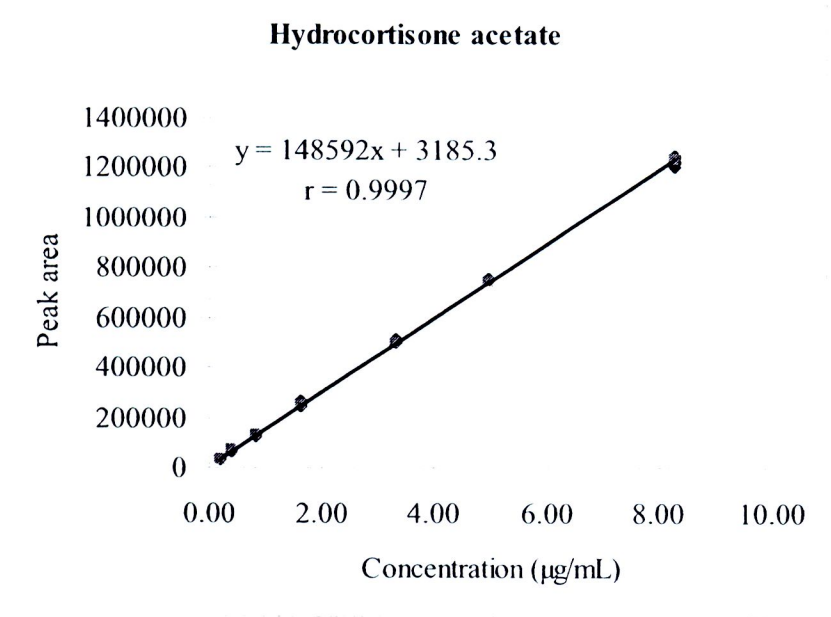


Figure 15 System linearity of hydrocortisone acetate

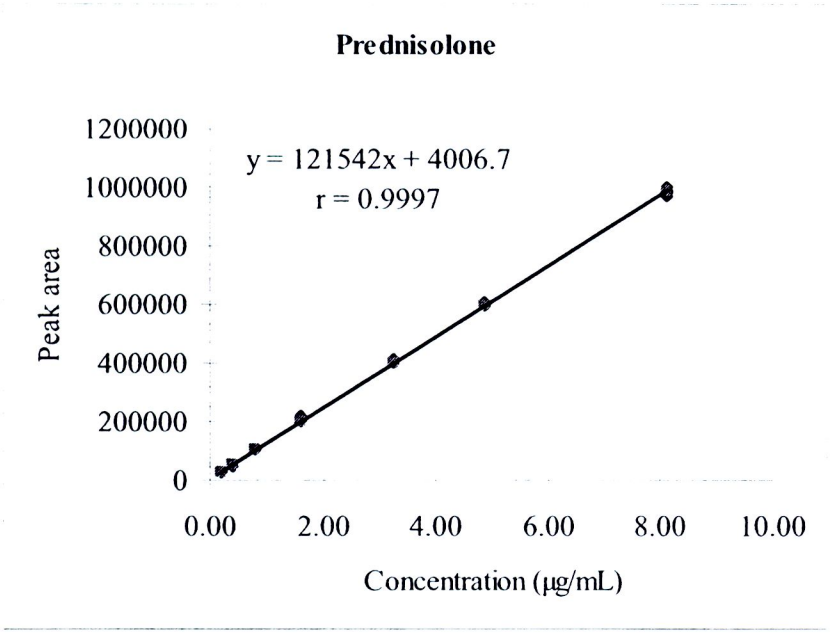


Figure 16 System linearity of prednisolone

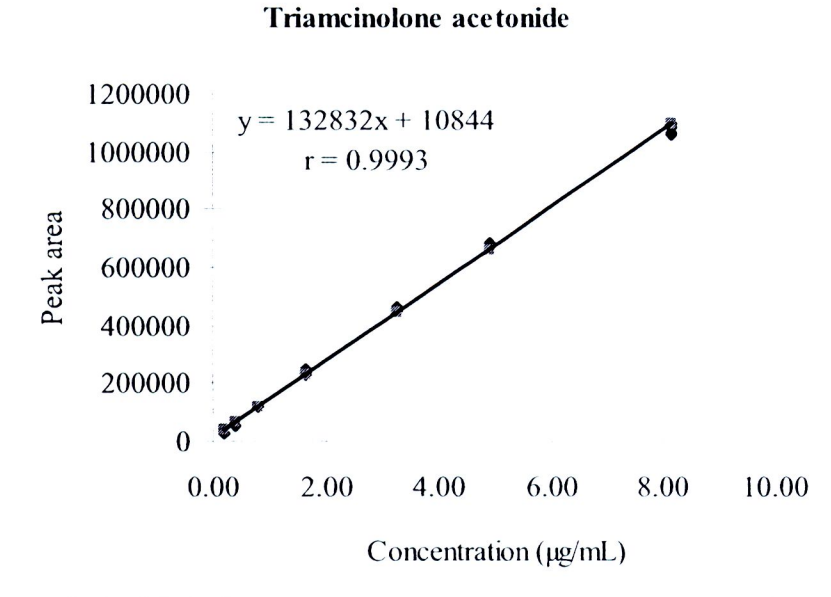


Figure 17 System linearity of triamcinolone acetonide

Table 14 Summary of slope, y-intercept and correlation coefficient (r) of all analytes for system linearity

No.	Analyte	Slope	y-intercept	r
1.	HQ	13617	21694	0.9953
2.	RA	564653	- 3356	0.9991
3.	BM	97171	3797	0.9998
4.	BMV	186792	- 4426	0.9998
5.	DM	109443	2405	0.9998
6.	HCA	148592	3185	0.9997
7.	PRL	121542	4007	0.9997
8.	TA	132832	10844	0.9993

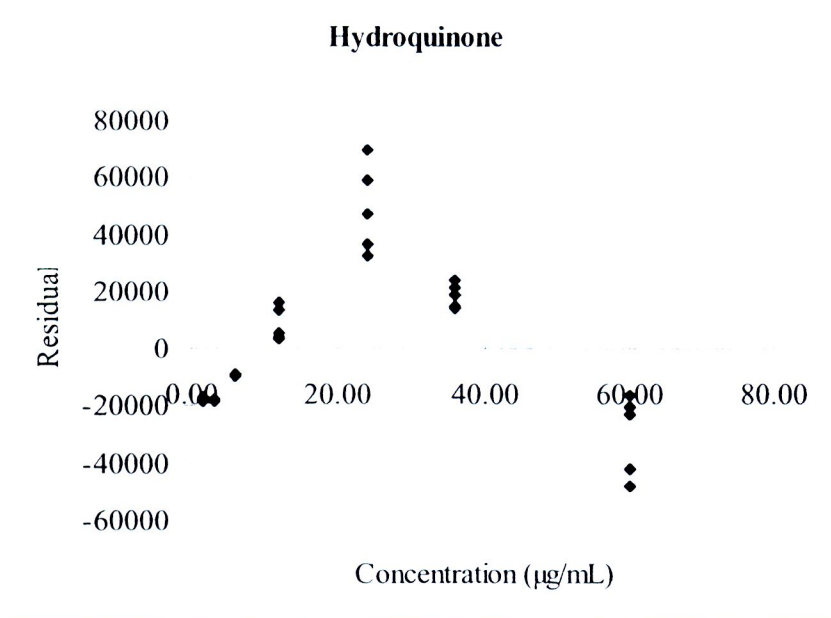


Figure 18 Residual plot of hydroquinone

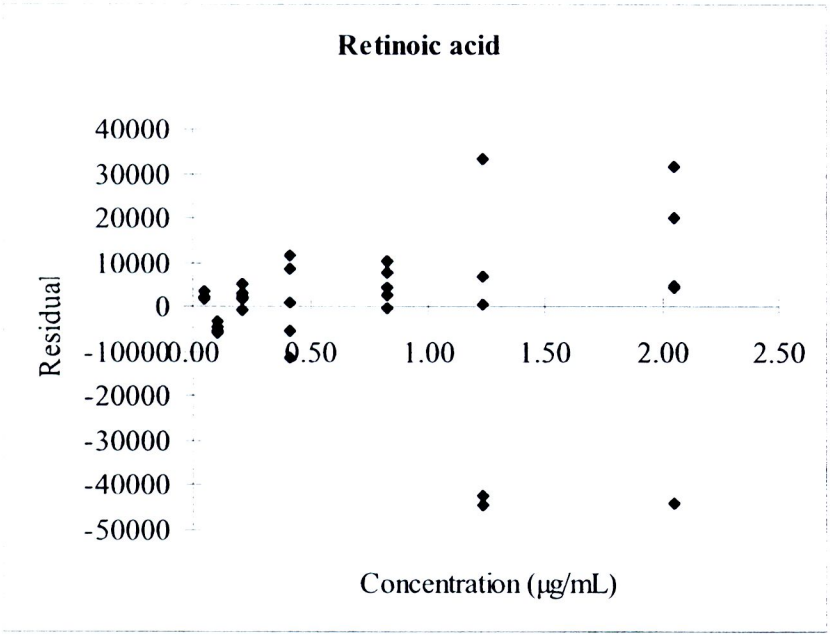


Figure 19 Residual plot of retinoic acid

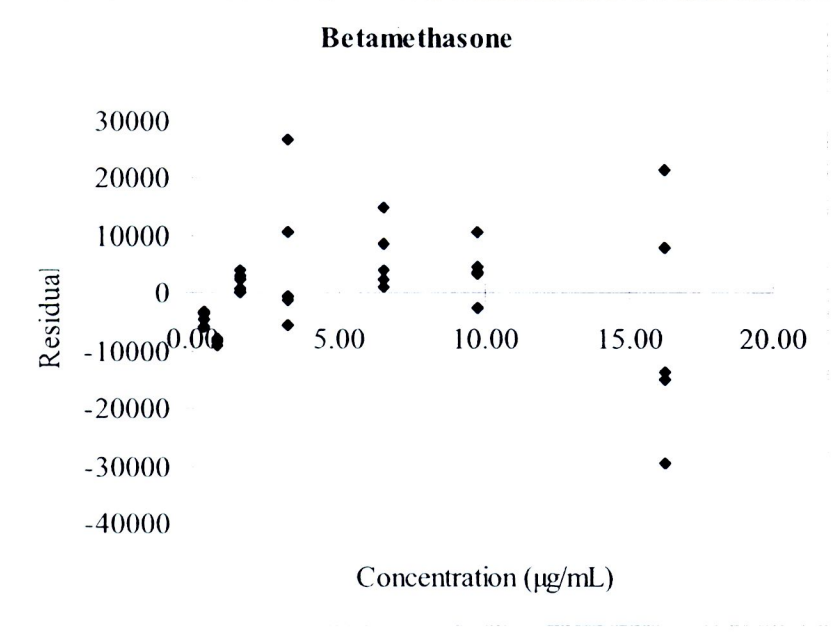


Figure 20 Residual plot of betamethasone

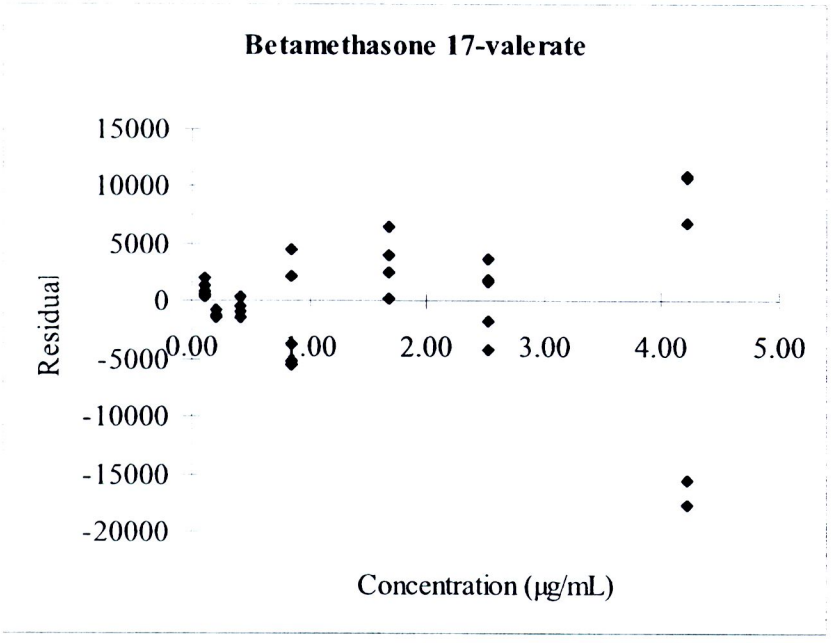


Figure 21 Residual plot of betamethasone 17-valerate

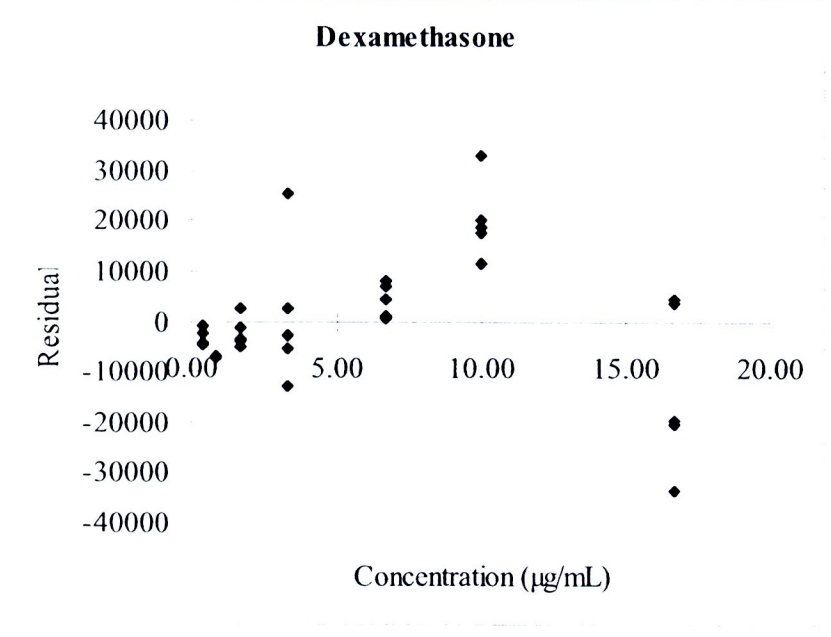


Figure 22 Residual plot of dexamethasone

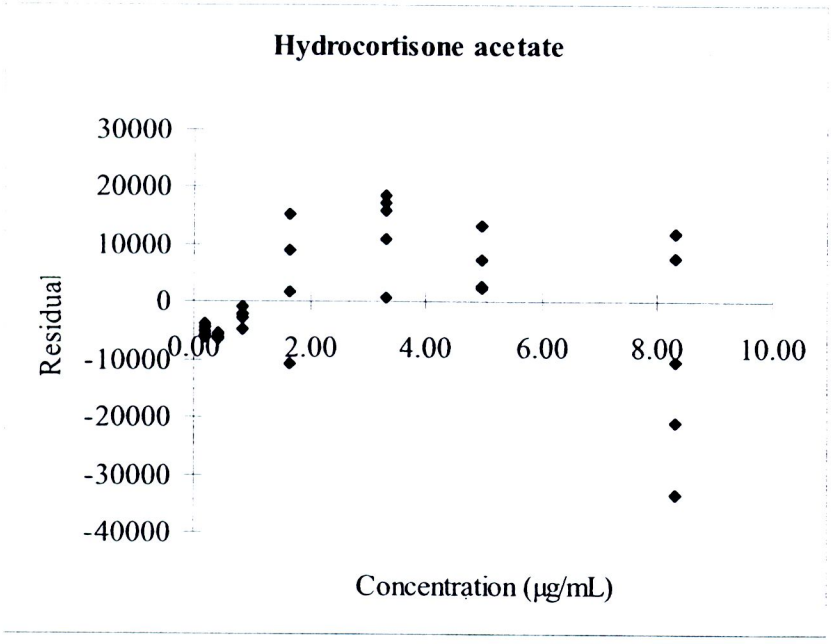


Figure 23 Residual plot of hydrocortisone acetate

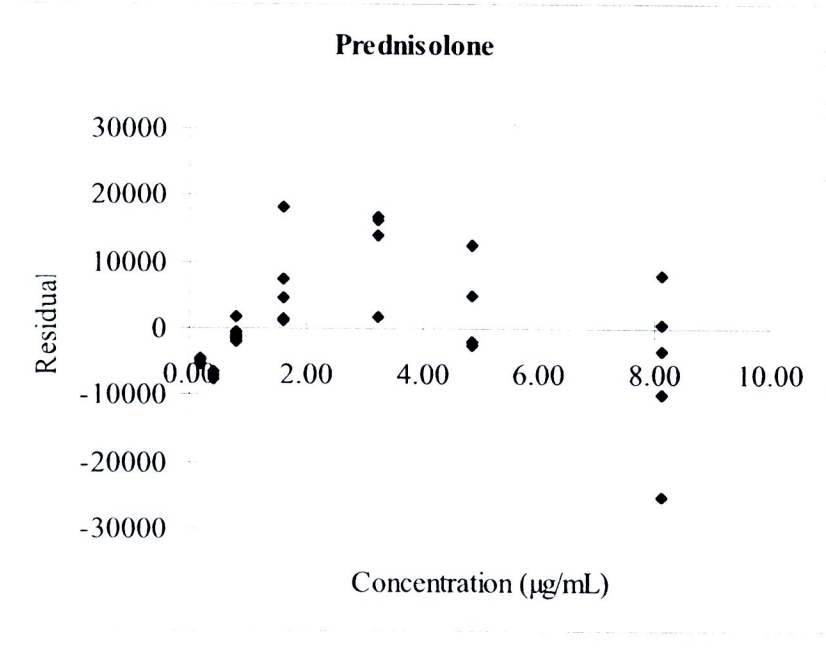


Figure 24 Residual plot of prednisolone

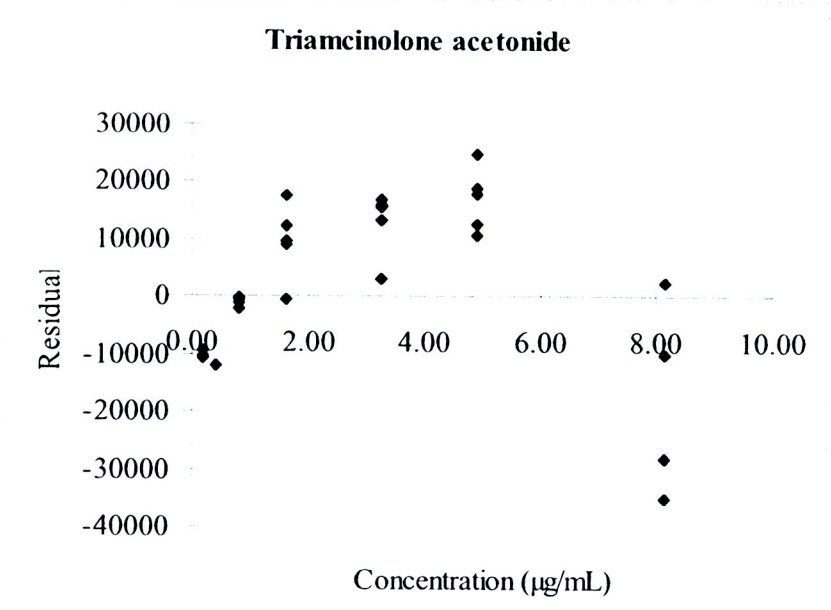


Figure 25 Residual plot of triamcinolone acetonide

2.2 Method linearity

The method linearity of the chromatographic response demonstrated by preparing three points for each analyte. To build these curves, an equation type $y = ax + b$ were used. All of the analytes gave correlation coefficient values greater than 0.998. Therefore, it is good in the range of tested concentrations, showing an acceptable correlation. As can be seen in Figure 26 to Figure 33 and Table 15.

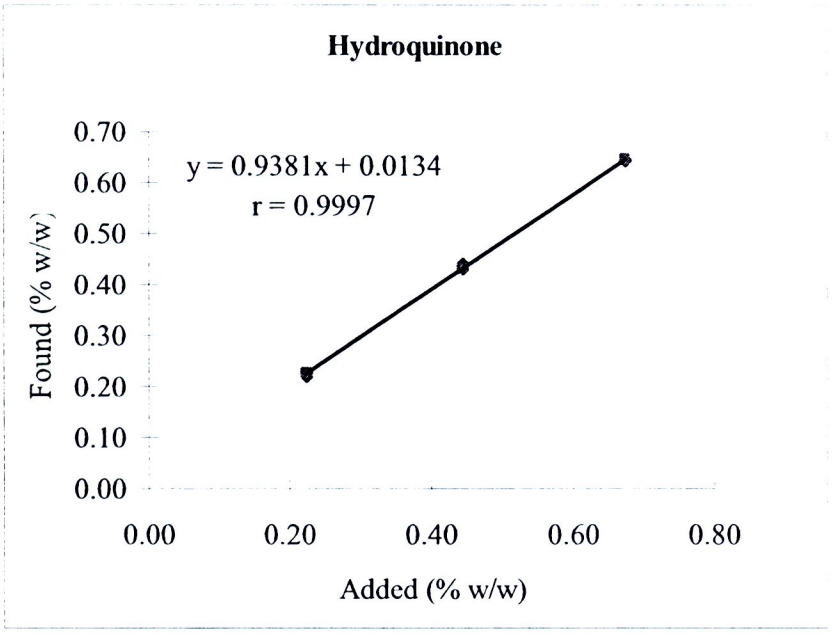


Figure 26 Method linearity of hydroquinone

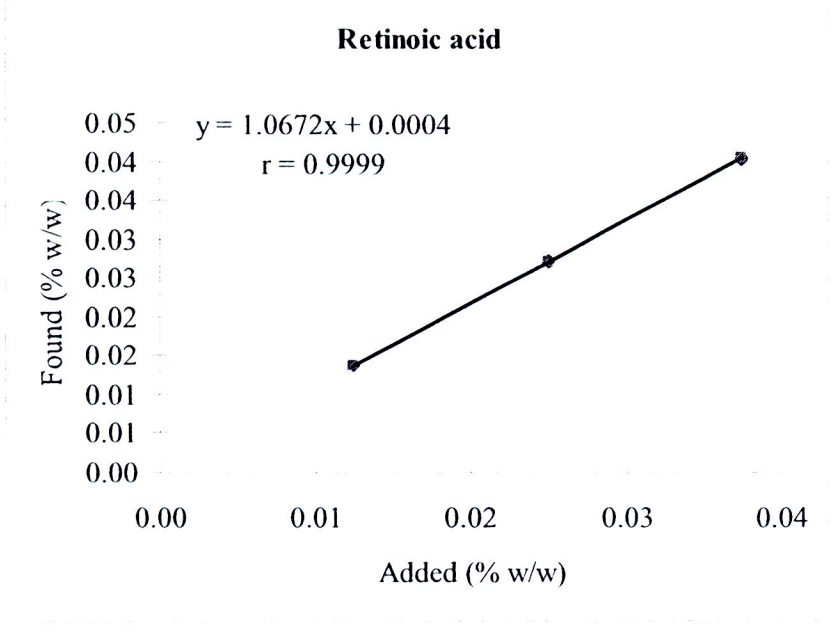


Figure 27 Method linearity of retinoic acid

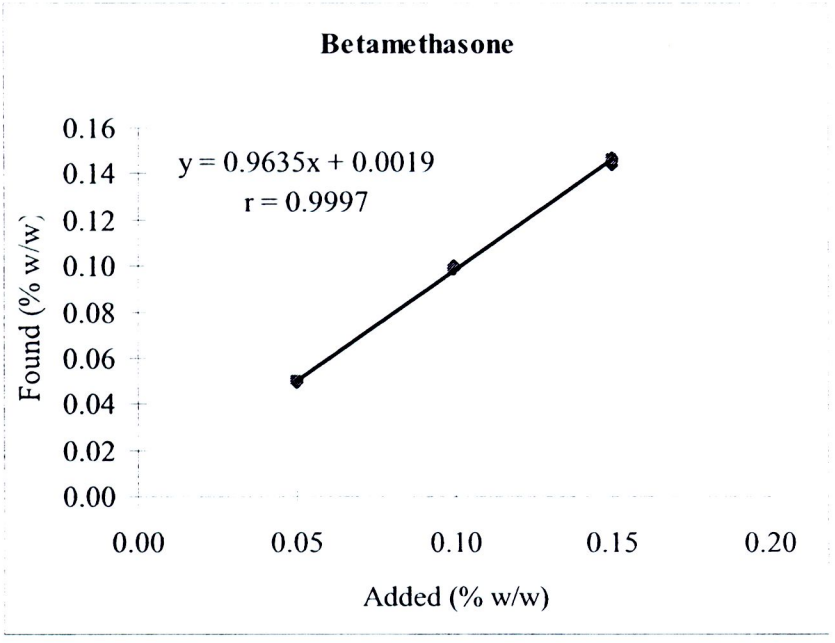


Figure 28 Method linearity of betamethasone

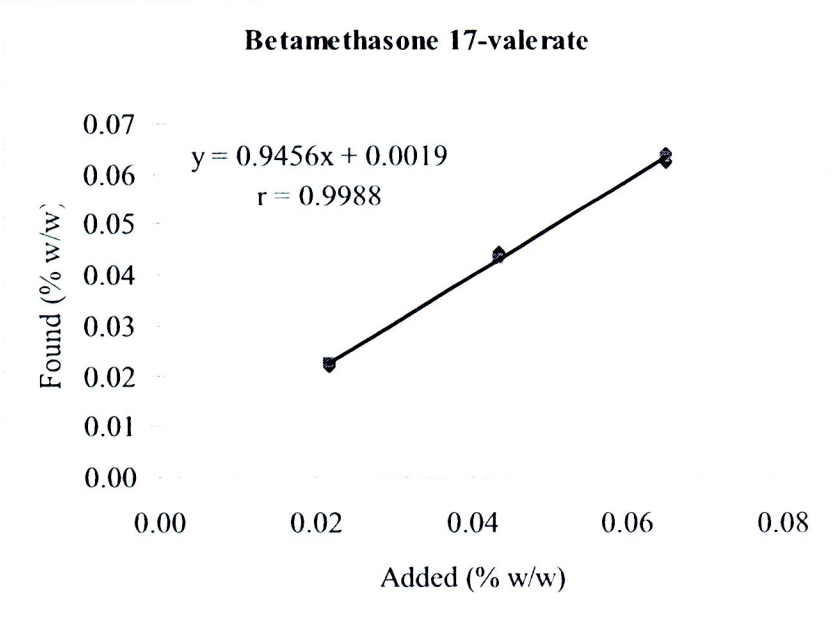


Figure 29 Method linearity of betamethasone 17-valerate

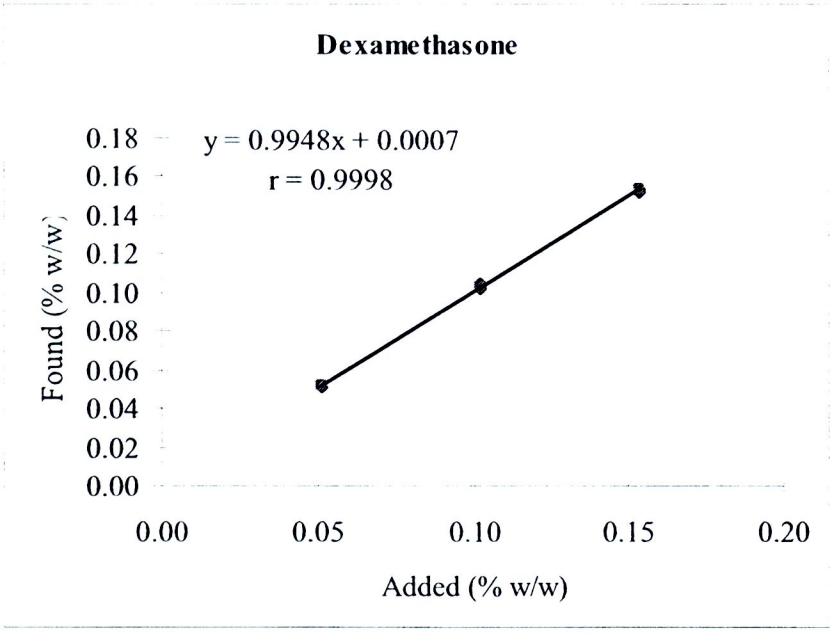


Figure 30 Method linearity of dexamethasone

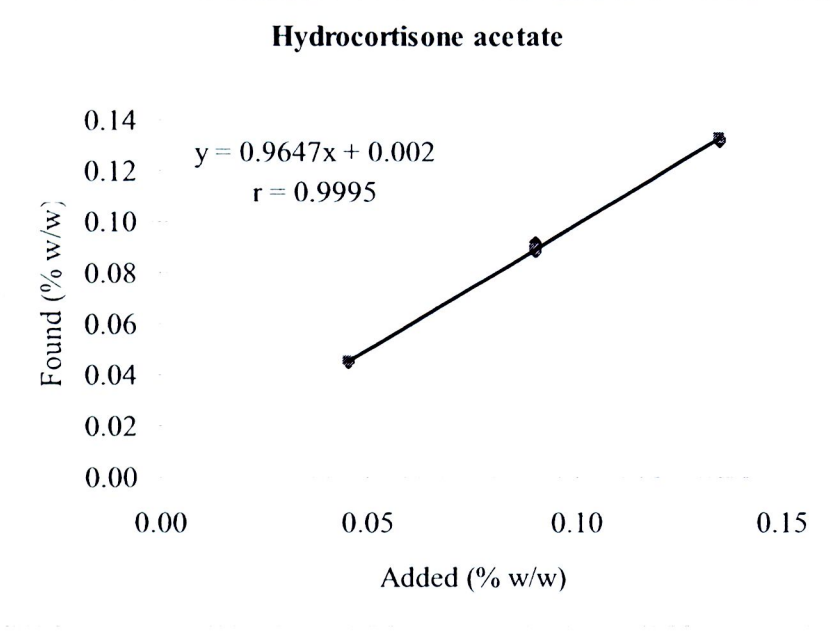


Figure 31 Method linearity of hydrocortisone acetate

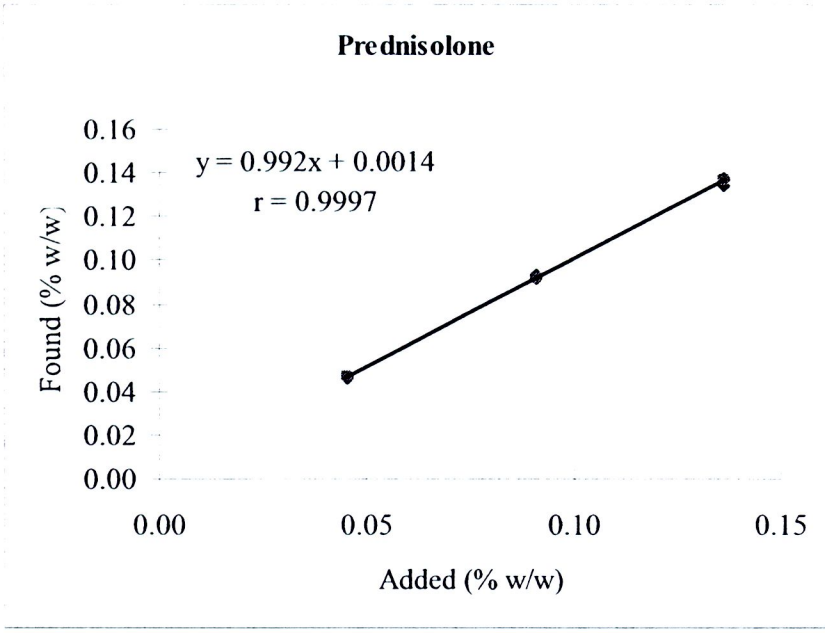


Figure 32 Method linearity of prednisolone

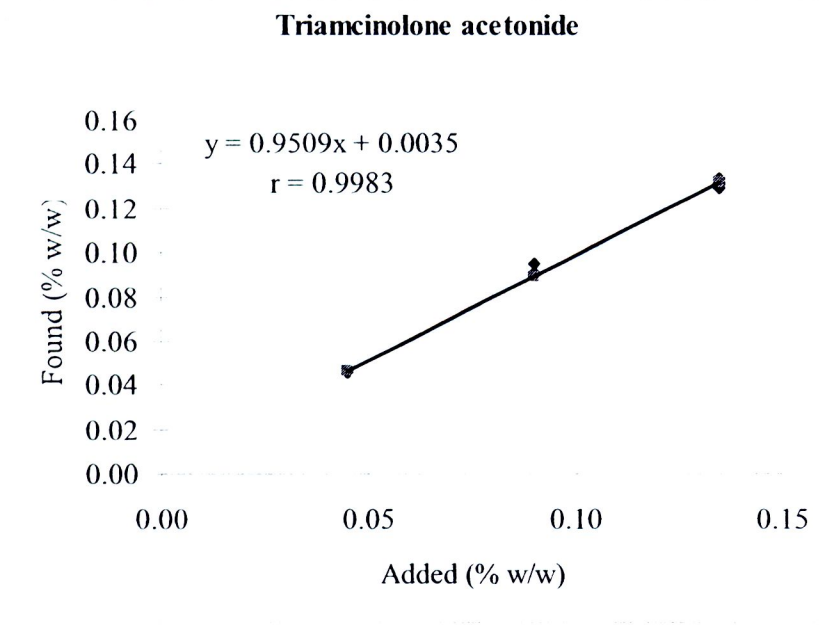


Figure 33 Method linearity of triamcinolone acetonide

Table 15 Summary of slope, y-intercept and correlation coefficient (r) of all analytes for method linearity

No.	Analyte	Slope	y-intercept	r
1.	HQ	0.9381	0.0134	0.9997
2.	RA	1.0672	0.0004	0.9999
3.	BM	0.9635	0.0019	0.9997
4.	BMV	0.9456	0.0019	0.9988
5.	DM	0.9948	0.0007	0.9998
6.	HCA	0.9647	0.002	0.9995
7.	PRL	0.9920	0.0014	0.9997
8.	TA	0.9509	0.0035	0.9983

3. Recovery

Recovery is the fraction of analyte added to the test sample (fortified or spiked) prior to analysis, which is measured by the method. It reflects extraction efficiency and handling losses. Accuracy was expressed as percentage of analytical recovery rates of the measured concentration against spiked-concentration for each analyte. The expect recovery % values depend on the analyte concentration as shown in Table 9.

The recovery was estimated by spiking standard solutions in 1 g of matrix blank at three concentration levels in five replicates. It was determined using peak areas as the agreement between the concentration of the target analytes detected and that spiked into matrix blank (blank cream). Mean recoveries of hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide were 95.0-100.7%, 106.9-109.6%, 96.1-100.2%, 95.5-103.0%, 98.8-102.7%, 96.9-102.3%, 98.6-103.1% and 95.6-105.2%, respectively. Each value is the mean of five determinations. The results were given in Table 16. These recovery values were within the range of acceptable % recovery. These recovery values indicate that no loss of analyte during the experiments as well as no interference with impurities occurred. However, hydroquinone can expose to light and air. Therefore, the slight lower recovery values

of hydroquinone might indicate a minor degradation of it most probably due to oxidation during the preparation. Recovery of retinoic acid was rather pool.

Table 16 Recovery (n=5) for each analyte added to laboratory-made cosmetic cream

Analyte	Spike (%w/w)	Found (%w/w)	Mean recovery (%) ± SD	Range (%)	RSD (%)
HQ	0.2249	0.2221	98.77±1.37	97.73-100.73	1.39
	0.4455	0.4356	97.78±0.97	96.18-98.76	0.99
	0.6746	0.6441	95.48±0.29	95.00-95.72	0.31
RA	0.0125	0.0137	109.28±0.44	108.80-109.60	0.40
	0.0250	0.0272	108.92±0.58	108.00-109.40	0.53
	0.0375	0.0404	107.57±0.42	106.93-108.00	0.39
BM	0.0500	0.0496	99.24±0.33	98.70-99.60	0.33
	0.0999	0.0989	99.03±0.73	98.35-100.15	0.74
	0.1498	0.1458	99.32±0.81	96.06-98.23	0.83
BMV	0.0217	0.0220	101.15±1.39	100.00-103.00	1.38
	0.0434	0.0438	100.99±1.24	100.00-102.88	1.22
	0.0651	0.0630	96.76±1.10	95.47-98.16	1.14
DM	0.0511	0.0512	100.27±0.18	100.10-100.49	0.17
	0.1021	0.1029	100.74±1.16	99.80-102.74	1.16
	0.1532	0.1528	99.75±0.64	98.83-100.52	0.61
HCA	0.0451	0.0450	99.69±0.63	99.22-100.78	0.63
	0.0901	0.0899	99.77±1.62	98.17-102.33	1.62
	0.1352	0.1319	97.54±0.42	96.89-98.08	0.43
PRL	0.0454	0.0462	101.74±0.87	100.88-102.97	0.86
	0.0909	0.0921	101.34±0.99	100.72-103.08	0.97
	0.1363	0.1376	100.04±0.68	98.64-100.95	0.86
TA	0.0450	0.0454	100.84±1.53	100.00-103.57	1.51
	0.0900	0.0910	101.16±2.33	99.67-105.22	2.30
	0.1350	0.1310	97.01±1.39	95.63-98.93	1.43

4. Precision

Calculated repeatability and intermediate precision values can be compared with those of existing methods. If there are no method with which to compare the precision parameters, theoretical relative reproducibility and repeatability standard deviations can be calculated from the Horwitz equation which is shown in Table 11. Hydroquinone was eluted well separate from the solvent front. The retention time observed (2.8 min) allows a rapid determination. The retention time of each analyte in different days is compared in Table 17, and this result is shown equilibration of it because of precision the retention time less than 2 percent RSD.

Table 17 Comparison the retention time of hydroquinone, retinoic acid and corticosteroids

Analyte	Retention time (min)					SD	% RSD
	Day 1	Day 2	Day 3	Day 4	Day 5		
HQ	2.82	2.80	2.82	2.78	2.79	0.02	0.64
RA	59.35	60.32	60.33	60.25	60.29	0.42	0.71
BM	26.02	25.45	25.87	25.26	25.10	0.39	1.54
BMV	47.55	47.19	47.46	47.09	47.19	0.20	0.42
DM	27.84	27.16	27.58	26.93	26.75	0.45	1.66
HCA	34.04	33.85	33.97	33.80	33.74	0.12	0.36
PRL	16.36	16.14	16.32	16.02	16.01	0.16	1.01
TA	33.43	33.19	33.34	33.13	33.06	0.15	0.46

4.1 Repeatability

Repeatability refers to the degree of agreement of results when conditions are maintained as constant as possible with the same analyst, reagents, equipment, and instruments performed within a short period of time. The repeatability of the method was determined by testing five independently sample in one level and was presented by % RSD. According to these results, the % RSD values were 1.48 at 0.56% w/w for hydroquinone; 1.81 at 0.03% w/w for retinoic acid; 0.84 at 0.12% w/w for betamethasone; 1.66 at 0.06% w/w for betamethasone 17-valerate; 1.23 at 0.12%

w/w for dexamethasone; 1.62 at 0.12% w/w for hydrocortisone acetate; 1.44 at 0.12% w/w for prednisolone and 1.50 at 0.11% w/w for triamcinolone acetonide. In these measurements, the standard deviations remained well within the recommended and accepted range the RSD of Horwitz. The relative standard deviations were always less than 5%. Overall, good repeatability was observed for all the analytes, because the results for each analyte were acceptable over the one day tested and that are summarized in Table 18.

4.2 Intermediate precision

The procedure was repeated on different days. Five spiked sample solutions were independently prepared and analyzed at each day for five consecutive days. Intermediate precision was calculated from data obtained from five different validation days. The relative standard deviations were below 5% and $p>0.05$, no significant difference was obtained from the p-value. The results of intermediate precision are summarized in Table 19 to Table 27.

Table 18 Repeatability evaluation of matrix blank spiked with hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide (n=5)

Analyte	Amount (% w/w)					% RSD
	No. 1	No. 2	No. 3	No. 4	No. 5	
HQ	0.5534	0.5744	0.5652	0.5571	0.5582	1.48
RA	0.0327	0.0331	0.0325	0.0319	0.0331	1.81
BM	0.1188	0.1198	0.1180	0.1171	0.1183	0.84
BMV	0.0593	0.0603	0.0596	0.0585	0.0578	1.66
DM	0.1241	0.1238	0.1203	0.1224	0.1230	1.23
HCA	0.1177	0.1169	0.1172	0.1148	0.1133	1.62
PRL	0.1190	0.1199	0.1198	0.1168	0.1163	1.44
TA	0.1157	0.1138	0.1145	0.1178	0.1123	1.50

Table 19 Intermediate precision evaluation of matrix blank spiked with hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide (n=5)

Analyte	Average amount (% w/w) (n=5)					% RSD	p
	Day 1	Day 2	Day 3	Day 4	Day 5		
HQ	0.5617	0.5584	0.5590	0.5582	0.5583	0.67	0.59
RA	0.0326	0.0328	0.0325	0.0323	0.0323	1.18	0.14
BM	0.1184	0.1187	0.1205	0.1187	0.1184	1.19	0.08
BMV	0.0591	0.0597	0.0589	0.0589	0.0590	1.16	0.33
DM	0.1227	0.1235	0.1241	0.1248	0.1240	1.00	0.07
HCA	0.1160	0.1179	0.1177	0.1181	0.1181	1.30	0.12
PRL	0.1183	0.1186	0.1175	0.1174	0.1183	0.91	0.28
TA	0.1148	0.1164	0.1152	0.1157	0.1154	1.21	0.48

Table 20 Intermediate precision for quantitation of hydroquinone in sample

Day	HQ, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.5534	0.5744	0.5652	0.5571	0.5582	0.5617	0.0083	1.4476	0.59
2	0.5554	0.5593	0.5598	0.5580	0.5596	0.5584	0.0018	0.3285	
3	0.5581	0.5592	0.5592	0.5596	0.5591	0.5590	0.0006	0.1015	
4	0.5581	0.5586	0.5577	0.5584	0.5585	0.5582	0.0004	0.0684	
5	0.5582	0.5589	0.5580	0.5583	0.5585	0.5583	0.0003	0.0589	

Table 21 Intermediate precision for quantitation of retinoic acid in sample

Day	RA, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.0327	0.0331	0.0325	0.0319	0.0331	0.0326	0.0005	1.5037	0.14
2	0.0332	0.0330	0.0324	0.0329	0.0326	0.0328	0.0003	0.9363	
3	0.0320	0.0324	0.0327	0.0329	0.0324	0.0325	0.0003	1.0307	
4	0.0321	0.0320	0.0320	0.0326	0.0328	0.0323	0.0004	1.1411	
5	0.0323	0.0322	0.0320	0.0327	0.0324	0.0323	0.0002	0.7343	

Table 22 Intermediate precision for quantitation of betamethasone in sample

Day	BM, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.1188	0.1198	0.1180	0.1171	0.1183	0.1184	0.0010	0.8438	0.08
2	0.1170	0.1165	0.1214	0.1195	0.1191	0.1187	0.0020	1.6735	
3	0.1184	0.1194	0.1207	0.1229	0.1214	0.1205	0.0017	1.4499	
4	0.1190	0.1185	0.1185	0.1186	0.1188	0.1187	0.0002	0.1827	
5	0.1183	0.1185	0.1183	0.1188	0.1183	0.1184	0.0002	0.1792	

Table 23 Intermediate precision for quantitation of betamethasone 17-valerate in sample

Day	BMV, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.0593	0.0603	0.0596	0.0585	0.058	0.0591	0.0010	1.6615	0.33
2	0.0600	0.0601	0.0604	0.0588	0.0591	0.0597	0.0007	1.1755	
3	0.0584	0.0587	0.0588	0.0599	0.0587	0.0589	0.0006	0.9656	
4	0.0590	0.0589	0.0597	0.0588	0.0580	0.0589	0.0006	1.0004	
5	0.0590	0.0590	0.0590	0.0585	0.0595	0.0590	0.0004	0.6305	

Table 24 Intermediate precision for quantitation of dexamethasone in sample

Day	DM, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.1241	0.1238	0.1203	0.1224	0.1230	0.1227	0.0015	1.2320	0.07
2	0.1240	0.1224	0.1239	0.1221	0.1250	0.1235	0.0012	0.9725	
3	0.1227	0.1225	0.1248	0.1257	0.1248	0.1241	0.0014	1.1605	
4	0.1247	0.1251	0.1245	0.1246	0.1253	0.1248	0.0003	0.2738	
5	0.1240	0.1242	0.1242	0.1244	0.1234	0.1240	0.0004	0.3243	

Table 25 Intermediate precision for quantitation of hydrocortisone acetate in sample

Day	HCA, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.1177	0.1169	0.1172	0.1148	0.1133	0.1160	0.0019	1.6212	0.12
2	0.1165	0.1184	0.1215	0.1171	0.116	0.1179	0.0022	1.8689	
3	0.1167	0.1165	0.1174	0.1188	0.1190	0.1177	0.0012	0.9808	
4	0.1181	0.1182	0.1186	0.1182	0.1175	0.1181	0.0004	0.3216	
5	0.1182	0.1182	0.1180	0.1180	0.1182	0.1181	0.0001	0.0868	

Table 26 Intermediate precision for quantitation of prednisolone in sample

Day	PRL, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.1190	0.1199	0.1198	0.1168	0.1163	0.1183	0.0017	1.4357	0.28
2	0.1182	0.1173	0.1201	0.1184	0.1193	0.1186	0.0010	0.8847	
3	0.1167	0.1165	0.1177	0.1184	0.1182	0.1175	0.0009	0.7239	
4	0.1177	0.1176	0.1183	0.1167	0.1170	0.1174	0.0006	0.5507	
5	0.1181	0.1179	0.1184	0.1179	0.1191	0.1183	0.0005	0.4196	

Table 27 Intermediate precision for quantitation of triamcinolone acetonide in sample

Day	TA, % w/w					Average	SD	%RSD	p
	No. 1	No. 2	No. 3	No. 4	No. 5	(%w/w)			
1	0.1157	0.1138	0.1145	0.1178	0.1123	0.1148	0.0021	1.8142	0.48
2	0.1182	0.1160	0.1170	0.1159	0.1150	0.1164	0.0012	1.0511	
3	0.1182	0.1141	0.1137	0.1157	0.1144	0.1152	0.0018	1.5748	
4	0.1154	0.1156	0.1165	0.1156	0.1152	0.1157	0.0005	0.4366	
5	0.1154	0.1157	0.1150	0.1145	0.1164	0.1154	0.0007	0.6163	

5. Limit of detection

The limit of detection is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantified as an exact value. LOD was determined by spiking ten samples at LOD concentrations and calculating signal-to-noise ratios (S/N). The signal-to-noise was calculated from the ratio between analyte peak signal to base line and peak-to-peak noise ratio. Values were still acceptable for all the analytes when the signal-to-noise ratios for spiked matrix blank were higher than three. The LOD values were 0.003% w/w for hydroquinone, 0.0001% w/w for retinoic acid, 0.0008% w/w for betamethasone, 0.0002% w/w for betamethasone 17-valerate, 0.0008% w/w for dexamethasone and 0.0004% w/w for hydrocortisone acetate, prednisolone and triamcinolone acetonide. The LODs and IDLs of all analytes ($S/N \geq 3$) are presented in Table 28.

Table 28 Limit of detection of all the analytes

Analyte	IDL (µg/mL)	LOD (% w/w)	S/N
HQ	0.60	0.003	13
RA	0.02	0.0001	51
BM	0.16	0.0008	13
BMV	0.04	0.0002	15
DM	0.16	0.0008	12
HCA	0.08	0.0004	22
PRL	0.08	0.0004	24
TA	0.08	0.0004	23

6. Limit of quantitation

The limit of quantitation of all analytes in matrix blank was the lowest concentration for which acceptable recovery and precision were obtained. The LOQ is always higher than the LOD. The LOQ values of the method at an S/N at least 10 were 0.0112% w/w for hydroquinone, 0.0004% w/w for retinoic acid, 0.0036% w/w for betamethasone, 0.0008% w/w for betamethasone 17-valerate, 0.0035% w/w for dexamethasone, 0.0017% w/w for hydrocortisone acetate, 0.0015% w/w for prednisolone and 0.0017% w/w for triamcinolone acetonide. These values of all analytes (S/N $\geq$ 10) are presented in Table 29.

Reactivities are highly variable among the analytes because of their very different structures.

Table 29 Limit of quantitation of all the analytes

Analyte	LOQ (μg/mL)	LOQ (% w/w)	Mean recovery ± SD (%)	Range (%)	% RSD	S/N
HQ	2.24	0.0112	93.89±0.54	92.66-94.69	0.58	33
RA	0.08	0.0004	96.70±0.39	96.29-97.61	0.41	109
BM	0.71	0.0036	95.83±0.23	95.42-96.16	0.24	37
BMV	0.16	0.0008	102.23±2.48	95.28-103.78	2.42	39
DM	0.70	0.0035	101.79±0.34	101.23-102.22	0.34	36
HCA	0.33	0.0017	99.43±0.98	98.50-101.88	0.99	38
PRL	0.30	0.0015	92.04±0.52	91.44-93.10	0.56	57
TA	0.33	0.0017	97.33±2.32	97.70-100.55	2.38	45

7. Measurement uncertainty

The measurement uncertainty (MU) was estimated by taking into account precision and recovery. For calculation of the expanded uncertainty a safety factor is needed. It must be reported with the quantitative result of the measurand because it provides valuable in formation about the quantitative result of the measurand.

The value of the measurement result from the assay is not the true value but rather an estimate of the value determined by the assay. The uncertainty of the value is a combination of uncertainties from the analytical procedure, the reference standard and the sample. In this study, the estimated MU at 95% confidence was less than 10%. The results are presented in Table 30, and raw data are shown in Appendix C, Table 43 to Table 50). The expanded relative measurement uncertainty was higher in hydroquinone than in the other analytes.

Table 30 Measurement uncertainty of all the analytes

Analyte	Amount (% w/w)	Expanded measurement uncertainty (% w/w)	Expanded relative measurement uncertainty
HQ	0.553	0.049	8.86
RA	0.033	0.001	3.03
BM	0.119	0.004	3.36
BMV	0.059	0.002	3.39
DM	0.124	0.004	3.23
HCA	0.118	0.004	3.39
PRL	0.119	0.007	5.88
TA	0.116	0.005	4.31

The application of optimized method in cosmetic samples

This successful method simultaneous determination hydroquinone, retinoic acid and corticosteroids using HPLC-MS was applied to cosmetic samples. The ten samples collected from Bureau of Cosmetics and Harzardous Substances, Department of Medical Sciences. We wanted to compare the new method with the routine method.

When analyzing cosmetic samples with the described HPLC-MS equipment and conditions, a different retention times were found when the mixture standard was analyzed. These retention times, from extracted ion chromatograms (EIC), gave values less than 10% RPD. However, these results were considered acceptable. Ten samples were analyzed for prohibited substances by using optimize HPLC-MS condition. It was found that five samples positive of prohibited substances, e.g., hydroquinone in two samples, retinoic acid in two samples and triamcinolone acetonide in one sample. The concentrations of hydroquinone were 0.51% w/w and 0.60% w/w. The concentrations of retinoic acid were 0.10% w/w and 0.10% w/w. The amount of triamcinolone acetonide was found in one sample, and the amount of triamcinolone acetonide was 0.028% w/w. Figure 34 shows chromatogram of cosmetic samples that found hydroquinone, retinoic acid and triamcinolone acetonide. Ten samples were also identify for prohibited substances by using TLC method of Bureau of Cosmetics and Hazardous Substances (DMSc SOP 02 002 [67], and SOP 06 02 186 [68]). The

results are listed in Table 31 to Table 32. LOD of hydroquinone, retinoic acid and corticosteroids using HPLC-MS have been lower than TLC and HPLC. However, the results for TLC and HPLC methods were consistent with new method.

Table 31 Analysis of hydroquinone, retinoic acid and corticosteroids in cosmetic samples using HPLC-MS

Analyte	Sample (% w/w)										LOD
	1	2	3	4	5	6	7	8	9	10	(%w/w)
HQ	0.51	0.60	ND	ND	ND	ND	ND	ND	ND	ND	0.003
RA	ND	ND	0.10	0.10	ND	ND	ND	ND	ND	ND	0.0001
BM	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0008
BMV	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0002
DM	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0008
HCA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0004
PRL	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0004
TA	ND	ND	ND	ND	0.028	ND	ND	ND	ND	ND	0.0004

ND = Not detected

Table 32 Analysis of hydroquinone, retinoic acid and corticosteroids in cosmetic samples using TLC and HPLC

Analyte	Sample										LOD
	1	2	3	4	5	6	7	8	9	10	(% w/w)
HQ	+	+	ND	ND	-	-	-	-	-	-	0.02
RA	ND	ND	+	+	-	-	-	-	-	-	0.02
BM	-	-	-	-	-	-	-	-	-	-	0.02
BMV	-	-	-	-	-	-	-	-	-	-	0.02
DM	-	-	-	-	-	-	-	-	-	-	0.02
HCA	-	-	-	-	-	-	-	-	-	-	0.02
PRL	-	-	-	-	-	-	-	-	-	-	0.02
TA	-	-	-	-	+	-	-	-	-	-	0.02

+ = Positive

ND = Not detected

- = Not tested

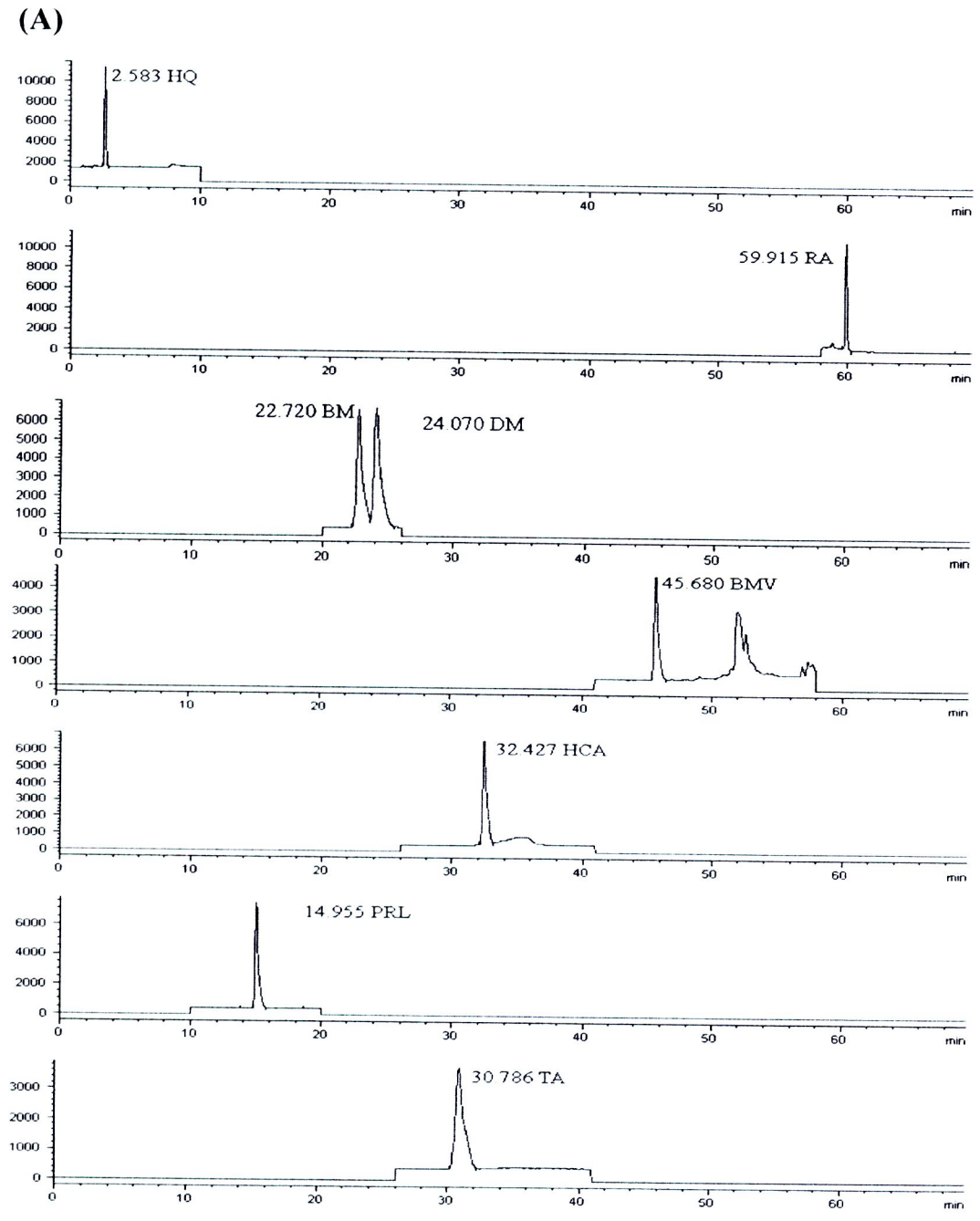


Figure 34 HPLC-ESI-MS EIC chromatograms of standard solution (A), matrix blank (B) and skin-whitening cosmetic samples (C-E), respectively

(B)

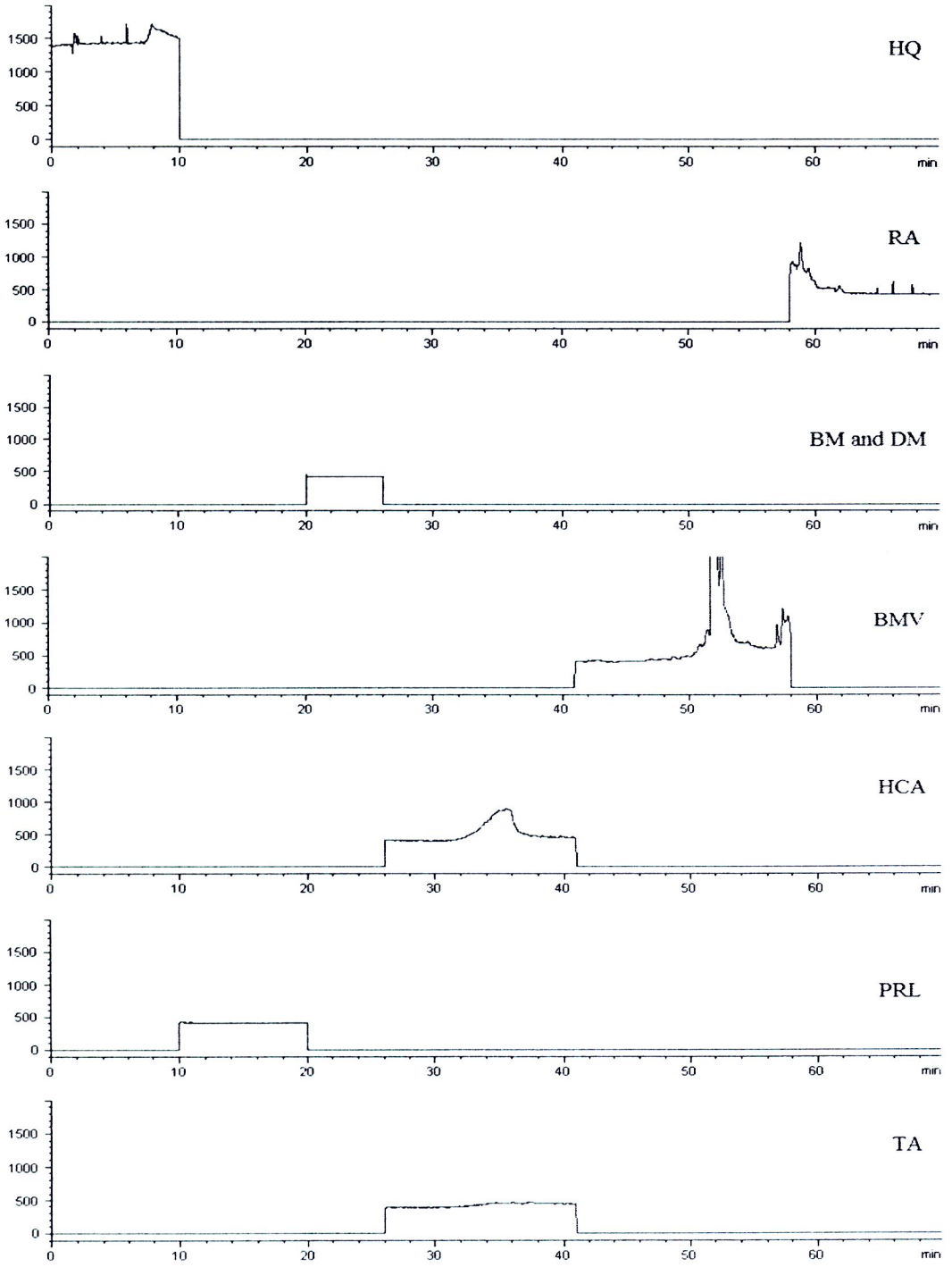
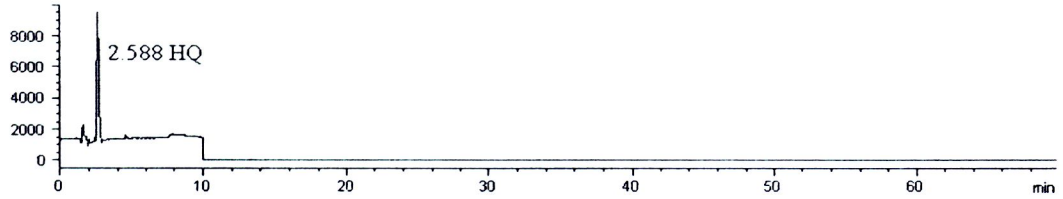
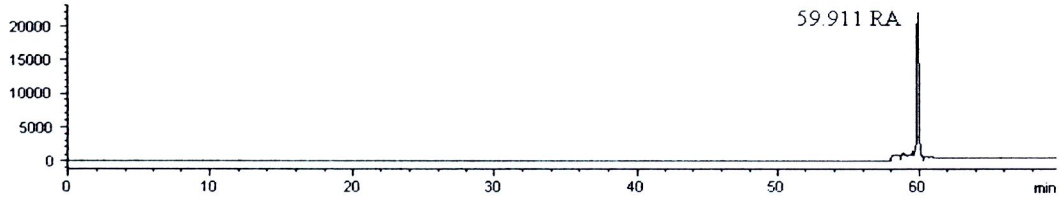


Figure 34 (Cont.)

(C)



(D)



(E)

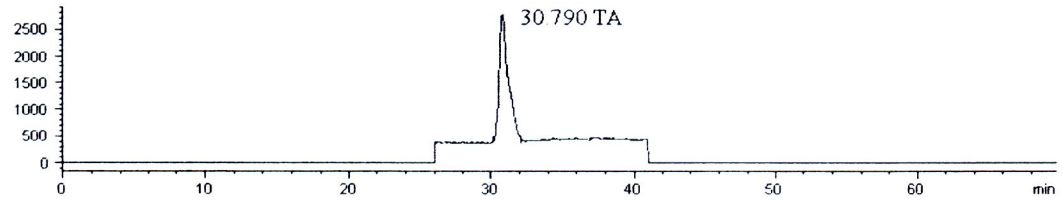


Figure 34 (Cont.)

Table 33 Pros and Cons of TLC and HPLC-MS techniques are compared to the instrumentation required

Parameter	TLC	HPLC-MS
Cost	800 baht/test/sample	2000 baht/test/sample
Time-consuming	4 days/10 samples	2 days/10 samples
Sensitivity	Low	High
Selectivity	Low	High
Resolution	Poor	Good
Litigation	Low efficiency	More efficiency
Instrument	Manual	Automatic
Sample	Too much of sample	Small of sample
Analysis	Qualitatiive analysis	Quantitative analysis