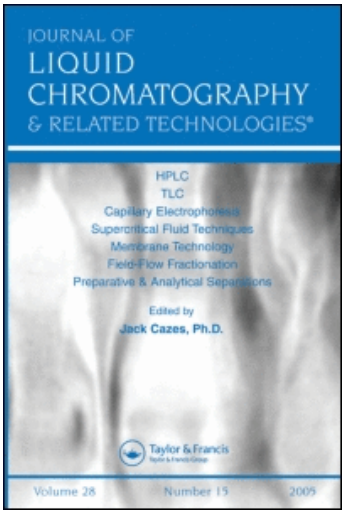

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SENSITIVE DETECTION AT LOW-WAVELENGTH FOR METHANOL GRADIENT ELUTION IN REVERSED-PHASE CHROMATOGRAPHY

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ABSTRACT

The use of a water-methanol gradient in conjunction with UV detection at low-wavelength in reversed-phase liquid chromatography was studied. In order to correct the baseline drift, formamide was added to the initial eluent. By using this initial eluent, gradient elution with water-methanol system could be performed at four times greater sensitivity. Sodium nitrate appeared to function similarly to formamide in enhancing the sensitivity. A wide selection of double wavelength detection was made possible by the simultaneous addition of formamide. This method seems to be convenient and practical since it allows the addition of acid and inorganic salt to the adjusted mobile phase. The simultaneous determination of 7 medical materials in cosmetics was carried out with this method, and satisfactory results were obtained both in the recovery and the variation coefficient.

INTRODUCTION

Gradient elution has been used in the field of modern high performance liquid chromatography (HPLC).

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This technique has the following advantages in overcoming general elution problems in HPLC (1). 1) Total analysis time can be significantly reduced. 2) Effective sensitivity becomes very high because of a negligible variation in the peak shape. 3) Elution and separation of multiple components having a wide difference in polarity can be accomplished simultaneously.

"General detection" of most of the organic compounds is carried out by UV absorption at low-wavelength near 210 nm. However, such a gradient technique at 210 nm can cause drastic baseline drifts. The elution technique of a methanol gradient combined with low-wavelength detection has been rarely reported because the absorption of methanol begins in that region. With methanol, therefore, a narrow gradient and low sensitive detection must usually be employed. V. Berry (2) has presented a sensitive gradient technique at low-wavelength using acetonitrile. In this system, detection at 210 nm with a sensitivity of 0.1 a.u.f.s. becomes feasible, baseline drift is less than 5%, and no ghost peaks appear. With his method, three problems related to methanol as eluent, namely, a mid-gradient hump, UV mismatch, and ghost peaks have been solved.

Otherwise, methanol is widely used because of its low price and toxity. The UV absorbance mismatch between initial and final eluents in the water-methanol system is greater than that in the water-acetonitrile system, because methanol

has an absorption at 183 nm. This paper shows how the addition of formamide to the initial eluent of the methanol gradient makes possible detection at 214 and 205 nm. This method can be performed conveniently and practically since it allows the addition of acid and inorganic salt to the adjusted eluents. All chromatographic profiles were obtained at high sensitivity with this method, and we show its application to the simultaneous determination of multiple medical materials in cosmetics.

EXPERIMENTAL

Reagents. Nine reagents in a series of phenones from acetophenone to tetradecanophenone were purchased from Tokyo Kasei (Tokyo, Japan). Methyl p-hydroxybenzoate, salicylic acid, butylated hydroxytoluene were purchased from Wako Pure Chemical (Osaka, Japan). Monoammonium glycyrrhizinate, tocopheryl acetate and diphenhydramine hydrochloride were purchased from Tokyo Kasei. Pantothenyl ethyl ether was purchased from Daiichi-Seiyaku (Tokyo, Japan). All these compounds were guaranteed reagent grade and were used without further purification. Methanol for HPLC (absorbance at 210 nm was 0.70 a.u. maximum and at 220 nm was 0.30 a.u. maximum) was purchased from Wako Pure Chemical and water was obtained from a Milli-R/Q-Reagent-Grade water system (Millipore, Bedford, MA, U.S.A.). Formamide and phosphoric acid were purchased from Wako Pure

Chemical and sodium perchlorate was purchased from Kanto Chemical (Tokyo, Japan).

Apparatus. The spectrophotometer used in this work was UVIDEC-610 (Japan Spectroscopic, Tokyo, Japan). The HPLC equipment consisted of two Waters 6000A pumps (Waters Assoc., Milford, MA, U.S.A.), a Waters 720 System Controller, a Waters U6K septumless loop injector, a Waters 441 UV detector and a Japan Spectroscopic UVIDEC100-2 variable wavelength UV detector. A column (6 mm id x 200 mm) packed with TSK-LS-410 (5μ) (Toyo Soda, Tokyo, Japan) was used and this column was preceded by a Brownlee Labs guard column (Rheodyne, Berkley, CA, U.S.A.).

Procedures. Methanol was allowed to stand in contact with air at room temperature for at least one day after cutting the seal. With gradients from 25 to 100% methanol, the initial eluent, a mixture of water and methanol (75/25) was first degassed by vacuum under ultrasonic waves. The flow rate was set at 1.5 ml/min and column temperature was maintained at 40°C with circulating warm water. It took 5.1 minutes to detect the eluent passed through the column after mixing two eluents. Therefore, the final eluent was held for 10 minutes after which the system controller indicated the end of gradient, by considering the equilibrium of the final eluent in the detector cell. The concentration of methanol added to each phenone was 100 μg/ml, and 15 μl of the mixture was injected. Table 1

TABLE 1. The symbols of medical materials in cosmetics

Ⓐ	Pantothenyl ethyl ether	■
Ⓑ	Methyl p-hydroxybenzoate	△
Ⓒ	Salicylic acid	○
Ⓓ	Diphenhydramine hydrochloride	□
Ⓔ	Butylated hydroxyanisol	◎
Ⓕ	Monoammonium glycyrrhizinate	▣
Ⓖ	Butylated hydroxytoluene	●
Ⓗ	Tocopheryl acetate	▲

shows the medical materials in cosmetics used in this work. The monoammonium glycyrrhizinate was dissolved in a mixture of ethanol and water (50/50) and the other 7 materials were dissolved in 95% ethanol at various concentrations. The injection volume was 20 μl.

RESULTS AND DISCUSSION

Methanol gradient at low-wavelength detection.

The UV mismatch on the detection at 214 nm was about 0.35 a.u. through the gradient from water to methanol. The baseline was concave upward against the linear gradient. The UV mismatch was about 0.02 a.u. through the gradient from water to 25% methanol, therefore, the analysis could be performed sufficiently at 214 nm with a sensitivity of 0.1 a.u.f.s.. It is clear that the UV mismatch takes place more than 25% methanol in the gradient. Therefore, at higher concentrations of methanol than 25% the UV mismatch must be corrected.

The correction method is discussed in the following section.

Selection of the additive to correct the baseline.

In general, the baseline drift caused by UV mismatch is diminished by the addition of another compound to the initial eluent. Such a compound must absorb light at the wavelength of the detector. The following points seem to be required by such an additive. First, the additive must be soluble in water and it must not affect any characteristics of mobile phase. Preferably the maximum absorption of the additive appears in the vicinity of the detecting region and its molar extinction coefficient is large. Second, the additive should not be retained on the column, and it should not slow down recovery time from the final eluent to the initial eluent. Acetone is one of the representative additives having a detection region of 229 to 254 nm. However, 60 ml of acetone was required in 1 L of the initial eluent to match the absorbance of the initial eluent (water : methanol = 75 : 25) and the final eluent (methanol) at 214 nm detection, which is too much to avoid change in the characteristics of the mobile phase. Taking into account the various requirements described above, formamide was chosen from the typical LC solvents (3-4). Fig.1 shows the differential UV absorption between the final eluent and the initial

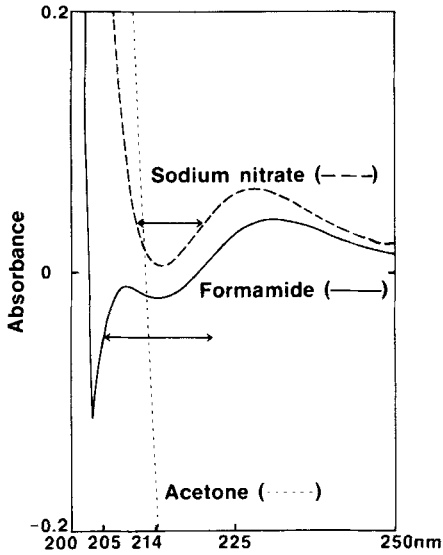

Fig.1 The differential UV absorption between the adjusted initial eluent and the final eluent at 214 nm. Sample side, the final eluent (methanol); reference side, the initial eluent (water:methanol=75:25) containing the additive;

eluent which was adjusted to near zero absorbance by addition of various additives at 214 nm. It is shown that the wavelength region is thereby expanded to a range of 205 to 222 nm for the permitted limit of ± 0.032 in UV absorption. The required volume of formamide per 1 L of the initial eluent was 125 μl. Sodium nitrate (5) which was used for the measurement of t_0 (void volume) was shown to be a good additive. It added at a rate of 340 μl of 1% aqueous solution per 1 L of the initial eluent. Fig.1 shows that the wavelength region is thereby expanded to a range of 211 to 221 nm for

the same permitted limit. The absorbance does not have to be matched precisely, because the absorbance of methanol is rising gradually in this range.

The effect of the gradient rate on the baseline.

Fig.2 shows the effect of the gradient rate on the baseline of a linear gradient of 25 to 100% methanol after matching the absorbance of the initial and final eluent. The absorption of formamide decreases linearly, whereas that of methanol increases concavely during the compositional change from the initial to final eluent, respectively. Therefore, the baselines at both wavelengths are concave. The curvature of the baseline increases remarkably with an increasing gradient rate. At a gradient rate of 5%/min or above was very the nonlinearity pronounced. The 2-component mobile phase does not seem to be at equilibrium in the detector cell at higher gradient rates. Additionary, a pronounced deviation of absorbance was observed at a methanol concentration of more than 75%. This is probably due to the interaction (6~7) of methanol molecules with residual oxygen molecules. From these results, the gradient rate has to be less than 2.5%/min to stabilize the baseline.

Reproducibility of peak heights for the quantitative analysis. It was shown that the use of formamide as an additive for baseline correction makes possible the use of a methanol gradient at low-wavelengths (214, 205 nm) and at high detector sensitivity (0.16 a.u.f.s.).

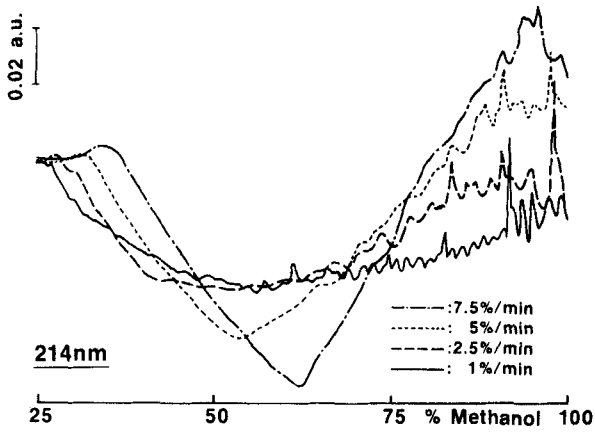

Fig.2 Effect of the gradient rate on the baseline.

Furthermore, elution profiles characteristic of multi component samples were obtained. To investigate the possibility of quantitative analysis, the reproducibility of peak height was further examined under these conditions. Following the suggestion of E.J.Kikta and A.E.Stange (8), phenones were employed as internal standard compounds with a wide range in polarity. Fig.3 shows the gradient elution profiles for 9 phenones at 214 and 205 nm detection. The variation coefficients of the peak heights of each component are summarized in Table 2. The variation coefficients of overscaled peaks should be at lower values, because ghost peaks appeared and baseline drift increased at methanol concentrations over 75%. The variation coefficients of peak heights were below 2%, except for the two highest phenones (n=10, 14). Therefore, quantitative analysis

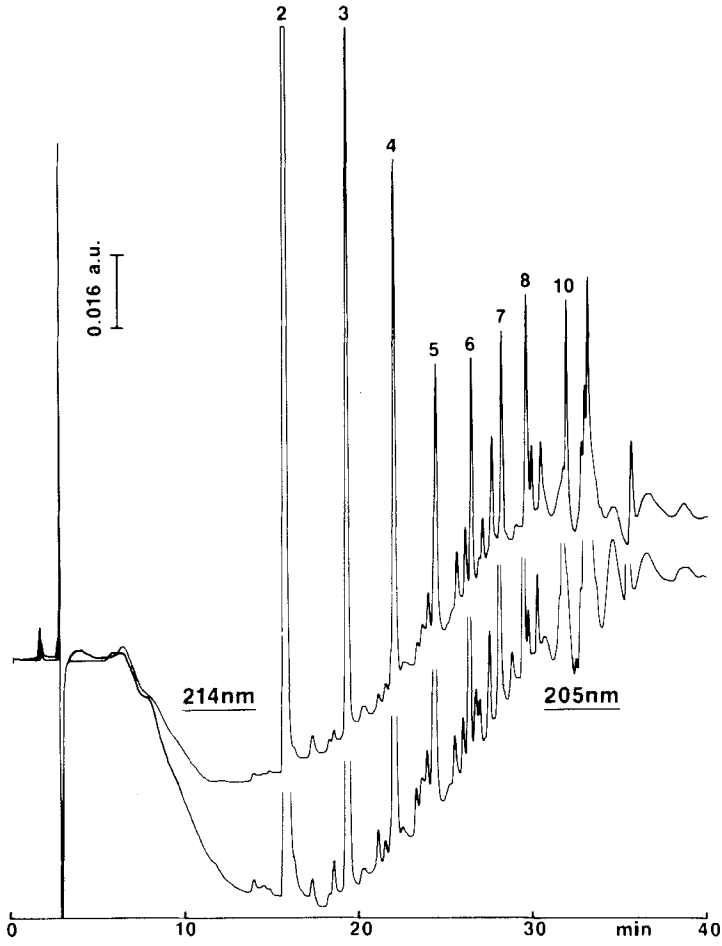

Fig.3 Gradient elution profile of 9 phenones. Mobile phase, 30 min linear gradient starting with a mixture of water:methanol=75:25, adjusted by formamide, and ending with methanol at a rate of 2.5%/min followed by 10 min of methanol. Sample size, 15 µl of each 100 µg/ml of 9 phenones; n = carbon side chain length for various phenones (for instance, n = 2 shows acetophenone).

TABLE 2. Variation coefficients of the peak heights of phenones

n	Detection wavelength	
	214 nm	205 nm
4	0.4	—
5	0.7	0.6
6	1.7	0.3
7	0.6	0.7
8	1.5	1.2
10	5.7	8.0
14	19.6	5.4 (%)

The reproducibility test was effected on repeating five times. n = carbon side chain length for various phenones.

seems possible within these limits. The reproducibility of peak height at 205 nm was more advantageous than that at 214 nm. This is probably because the sensitivity of phenone at 205 nm is higher than that at 214 nm. The solvents used for the mobile phase must be thoroughly purified for accurate analyses (9-10).

Application to the simultaneous determination of multiple medical materials in cosmetics. In general, various medical materials are contained in cosmetics and their amount is very small. Their solubilities differ from each other. Some materials are soluble in water and others are soluble in oil. It is convenient to apply the just described method to the simultaneous determination of multiple medical materials in cosmetics. Fig.4 shows the gradient elution profile for typical

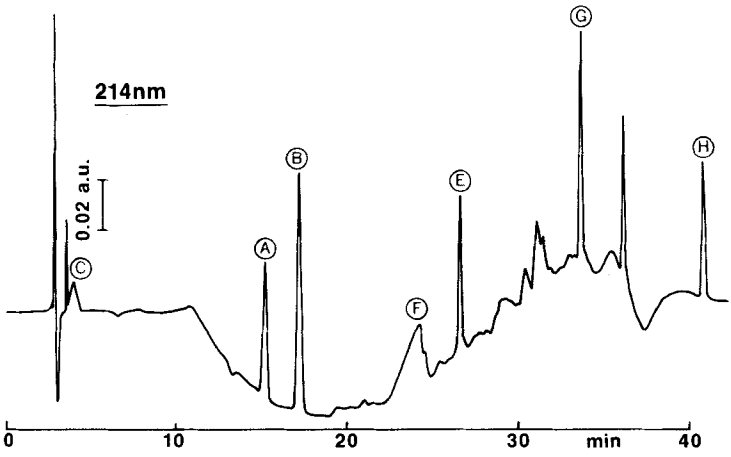

Fig.4 Gradient elution profile of typical medical materials.
Sample size, 20 μl of each concentration is Ⓐ : 300 μg/ml, Ⓑ : 20 μg/ml, Ⓒ : 10 μg/ml, Ⓓ : 20 μg/ml, Ⓔ : 20 μg/ml, Ⓕ : 750 μg/ml, Ⓖ : 20 μg/ml, Ⓗ : 25 μg/ml; the other conditions are the same as in Fig.3.

medical materials in cosmetics. The sample consists of 8 components. In this case, it is difficult to accomplish the elution and the separation by means of only the water-methanol eluent system. Actually, salicylic acid was hardly retained, monoammonium glycyrrhizinate did not show a sharp peak, and diphenhydramine hydrochloride was not eluted at all because of its adsorption on the column. Next, the addition of an acid and an inorganic salt into the mobile phase containing the corrective additive was attempted. Phosphoric acid was used since it has no absorption at low wavelength. This acid was added to the corrected initial eluent and the solution

was adjusted to pH 2.5. Taking into account the recovery time from the final eluent to the initial eluent, phosphoric acid was added to the final eluent at the rate of 1 ml per L. Sodium perchlorate was selected since it was very soluble in methanol. When a large amount of sodium perchlorate was contained in the eluent, an additional absorption appeared at low-wavelength. Therefore, the same amount of this salt was added into the initial and the final eluents. Fig.5 shows the separation of 8 components as mentioned above. Clearly, good elution and separation of the 8 components were obtained. With a mixture containing only phosphoric acid, the peak shape of monoammonium glycyrrhizinate was improved, salicylic acid and diphenhydramine hydrochloride were retained, but the two components were not separated. The addition of acetic acid allows to correct the baseline and to make an acidic mobile phase. In this case, the mobile phase has to be strictly pH 3.5 at 214 nm detection. Fig.5(b) shows the gradient profile for 8 components obtained with initial eluent without formamide. The difference between Fig.5(a) and (b) clearly shows the effect of a corrective additive. In Fig.5(b), it is difficult to determine and identify the trace peaks because of a sudden rise of the baseline. When 0.5 mol sodium perchlorate was added to the eluent, unknown peaks appeared at methanol concentrations over 75%. However, such peaks did not appear on the addition

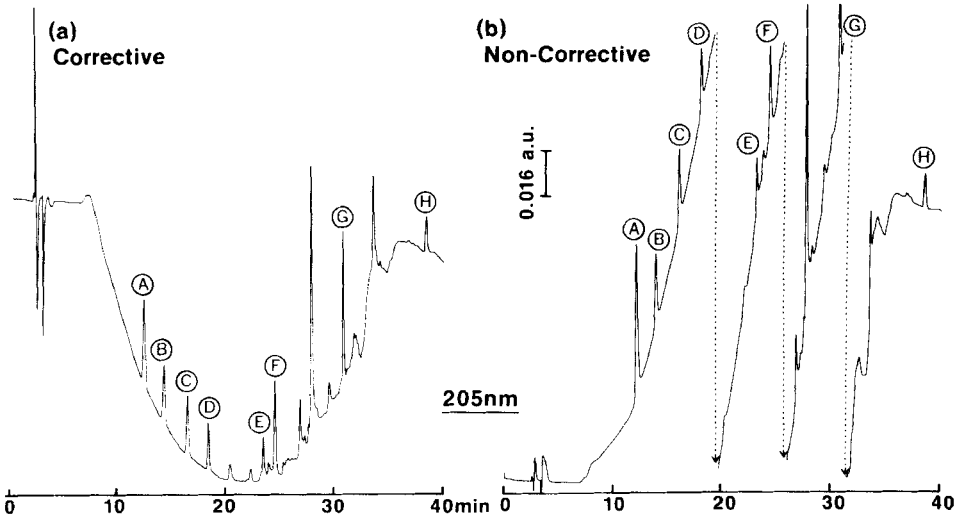

Fig.5 Separation of typical medical materials used in cosmetics.
Mobile phase, (a): 30 min linear gradient starting with 0.1 mol sodium perchlorate (water:methanol: phosphoric acid:formamide=750:250:5:0.125), and ending with 0.1 mol sodium perchlorate (methanol: phosphoric acid=1000:1) followed by 10 min of the final eluent, (b) excepts formamide from the initial eluent, and the other conditions are the same as in (a). Sample size, 20 µl of each concentration is Ⓐ : 100 µg/ml, Ⓑ : 5 µg/ml, Ⓒ : 2.5 µg/ml, Ⓓ : 5 µg/ml, Ⓔ : 5 µg/ml, Ⓕ : 250 µg/ml, Ⓖ : 5 µg/ml, Ⓗ : 5 µg/ml;

of 0.1 mol sodium perchlorate. Further, the baseline drift was reduced by the addition of inorganic salt. This eluent system was chosen for the final analysis. Fig.6 shows the calibration curves of 8 components under these conditions. Each calibration curve had good linearity and the correlation coefficients were greater than 0.998 at 214 nm and greater than 0.997 at 205 nm. The analysis required 55 minutes including

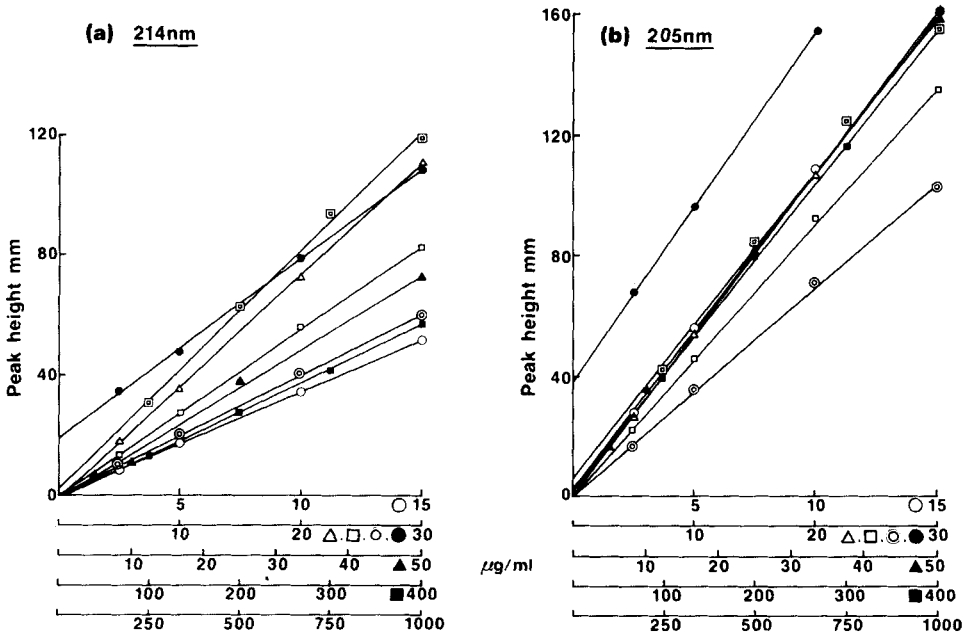

Fig.6 Calibration curves of 8 components for medical materials.
Sample size, 20 μl of mixed solution for various concentrations; the other conditions are the same as in Fig.5(a);

the recovery time of 15 minutes from the final eluent to the initial eluent. With manual injection, it seems to be difficult to complete all analyses in one day. The operation was repeated using new solvents under the same conditions. Good linearity was again observed on these data and the correlation coefficient was lower by 0.01 at both wavelengths. Only the calibration curve of butylated hydroxytoluene had a positive Y-intercept, and this was the case because the elution of butylated

TABLE 3. Recovery test from a known sample

Sample	Added (ug/5g)	No.1	No.2	No.3	No.4	No.5	Recovery(%)	c.v.(%)
Ⓐ	2500	2505	2518	2544	2531	2531	101.2	0.8
		2491	2594	2526	2526	2526	101.3	1.5
Ⓒ	75	80.7	83.5	79.7	81.6	82.6	108.8	1.8
		81.3	82.8	78.4	81.3	81.3	108.1	2.0
Ⓓ	150	158.1	162.5	158.1	157.0	161.4	106.3	1.5
		160.5	164.2	158.7	158.7	164.2	107.5	1.7
Ⓔ	150	150.2	153.1	150.2	151.7	148.8	100.5	1.1
		148.4	150.9	148.4	148.4	145.9	98.9	1.2
Ⓕ	5000	5085	5085	5052	5085	4987	101.2	0.8
		5152	5109	5067	5067	5109	102.0	0.7
Ⓖ	150	165.7	153.6	151.9	151.9	153.6	103.6	3.8
		168.8	152.0	150.3	148.6	148.6	102.4	5.6
Ⓗ	250	245.8	247.4	244.2	250.6	241.0	98.3	1.5
		———	260.5	253.8	263.8	250.4	102.9	2.4

c.v. : variation coefficient;
up line : 205 nm detection / down line : 214 nm detection;

hydroxytoluene took place on a ghost peak. The retention time and the peak height of this ghost peak were reproducible, and good linearity was obtained for the calibration curve consequently. The medical materials were added to a known lotion at a certain concentration and the recovery test was carried out with these calibration curves. Table 3 shows the result of the recovery test for 7 components. The result of methyl p-hydroxybenzoate was left out of the table since it was included in other cosmetic materials. Satisfactry results were obtained for both the recoveries and the coefficients of variation except for butylated hydroxytoluene. However, many practical problems will arise with the simultaneous deter-

mination of multiple components. For instance, in the case of extraction from oil rich cosmetics, effective extraction will be required for all components. So, simultaneous determination will be difficult for extreme differences of component ratios or response ratios. This problem may be overcome by improving the UV detector of the HPLC system.

CONCLUSION

The addition of a corrective additive to the initial eluent has proved to be very effective for stabilizing the baseline. This technique made possible the use of the methanol gradient technique with sensitive low-wavelength detection. Especially formamide was a good corrective additive. This method could be applied to the simultaneous determination of multiple micro components of cosmetics. The sensitivity of the analysis could be enhanced by more than four times by the addition of formamide into the initial eluent. It was possible to add acid and inorganic salt to the adjusted eluent. Double wavelength detection could be performed simultaneously on the basis of the exactly measured absorption of formamide. This analysis makes possible the detection of trace components like antiseptics contained in raw materials for cosmetics. This method should also be useful for profile analyses of natural medicines which consist of complex mixtures.

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