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간접 분광광도 측정에 의한 비발색단의 액체크로마토그래피

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Liquid Chromatography of Non-Chromophores by Indirectly Photometric Detection

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요 약. 역상 액체크로마토그래피에 의한 지방족 알코올, 케톤 및 에스테르를 분리하는데 검출 시약으로 brilliant green에 관하여 연구하였다. 발색단을 가지고 있지 않은 이들 시료들을 brilliant green을 사용함으로서 가시영역인 625 nm에서 분광광도 검출기로서 간접적으로 검출할 수 있었다. 또한 몇가지의 혼합시료들을 좋은 분리도와 비교적 높은 감도로서 분리 및 검출할 수 있었다.

ABSTRACT. Brilliant green was examined as a detection reagent in the separation of some aliphatic alcohols, ketones and esters by the reverse-phase liquid chromatography. These samples not possessing chromophore could be indirectly detected by photometric detector with the assistance of brilliant green in visible range (625nm). Some mixtures of samples were also able to separated and detected with good base line resolution and comparatively high sensitivity.

INTRODUTION

A major instrumentation requirement in modern liquid chromatography is a sensitive detector for continuously monitoring the column effluent. The most widely used detectors in high performance liquid chromatography are refractometer based on the difference in refractive index between pure reference mobile phase and the column effluent and photometer based on UV-visible absorption. These detectors have each some advantages and limitations. The RI detector responses to all solutes but its sensitivity is comparatively lower than other detectors. The UV-visible detector has a high sensitivity for many solutes but non-absorbing compounds in sample are not detected.

In 1973, G. Schill *et al*[1]., introduced first that ionizable species could be separated using ion-pair chromatographic method. In this method, even non-UV detector absorbing species could be responsed on UV detector by the addition of a UV absorbing compound as ion-pair reagent to the eluent. Thereafter, there have appeared a number of papers on the determination of non-absorbing organic or inorganic species using ion-pair chromatography systems[2~6].

DiNunsio and Freiser[7] extended this idea to

visible range, using brilliant green as the stationary phase and hexane–dichloromethane as eluent in normal-phase chromatography and were able to separate aliphatic acids with higher sensitivity at 630nm wavelength. Gnanasambandan and Freiser[8,9] were also able to detect a series of aliphatic alcohols and sugars not possessing chromophores in visible range at submicrogram levels using methylene blue dye as eluent in reverse-phase chromatography.

This paper describes which demonstrate that non-absorbing aliphatic alcohols, ketones and esters can also be indirectly detected with dye assisted chromatographic technique. The dye used in this work was the hydrogen sulfate salt of brilliant green that absorbs strongly invisible range.

EXPERIMENTAL

Materials. The aliphatic alcohols, ketones and esters used in this study were obtained from Sigma Chemical Co., B. D. H Aldrich and Tokyo Kassei. Double distillated and deionized water was degassed under vacuumat 50 °C. Reagent grade methanol was used without further purification. Brilliant green bisulfate(Eastman, dye content 96%) was used after purification from ethanol–ether.

Apparatus. A high performance liquid chromatograph consisting of Pye-Unicam, dual pump (PU 4010), a variable wavelength, UV-visible detector (PU 4020), a 20μm loop rotary valve injector and 10mv strip chart recorder(Philips, PM 8251) or CDP 4 computing recorder was used.

The column used was a 25cm×4.6mm, Partisil 10 ODS.

The mobile phase used in this study consisted of mixtures of methanol–water(from 10 to 50V/V%) containing 0.1m*M* brilliant green. The pH of mobile phase ranged from 4.2 to 4.4. Before using, they are filtered through a 0.45 μm millipore filter membrane.

Procedure. The column was thermostated at 30±1 °C with a column jacket and a Haake FE temperature controller. The average flow rate was maintained at 1.0m*l*/min.

Before injecting the samples, the column was equilibrated with mobile phase containing brilliant green by passing mobile phase through the column until the effluent had same absorbance as that of original mobile phase. When the pateau or background level absorbance was achieved, samples were injected.

The Separation behavior of the analytes were characterized by the capacity factor. The capacity factor were calculated by using equation, $k' = (V_r - V_0)/V_0$ where V_r is the apparent retention volume of a solute and V_0 is the void volume of column.

RESULTS AND DISCUSSION

A considerable amount of work has been reported the aggregation organic dyes[10~12]. In generally, the solution of dye in water does not obey Beer's law and large deviations from the law are observed. According to the spectrophotometric studies for many classes of dye, the molar extinction coefficient in aqueous solution becomes weaker as concentration increases.

This phenomena is attributed to aggregation of the dye molecules to form dimers and higher polymers. In the other hand, it is found that the molar extinction coefficient of dyes in organic solvent is larger than in aqueous solution, because the dyes are dissociated to monomers from dimers or higher polymers by the formation of adduct between dye molecules and organic solvent molecules.

Gnanasambandan and Freiser[8,9] have already demonstrated that non-chomophores aliphatic alcohols could be detected with remarkable sen-

sitivity at 651nm by use of methylene blue dye as eluent. They proposed that dye and alcohol in effluent forms an adduct whose absorptivity increased larger than the inherent molar absorptivity of the original mobile phase.

In this study, the determination of some aliphatic alcohols, ketones and esters based on this principle is attempted.

At first, the spectra was investigated to find the molar extinction coefficient (ε_{max}) and maximum absorption wavelength (λ_{max}) of brilliant green dye. λ_{max} was 625 nm in visible range and ε_{max} was changed according to the concentration of dye. ε_{max} was 4.32×10^4 *L*. $mol^{-1}cm^{-1}$ at 0.075mM dye in pure aqueous solution, but this value decreased with increasing of the concentration of dye since the dye molecules are aggregated to form dimers or higher polymers. Also, the molar extinction coefficients were determined in various organic aqueous mixture solvent. As can be seen in *Table* 1, ε_{max} increased as the percentage of organic solvents increased. As being already explained at the above, this phenomena shows that the dye shifts from dimer to monomer.

Therefore, even though samples have no chromophore, they can be detected indirectly since the absorbance of mobile phase increases when sample molecules coelute with mobile phase. *Fig.* 1 shows the chromatogram of *t*-butanol and *n*-pentanol. The two peaks are very sharp and sensitive. The peak height of *n*-pentanol is larger than that of *t*-butanol. This is because more hydrophobic molecule has larger ε_{max} than less hydrophobic molecule as can be seen in *Table* 1. In this study, the concentration of brilliant green fixed to 0.1 mM because the sensitivity of detector was weaker at lower concentration and, at higher concentration than 0.1 m*M*, the background absorbance to detector signal was too high to offset electrically.

Table 1. Molar extinction coefficient of brilliant green in various organic-water solvent

Organic solvent	Molar extinction coefficient ($\varepsilon_{max}\times10^4$)		
	10%	20%	30%
Methanol	6.28	7.97	9.37
Ethanol	6.49	8.58	9.97
Propanol	7.17	9.88	10.2
Acetone	6.98	8.99	9.85

Detection: 625 nm. Concentration of Brilliant green: $7.5\times10^{-5}M$. ε_{max} in Aqueous Solution: 4.32×10^4 *L*. $mol^{-1}cm^{-1}$. Length of Cell: 0.200cm.

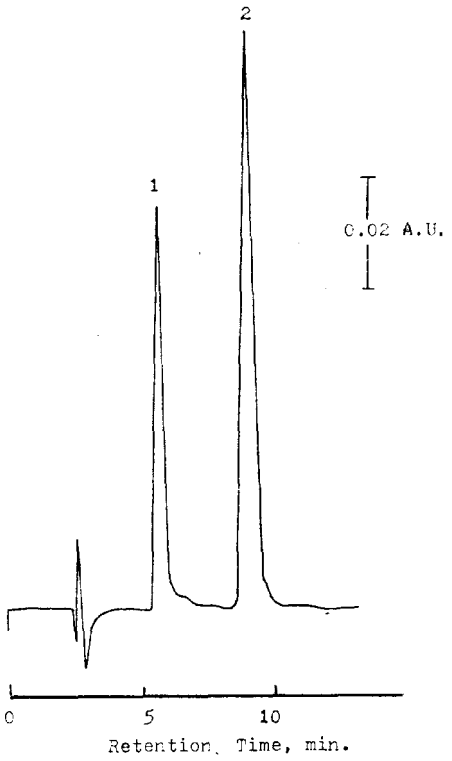

Fig. 1. Chromatogram of *t*-butanol and *n*-Pentanol. 1; *t*-Butanol, 2; *n*-Pentanol. Injected volume: 20 μL containing 100 μg, Mobile phase: 20 % (V/V) MeOH-H_2O containing 0.1 m*M* Brilliant green, Flow rate: 1.0 m*l*/min Temp.: 30 °C, Detection: 625 nm.

In order to find optimal chromatographic conditions, the capacity factors were determined us-

Table 2. Capacity factor of alcohols, ketones and esters

Compound	Capacity factor, k'				
	10%	20%	30%	40%	50%
Ethanol	0.38	0.24	0.12	—	—
n-Propanol	0.74	0.67	0.42	0.28	—
i-Propanol	0.51	0.44	0.31	0.20	—
n-Butanol	2.23	1.60	0.98	0.85	0.63
t-Butanol	1.87	1.17	0.83	0.52	0.46
n-Pentanol	3.27	2.30	1.93	1.42	1.03
n-Hexanol	10.6	7.81	6.15	4.13	3.22
Acetone	0.40	0.33	0.26	—	—
2-Butanone	1.15	0.98	0.94	0.81	0.59
2-Pentanone	4.01	3.27	2.76	2.09	1.24
4-Methyl 2-pentanone	11.4	8.51	6.41	4.30	2.20
3-Dimethyl 2-butanone	9.65	7.76	5.94	3.72	2.18
2-Heptanone	—	—	22.5	14.3	5.54
5-Methyl 2-hexanone	—	—	19.2	11.7	4.76
3-Heptanone	—	—	22.0	13.6	5.35
Methyl acetate	1.01	0.93	0.84	0.66	0.44
Methyl propanoate	3.06	2.81	2.12	1.80	1.06
Methyl butanoate	9.82	8.13	6.09	3.80	2.27
Methyl pentanoate	—	—	16.7	9.90	4.67

Column: Partisil 10 ODS, 25 cm×4.6 mm. Mobile phase: 0.1 m*M* Brilliant green and Methanol, pH= 4.3. Flow rate: 1.0m*L*/min. Temp: 30°C Detection: 625nm.

ing a various mixture of methanol water containing brilliant green at 0.1 m*M*. As can be seen in *Table* 2, k' values decreased with increasing concentration of methanol of mobile phase and also increased with increasing molecular weight or hydrophobility of samples. The order of k' value of isomers was normal>iso> tertiary according to its hydrophobility. The less hydrophobic alcohols are readily eluted using 10 to 20% of methanol, whereas the more hydrophobic ketones and esters require 40 to 50% of methanol for elution. In case of esters, those whose molecular weight are greater than methyl pentanoate seemed to be hydrolyzed during the elution through the column. Therefore, the capacity factor of such a compounds could not be

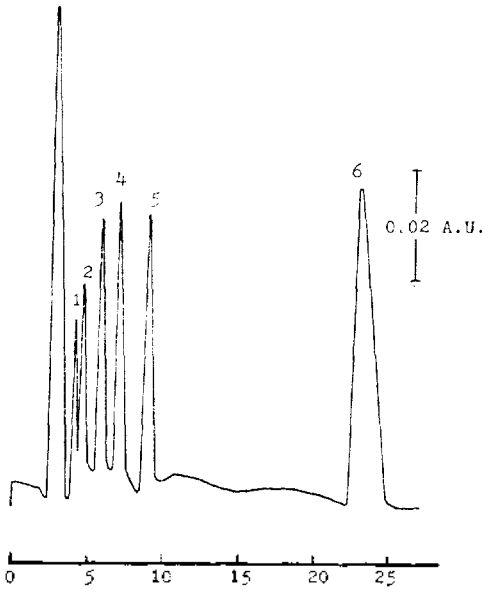

Fig. 2. Separation of a Mixture of some Alcohols. Samples: 1; *i*-Propanol, 2; *n*-Propanol, 3; *t*-Butanol, 4; *n*-Butanol, 5; *n*-Pentanol, 6; *n*-Hexanol, Injected volume: 20 μl containing 50 μg, Other experimental conditions are same as in *Fig.* 1.

measured because the k' values were not reproducible and two or three peaks appeared in the chromatogram.

Some mixture of alcohols, ketones and esters were attempted to separate under optimum condition. *Fig.* 1, 2 and 3 represent the separation of a mixture of some alcohols, ketones and esters. The mixtures, as can be seen in figures, are chromatographically well separated with good base line resolution and modest sensitivity. The peak height and sensitivity increased with increasing the molecular weight as was observed in the separation of alcohols with methylene blue[8].

The quantitation and detection limit were also investigated by preparing various calibration curves of some samples. The calibration curves were prepared by injecting a synthetic mixture of compounds that are completely separated and

Table 3. Calibration curves for ketones and esters

Compound	Linear regression parameters			
	Slope±sd	Intercept±sd	Correl. coef.	Range of conc. μg
[a] 2-Butanone	0.229±0.005	−0.235±0.138	0.998	25~150
2-Pentanone	0.355±0.005	0.508±0.406	0.999	20~150
4-Methyl 2-butanone	0.543±0.009	0.531±0.360	0.997	10~150
2-Heptanone	0.864±0.037	−1.101±0.693	0.996	10~100
[b] Methyl acetate	0.175±0.004	−0.130±0.029	0.998	25~200
Methyl propanoate	0.208±0.003	0.317±0.064	0.997	20~200
Methyl butanoate	0.297±0.006	0.386±0.175	0.996	10~100
Methyl pentanoate	0.479±0.017	0.895±0.942	0.996	10~100

Slope values are expressed as peak height *vs.* μg injected, Experimental condition, a: same as in *Fig.* 3, b: same as in *Fig.* 4.

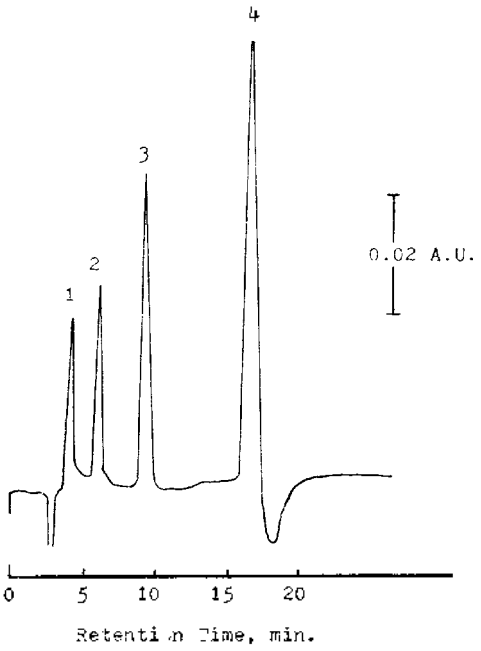

Fig. 3. Separation of a Mixture of some Ketones. Samples: 1; 2-Butanone, 2; 2-Pentanone, 3; 4-Methyl 2-butanone, 4; 2-Heptanone, Injected volume: 20 μl containing 50μg, Mobile phase: 50% (v/v) MeOH-H_2O containing 0.1mM Brilliant green, Other experimental conditions are same as in *Fig.* 1.

measured by a plot of peak height(m. a. u.) versus microgram of sample injected. The linear regression parameters for calibration curves are listed in *Table* 3, showing sensitivity, linearity and precision for each samples. values and uncertainties are expressed as milliabsorbance unit. As can be seen from this data, the regression parameters for calibration curves exhibit linearity in the given concentration range correlation coefficient 0.996~0.999).

Table 4. The detection limit of ketones and esters

Compound	Detection limit μg	
	30%	50%
2-Butanone	7.7	11.2
2-Pentanone	4.6	6.8
4-Methyl 2-butanone	2.3	3.5
2-Heptanone	1.6	2.4
Methyl acetate	7.4	10.0
Methyl propanoate	4.8	7.0
Methyl butanoate	1.8	2.2
Methyl pentanoate	1.5	2.9

Mobile phase: 0.1 m*M* Brilliant green and Methanol Detection: 625nm, Temp.: 30 °C.

The qualitative detection limit was determined in order to compare the sensitivity each other. The detection limit was obtained from the peak height equivalent to twice the noise level of detector. The data in *Table* 4 show that sensitivity increased with increasing molecular weight and decreasing percentage of methanol.

In conclusion, this technique provides that even a compound of non-chromophore or poorly absorbing chromophore can be detected enhanced

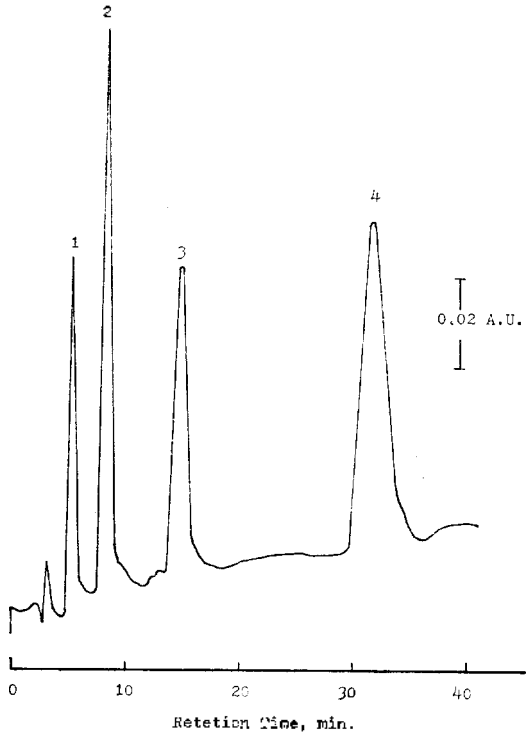

Fig. 4. Separation of a Mixture of some Esters. Samples: 1; Metyhl acetate (60 μg), 2; Methyl propanoate (60 μg), 3; Methyl butanoate (50μg), 4; Methyl Pentanoate (50 μg), Mobile phase: 40% (v/v) MeOH-H_2O cotaining 0.1 mM Brilliant green. Other experimental conditions are same as in *Fig.* 1.

their sensitivity with UV-visible detector by the assistance of dye.

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REFERENCE

1. S. Eksborg, P. O. Lagerstöm, R. Modin, and G. Schill, *J. Chromatogy.*, **83**, 99 (1973).
2. N. Parris, *Anal. Biochem.*, **100**, 260 (1979).
3. M. Denkert, I. Hackzell, G. Schill, and E. Sjögren, *J. Chromatogr.*, **213**, 31 (1981).
4. B. Sachok, S. N. Deming and B. A. Bidlingmeyer, *J. Liquid Chromatogr.*, **5**(3), 389 (1982).
5. R. A. Cochrane and D. E. Hillman, *J. Chromatogr.*, **241**, 392 (1982).
6. William E. Barber and Peter W. Carr, *J. Chromatogr.*, **260**, 89 (1983).
7. J. DiNunzio and H. Freiser, *Talanta*, **26**, 587 (1979).
8. T. Gnanasambandan and H. Freiser, *Anal. Chem.*, **54**, 1282 (1982).
9. T. Gnanasambandan and H. Freiser, *Anal. Chem.*, **54**, 2379 (1982).
10. W. West and Sandra Pearce, *J. Phys. Chem.*, **69**, 1894 (1965).
11. G. R. Haugen and E. R. Hardwick, *J. Phys Chem.*, **67**, 725 (1963).
12. G. Blauer, *J. Phys. Chem.*, **65**, 1457 (1961).