Reversed-Phase High-Performance Liquid Chromatography for Separation of Carboxylates Using On-Line Recycled Eluent and Consecutive Determinations of Citrate in Beverages

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Reversed-phase high-performance liquid chromatography (RPLC) for separation of seven carboxylates (tartrate, malate, malonate, acetate, citrate, succinate, and propionate) using on-line recycled eluent was investigated. The RPLC system consisted of octadecyl silica gel column as a separation column, graphitic carbon column as a column to eliminate the injected carboxylates in a sample from an eluent, and 5 ml of 5 mM ammonium phosphate buffer at pH 3.4 as the eluent. The graphitic carbon column could eliminate the injected malonate and citrate in the sample from the eluent. The performance of the RPLC using the on-line recycled eluent with the eliminating column was the same as that of the conventional RPLC. The relative standard deviation values of retention times and peak heights of the carboxylates in this method were below 0.67 and below 5.8%, respectively. This method was applied to determinations of the citrate in some commercial beverages. These results corresponded to those of the conventional RPLC for over 50 times consecutive determinations.

1. Introduction

Reversed-phase high-performance liquid chromatography (RPLC) using an octadecyl silica gel (C_{18}) column is a well-used technique for a separation of carboxylates in a beverage [1]. A consecutive determination of the carboxylates in the beverage to

control a quality of beverage has been required in a beverage industry. However, the large eluent volume would be generally required in the RPLC technique, when an analyst must determine some analytes in many samples. A decrease of waste volume, such as an effluent volume, in the RPLC measurement, would be claimed in order to prevent an environmental pollution. Also, if the eluent could continue to be flowed in the RPLC system by a circulation of the eluent, the RPLC measurement would be possible at any time without waiting for stabilizing the system, because the eluent is always flowing in the RPLC system without decreasing the eluent volume and without changing the composition of the eluent.

Several flow injection analyses (FIA) using on-line recycling reagent solution have been recently established [2-9]. Large decreasing the volume of reagent solution used in the FIA without changing the compositions of the reagent solution was achieved, as compared to that in the conventional FIA. However, there are few reports for LC using the on-line recycling eluent. In the case of FIA, it is especially successful for re-using of the reagent solution to introduce an eliminating column to eliminate an injected sample from the reagent solution into the system [6,7]. The system to utilize the eliminating column might be applied to an LC system.

A separation of some anions by anion chromatography using on-line recycled eluent in presence of mixed ion-exchange resins has been achieved [10]. The eluent volume has been decreased to one tenth less than that in a conventional anion chromatography. The reproducibility and precision were the similar as those in the conventional anion chromatography.

An analytical reliability of simply mobile-phase recycling in RPLC with UV detector has been reported [11]. This report suggests that a new system for the mobile-phase recycling in RPLC would be required, because the background increased with increasing injection numbers of the carboxylates in the simply recycling system. Therefore, the determination and separation of the carboxylates in RPLC using on-line recycled eluent were investigated.

Generally, almost all the commercial beverages contain the citrate, which gives an acidic taste into the beverage. It is, therefore, important to determine the citrate in the beverage in respect to a control of its quality. Accordingly, this RPLC using the on-line recycled eluent was applied to determination of the citrate in some beverages.

2. Experimental
2.1. Instrumental

An RPLC system was used, consisting of a Tosoh CCPD pump and UV-8000 UV detector (detection wavelength, 210 nm), and Rheodyne 7725 injector with 20-μl sample loop. A C_{18} column (4.6 mm i.d. × 150 mm, Tosoh TSK-GEL ODS-80T$_M$) was kept in air-oven (Tosoh CO-8010) at a constant 35°C. A flow rate 0.8 ml min^{-1} was used for the C_{18} column [1].

2.2. Reagents

An eluent of 5 mM ammonium phosphate at pH 3.4 adjusted by phosphoric acid (Wako, Osaka, Japan) was used. A graphitic carbon column (Hypercarb 4.6 mm i.d. × 50 mm, Thermo, Waltham, MA) was purchased for use in the elimination of carboxylates from the eluent. Tartaric, malic, malonic, acetic, citric, succinic, and propionic acids were chosen as sample, as appeared in ref. [1]. All the sample solutions of the carboxylates were prepared from 100 mM of stock solutions, using reagent grade materials (Wako, Osaka, Japan). All water used was purified using Milli-Q water system (Millipore, Bedford, MA), after a distillation through a mixed ion-exchange resin. All solutions were filtered through a 0.45-μm mixed cellulose membrane (Advantec, Tokyo) before use. All six commercial beverages A – F used as real samples were diluted to the ratio of 1:10 by the water.

3. Results and discussion
3.1. Simply on-line recycled eluent system

The 5 mM ammonium phosphate buffer at pH 2.4 as the eluent under the C_{18} column manufacture's conditions has been used [1]. However, resolution of the seven carboxylates on the 5 mM ammonium phosphate buffer at pH 3.4 was better than that at pH 2.4. Therefore, the eluent at pH 3.4 in further study was used. Fig. 1 shows the typical chromatogram of seven carboxylates under the modified C_{18} column manufacturer's conditions. The separation of the seven carboxylates was observed. In order to
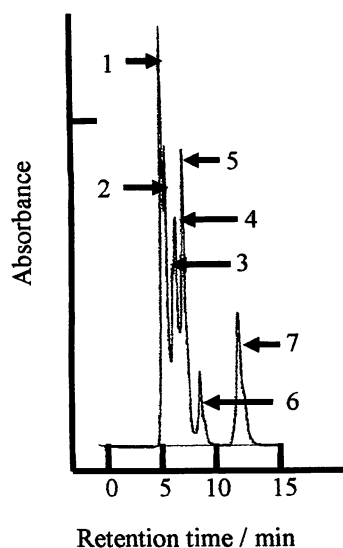
Fig. 1. Chromatogram of seven carboxylates on C_{18} column. Conditions: eluent, 5 mM ammonium phosphate at pH 3.4; flow rate, 0.8 ml min^{-1}; sample volume, 20 μl; temperature, 35°C; detection, 210 nm. Peaks: 1, 1 mM tartrate; 2, 1 mM malate; 3, 1 mM malonate; 4, 5 mM acetate; 5, 1 mM citrate; 6, 1 mM succinate; 7, 5 mM propionate.

reproduce the separation of the carboxylates by re-using the effluent during the chromatographic measurements, the RPLC system using on-line recycled eluent under the same chromatographic conditions was investigated. The one is a simple system to return the effluent during the chromatographic measurements to an eluent reservoir without a column to eliminate the injected carboxylates in sample from the eluent. The other is the system to return it via the column to eliminate the injected carboxylates in sample from the eluent, as shown in Fig. 2. In both the systems, the eluent reservoir was sealed by paraffin film during chromatographic measurements, because of preventing a volatilization of the eluent. Also, the eluent was always stirred by magnetic stirring during the chromatographic measurements, because of keeping the homogeneous eluent. Fig. 3 shows typical chromatograms of seven carboxylates using on-line recycled eluent volumes of 5 and 50 ml in the simple system without the eliminating column. In case of 50 ml, even chromatogram of the fifty times measurement corresponded to that of the conventional RPLC. However, in case of 5 ml, the
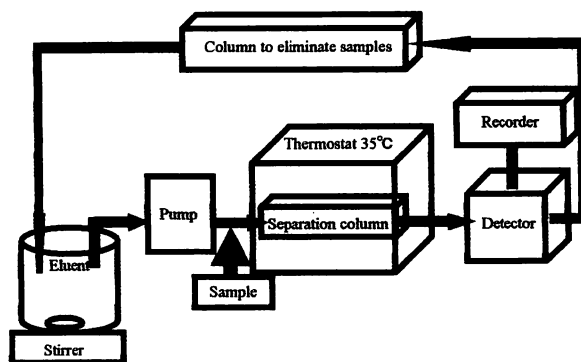

Fig. 2. RPLC system using on-line recycled eluent with column to eliminate the injected carboxylates in sample from eluent.
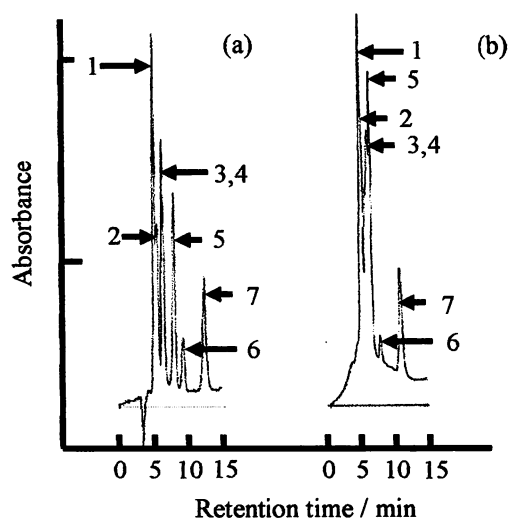

Fig. 3. Chromatograms of seven carboxylates using on-line recycled eluent. Eluent volume (ml) and injections (times): (a), 50 and 50; (b) 5 and 2. Other conditions and identification of peaks as in Fig. 1.

increased background on chromatogram in the second chromatographic measurement was observed.

3.2. On-line recycled eluent system with eliminating column

Fig. 4 shows chromatogram of the seven carboxylates using a graphitic carbon column as the separation column under the same conditions. Although the peaks of five carboxylates were observed, both the peaks of malonate and citrate were not observed. This shows that the graphitic carbon column could eliminate the malonate and citrate in the injected sample from the eluent in the RPLC

system using the on-line recycled eluent with its column as an eliminating column in Fig. 2.

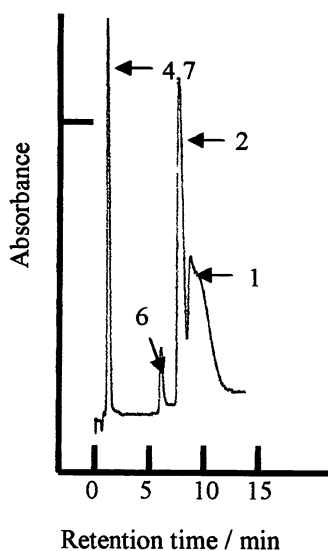Adsorption of the malonate and citrate onto the

Fig. 4. Chromatogram of seven carboxylates on graphitic carbon column. Conditions and identification of peaks as in Fig. 1.

graphitic carbon in the 5 mM ammonium phosphate buffer at pH 3.4 was strong. It has been reviewed on a difference of retention mechanism between the C_{18} and the graphitic carbon column [12]. In case of the graphitic carbon column, both the hydrophobic and electrostatic interactions of π-electron on the graphitic carbon give influence on the retention mechanism. In case of the C_{18} column, the hydrophobic interaction without the electrostatic interaction of π-electron gives influence on the retention time. However, since the retention mechanism of the carboxylates on the graphitic carbon column in the 5 mM ammonium phosphate buffer at pH 3.4 is complicated, the difference of retention for the seven carboxylates between the C_{18} and the graphitic carbon column in this study could not be obvious.

Fig. 5 shows chromatograms of the seven carboxylates using the on-line recycled eluent in the RPLC system with the graphitic carbon column to eliminate the malonate and citrate from the eluent. A slight difference of resolution of the malate and malonate between this RPLC system and the conventional RPLC was observed. This might cause to the small eluent volume of 5 ml. The detail reason
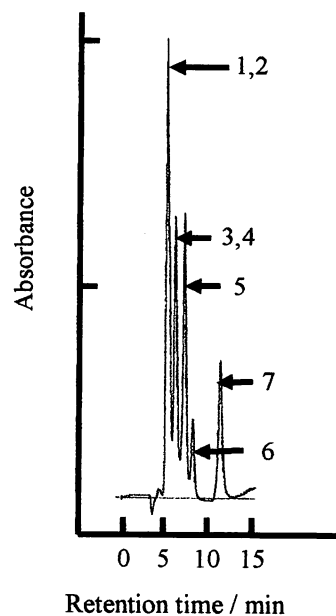

Fig. 5. Chromatogram of seven carboxylates using on-line recycled eluent with graphitic carbon column to eliminate carboxylates from eluent. Eluent volume: 5 ml; injections, 50 times. Other conditions and identification of peaks as in Fig. 1.

could not be found. The resolution of carboxylates after 50 injections on the 5 ml eluent volume was the same as that of first injection and that in the conventional RPLC. The relative standard deviation (RSD) values of retention times and peak heights of carboxylates in the conventional RPLC and in the RPLC using the on-line recycled eluent without and with the eliminating column are summarized in Tables 1 and 2. The RSD values of retention times of carboxylates in the RPLC using the on-line recycled eluent with the eliminating column were almost the similar to those in the conventional RPLC. Especially, the RSD value of retention time of citrate was improved from that without the eliminating column. The RSD values of peak heights of carboxylates in the RPLC using the on-line recycled eluent with the eliminating column were 2.0 to 5.8%. The RSD values were not so high, as compared to those from 0.82 to 5.1% in the conventional RPLC. Therefore, the RSD values are enough to determine the carboxylates. Surprisingly, the peaks of tartrate, malate, acetate, succinate, and propionate, which could not be eliminated from the eluent by the

Table 1 RSD values (%) of retention times of carboxylates for 50 times consecutive chromatographic measurements

Carboxylate	Coventional RPLC	RPLC using on-recycling eluent without eliminating column		RPLC using on-recycling eluent with eliminating column
		50 ml of eluent volume	5 ml of eluent volume	5 ml of eluent volume
Tartrate	0.26	0.30	0.30	0.21[a]
malate	0.30	0.34	0.42	0.21[a]
malonate	0.42	0.46	0.66	0.23[a]
acetate	0.27	0.26	0.19	0.23[a]
citrate	0.60	0.64	1.6	0.67
succinate	0.58	0.63	0.84	0.15
propionate	0.38	0.40	0.32	0.65

a. Not separated.

Table 2 RSD values (%) of peak heights of carboxylates for 50 times consecutive chromatographic measurements

Carboxylate	Conventional RPLC	RPLC using on-recycling eluent without eliminating column		RPLC using on-recycling eluent with eliminating column
		50 ml of eluent volume	5 ml of eluent volume	5 ml of eluent volume
Tartrate	1.3	0.91	3.3	2.0[a]
malate	5.1	4.7	8.1	2.0[a]
malonate	2.6	3.4	8.7	5.2[a]
acetate	1.1	1.8	9.7	5.2[a]
citrate	1.4	1.3	11.3	5.0
succinate	2.4	4.3	11.7	3.3
propionate	0.82	1.9	5.3	5.8

a. Not separated.

eliminating column, did not give large influence on these RSD values, as compared to those of the conventional RPLC. The reason could not be obvious.

Consequently, using 5 ml of the eluent volume, over 50 times consecutive determinations of the seven carboxylates using the on-line recycled eluent were achieved. The over 50 times consecutive determinations would require the over 600 ml eluent volume in a conventional RPLC, except for time to stabilize the chromatographic system, because the flow rate and the measurement time was 0.8 ml min^{-1} and 15 min, respectively. Also, using the graphitic carbon column to clean and to recycle the eluent, the over 50 times consecutive determinations of the carboxylates on only the 5 ml eluent volume were achieved without significant changes of these retention times and peak heights. Furthermore,

although the pH values of samples were 2.9 to 3.1, the small change of pH of the eluent after the 50 times consecutive measurements did not give significant influence on the 50 times consecutive determinations of the carboxylates, because the pH of the eluent was 3.4.

3.3. Determination of citrate in beverages

Fig. 6 shows a typical chromatogram of a commercial beverage A. Two unknown peaks and a peak of citrate were observed. The peaks of acetate, malonate, succinate, and propionate in beverages A – F were not observed. Although the peaks of tartrate and malate in the beverages B, D, and F were observed, the good resolution of these peaks was not observed. The peak of citrate in all the beverages was observed. The results of determinations of the citrate in the six commercial beverages A – F are summarized in Table 3. All the results corresponded
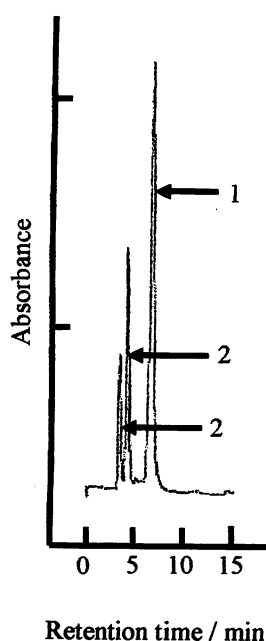
Fig. 6.   Typical chromatogram of citrate in beverage. Sample: 10-fold dilution of beverage A. Peaks: 1, citrate; 2, unknown. Other conditions as in Fig. 1.

Table 3 Determination of citrate in beverages[a]

Beverage	[Citrate] / mM	
	Conventional RPLC	This method[b]
A	21.7 ± 0.3	20.1 ± 2.6
B	16.4 ± 0.1	14.7 ± 2.0
C	17.3 ± 0.1	17.8 ± 1.3
D	12.4 ± 0.2	17.2 ± 0.9
E	-[c]	-[c]
F	11.7 ± 0.2	13.8 ± 1.1

a. Errors are standard deviations (n = 3).

b. Eluent volume is 5 ml.

c. Not separated with unknown peak.

to those in the conventional RPLC within errors. All the RSD values of retention times of the citrate in this method and in the conventional RPLC for 50 times consecutive determinations in the beverages were below 1.2 and below 0.58%, respectively. The matrix, such as amino acids, in the beverages did not give influence on the 50 times consecutive determinations of the citrate in this method. Furthermore, since pH values of samples were 3.5 to 3.9, the small change of pH of the eluent after the 50 times consecutive measurements did not give

significant influence on the determinations of the citrate in the beverages.

The C_{18} and graphitic carbon columns were washed by water and methanol, after the chromatographic measurements. Resultantly, those columns without degradation of column performances during this study could be used.

4. Conclusions

The over 50 times consecutive determinations of citrate in the conventional RPLC were achieved by only the eluent volume of 5 ml in the proposed RPLC using the on-line recycled eluent with the graphitic carbon column to eliminate the injected malonate and citrate in the sample from the eluent. The proposed method could apply to the over 50 times consecutive determinations of the citrate in the commercial beverages without significant changes of its retention time and peak height.

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