Journal of Chromatography, 457 (1988) 333–343
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 946

SIMULTANEOUS DETERMINATION OF NINE FOOD ADDITIVES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

YOSHITOMO IKAI*, HISAO OKA, NORIHISA KAWAMURA and MASUO YAMADA
Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita-ku, Nagoya 462 (Japan)
(First received April 12th, 1988; revised manuscript received August 29th, 1988)

SUMMARY

A technique for the simultaneous determination of sorbic acid, benzoic acid, dehydroacetic acid, *p*-hydroxybenzoic acid esters (ethyl, isopropyl, *n*-propyl, isobutyl and *n*-butyl *p*-hydroxybenzoate) and saccharin sodium using ion-pair reversed-phase high-performance liquid chromatography is described. The nine food additives were separated on a Nucleosil $3C_{18}$ (3 μm) column (75 × 4.6 mm I.D.) using methanol–acetonitrile–0.05 *M* aqueous acetonic acid solution (pH 4.5) (1.5:1:3.1) containing 2.5 m*M* cetyltrimethylammonium chloride as the mobile phase at a flow-rate of 1.0 ml/min, and detected at 233 nm. The food additives were separated within 18 min and their calibration graphs were linear between 2 and 200 ng.

INTRODUCTION

Sorbic acid (SOA), benzoic acid (BA), dehydroacetic acid [DHA, 3-acetyl-6-methyl-2*H*-pyran-2,4(3*H*)-dione] and *p*-hydroxybenzoic acid esters (ethyl-, isopropyl-, *n*-propyl-, isobutyl- and *n*-butyl-PHBA) as preservatives and saccharin sodium (SA) as a sweetener are food additives commonly used in Japan. One of the most important duties of a public health agency is to check quantitatively these food additives in foods. They are usually analysed according to individual methods, which is very inefficient from the point of view of analytical processing time and materials, so a simple and simultaneous analytical method for additives in various foods is required.

As the food additives differ considerably in polarity, their simultaneous determination by gas or thin-layer chromatography is difficult. However, we considered that high-performance liquid chromatography (HPLC) using solvent gradient and ion-pair techniques might be a more promising approach. Although some HPLC systems for the simultaneous determination of food additives except DHA have already been reported using these HPLC techniques[1–6], no system for all of the above food additives has been established. We attempted to modify the reported HPLC systems[3,4] so as to make them applicable to the determination of the nine food additives including DHA, but satisfactory results could not be obtained because DHA appeared as an extremely tailing peak on the chromatogram. Therefore, we investigated the control of the tailing of DHA and established a simultaneous method

0021-9673/88/$03.50

for the nine food additives using an HPLC system. This paper describes techniques for the simultaneous determination of the nine food additives including DHA using ion-pair reversed-phase (RP) HPLC.

EXPERIMENTAL

Materials

Acetic acid, acetonic acid (2-hydroxyisobutyric acid), acetonitrile, acetylacetone, adipic acid, anhydrous sodium sulphate, citric acid, diethyl ether, malonic acid, methanol, oxalic acid, phosphoric acid, propionic acid, sodium chloride, sodium hydroxide, sulphuric acid and tartaric acid were of analytical-reagent grade.

p-Hydroxybenzoic acid and its esters (methyl, ethyl, isopropyl, *n*-propyl, *sec.*-butyl, isobutyl and *n*-butyl *p*-hydroxybenzoate), DHA, salicylic acid, thiabendazole (TBZ), *o*-phenylphenol (OPP), diphenyl (DP), butylhydroxyanisole (BHA), dibutylhydroxytoluene (BHT), cetyltrimethylammonium chloride (CTA), *n*-tetradecyltrimethylammonium bromide (TTA) and tetrabutylammonium bromide (TBA) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and SOA, BA, SA, ascorbic acid, erithorbic acid, nicotinic acid and nicotinamide from Wako (Osaka, Japan).

Apparatus

A chromatograph equipped with a constant-flow pump (LC-5A, Shimadzu, Kyoto, Japan) was used together with a variable-wavelength detector (Shimadzu SPD-2AM).

Separations were performed on Nucleosil $3C_{18}$ (3 μm) (Macherey-Nagel, Düren, F.R.G.) packed in a stainless-steel column (75 × 4.6 mm I.D.).

Preparation of mobile phase

The appropriate amounts of methanol, acetonitrile and aqueous solutions were mixed, then the ion-pair reagents were dissolved in the mixture to give the calculated concentrations. The pH values of aqueous solutions were adjusted with sodium hydroxide.

RESULTS AND DISCUSSION

As described in the Introduction, SOA, BA, DHA and SA have very different polarities to PHBAs. In order to separate such analytes by RP-HPLC, solvent gradient and ion-pair techniques are generally used. However, the solvent gradient method is unsuitable for routine analysis, because it is time consuming and requires expensive apparatus. Therefore, we tried to separate the nine food additives using an ion-pair technique. After various studies of the retention behaviour of the additives, we achieved their baseline separation. A typical HPLC trace under the optimal conditions is shown in Fig. 1. Methanol–acetonitrile–0.05 *M* aqueous acetonic acid solution (pH 4.5) (1.5:1:3.1) containing 2.5 m*M* CTA was used as the mobile phase and Nucleosil $3C_{18}$ (3 μm) packed in short column (75 × 4.6 mm I.D.) was used as the stationary phase. The flow-rate and measurement wavelength were adjusted at 1.0 ml/min and 233 nm, respectively.

In order to optimize the HPLC conditions described above, the following pa-

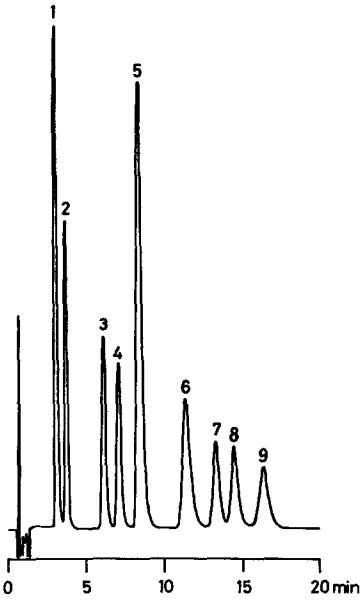

Fig. 1. Typical high-performance liquid chromatogram of food additives under the optimal conditions. 1 = DHA; 2 = ethyl-PHBA; 3 = isopropyl-PHBA; 4 = *n*-propyl-PHBA; 5 = SOA; 6 = BA; 7 = isobutyl-PHBA; 8 = *n*-butyl-PHBA; 9 = SA (100 ng each). HPLC conditions: column, Nucleosil $3C_{18}$ (3 μm) (75 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.05 *M* aqueous acetonic acid solution (pH 4.5) (1.5:1:3.1) containing 2.5 m*M* CTA; flow-rate, 1.0 ml/min; detection, 233 nm.

rameters were examined: (1) effect of acetonic acid, (2) effect of ion-pair reagent, (3) proportion of methanol and acetonitrile and (4) proportions of organic solvents and aqueous solution. The results of these examinations are described below. In interpreting the chromatograms, the parameters used are (a) the asymmetry factor (A_s), which is the ratio of the length of the rear (tailing edge) to that of the front (leading edge) of the peak along a line parallel to 10% of its height above the baseline, and (b) the capacity factor, $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the sample peak and t_0 that of a non-retained peak.

Effect of acetonic acid

When SOA, BA, DHA and SA are determined by RP-HPLC, the pH of the mobile phase is one of the most important factors for the separation. In order to stabilize the pH of the mobile phase, phosphate and acetate buffer solutions are generally used. However, we could not obtain satisfactory results in attempts to separate the nine food additives using these buffer solutions. Between pH 3.0 and 5.0, the determination of DHA was prevented by its extreme peak tailing. On the other hand, outside that pH range, satisfactory separation of the additives could not be achieved under any HPLC conditions, although DHA did not appear as a tailing peak. We therefore considered that these pH buffer solutions are unsuitable for the determination of the nine food additives including DHA.

In a previous study[7], we were able to determine successfully some tetracycline antibiotics that are similar to DHA in their HPLC behaviour, using a mobile phase

containing 0.01 *M* aqueous oxalic acid solution. We tested the effect of oxalic acid for DHA using methanol–0.01 *M* aqueous oxalic acid solution (1:2) as the mobile phase, and good chromatograms of DHA were obtained even when aqueous oxalic acid solutions adjusted to pH 3.0–5.0 were used. We assume that the tailing is due to the interaction between DHA and residual silanol groups of the stationary phase and that oxalic acid has the ability to block such silanol groups. It seemed likely that the tailing of DHA could be controlled by other compounds that have similar structures to oxalic acid, and several compounds were tested. The A_s values of DHA were investigated using aqueous solutions of these compounds at concentrations in the mobile phase of 0.005–0.05 *M* and typical values for DHA are given in Table I. Although the A_s values of DHA improved with increasing concentration of the test compounds, satisfactory results were not obtained at any concentrations except with acetylacetone, oxalic acid and acetonic acid. Acetylacetone was most effective in controlling the tailing of DHA in spite of the low concentration; however, it was not suitable for practical analysis, because it did not give a stable baseline. Oxalic acid was also unsuitable for high-sensitivity determinations, because it shows a strong absorption band at wavelengths shorter than 250 nm. Therefore, we used acetonic acid solution as the aqueous component in the mobile phase in subsequent work.

The influence of the concentration of acetonic acid was examined. As shown in Fig. 2, the A_s values of DHA were almost constant above a concentration of 0.05 *M*. Fig. 3 shows that the k' values of SOA, BA, DHA and SA decreased with increase in concentration of acetonic acid, whereas those of PHBAs were independent of concentration. When 0.05 *M* aqueous acetonic acid solution was used, a satisfactory separation was obtained.

Next, the influence of the pH of the aqueous acetonic acid solution was examined. As shown in Fig. 4, the k' values of PHBAs were not influenced very much, but those of SOA, BA, DHA and SA were influenced significantly by pH. A satisfactory

TABLE I

COMPARISON OF ASYMMETRY FACTORS OF DHA USING VARIOUS AQUEOUS SOLUTIONS IN THE MOBILE PHASE

Column, Nucleosil $3C_{18}$ (3 μm) (75 × 4.6 mm I.D.); mobile phase, methanol–aqueous solution (1:2); flow-rate, 1.0 ml/min; detection, 225 nm.

*Aqueous solution**	*Asymmetry factor of DHA*
0.01 *M* phosphoric acid	7.0
0.01 *M* acetic acid	6.0
0.01 *M* tartaric acid	4.6
0.01 *M* citric acid	4.3
0.01 *M* malonic acid	3.4
0.01 *M* lactic acid	3.2
0.01 *M* oxalic acid	2.5
0.01 *M* acetonic acid	2.0
0.001 *M* acetylacetone**	1.8

* adjusted to pH 4.5 with sodium hydroxide.

** 0.01 *M* acetic acid solution.

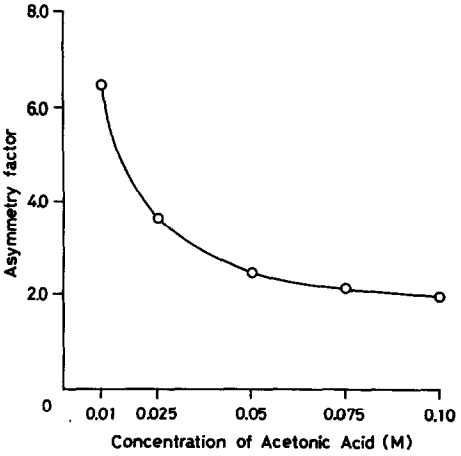

Fig. 2. Influence of concentration of acetonic acid on asymmetry factor of DHA. Mobile phase, methanol–acetonitrile–aqueous acetonic acid solution (pH 4.5) (1.5:1:3.1) containing 2.5 m*M* CTA.

separation was obtained when the aqueous acetonic acid solution was adjusted to pH 4.5, and in subsequent work we used 0.05 *M* aqueous acetonic acid solution adjusted to pH 4.5.

Effect of ion-pair

As mentioned above, the polarities of SOA, BA, DHA and SA are much higher than those of PHBAs. Accordingly, the retention times of SOA, BA, DHA and SA are so short that they cannot be determined together with PHBAs on the same chro-

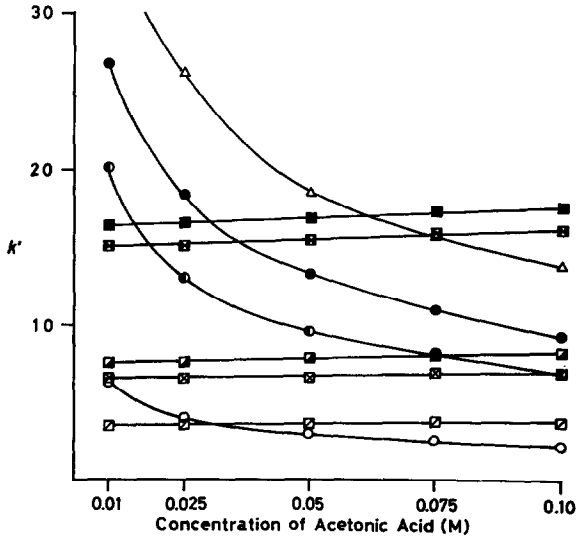

Fig. 3. Influence of concentration of acetonic acid on *k'* values of food additives. ○, DHA; ◐, SOA; ●, BA; △, SA; ⊡, ethyl-PHBA; ⊠, isopropyl-PHBA; ◪, *n*-propyl-PHBA; ⋈, isobutyl-PHBA; ■, *n*-butyl-PHBA. Mobile phase as in Fig. 2.

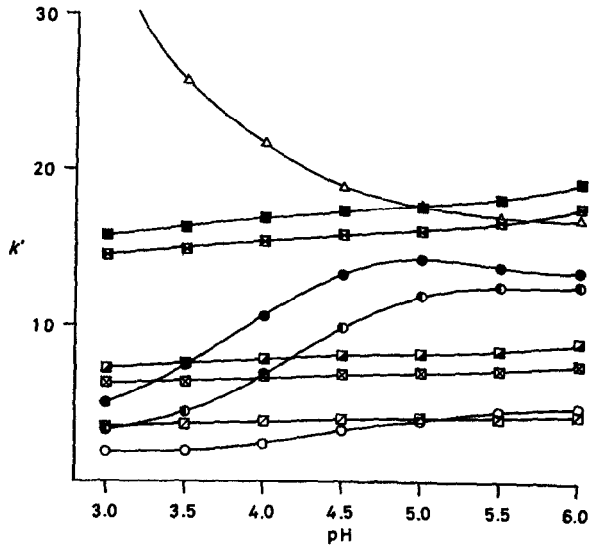

Fig. 4. Influence of pH of aqueous acetonic acid solution on k' values of food additives. Symbols as in Fig. 3. Mobile phase, methanol–acetonitrile–0.05 *M* aqueous acetonic acid solution (1.5:1:3.1) containing 2.5 m*M* CTA.

matogram using RP-HPLC. Therefore, we attempted to increase the retention of SOA, BA, DHA and SA to the level of those of PHBAs by adding an ion-pair reagent to the mobile phase. In order to select a suitable ion-pair reagent, the retention times of SOA, BA, DHA and SA were compared with those of PHBAs using TBA, TTA

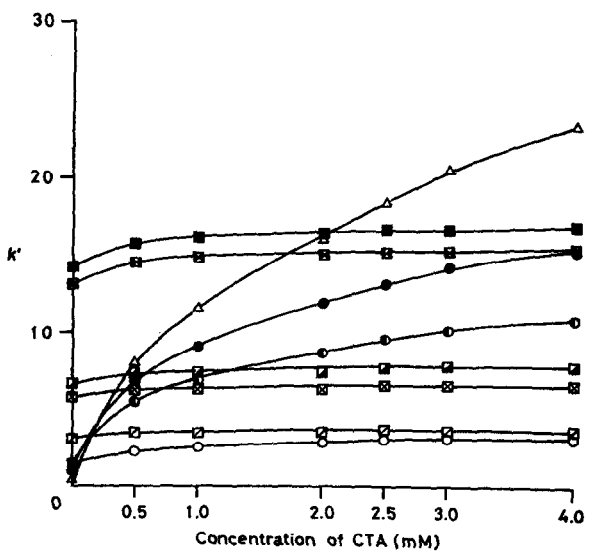

Fig. 5. Influence of concentration of CTA on k' values of food additives. Symbols as in Fig. 3. Mobile phase, methanol–acetonitrile–0.05 *M* aqueous acetonic acid solution (pH 4.5) (1.5:1:3.1) containing CTA.

and CTA as ion-pair reagents. Although CTA was effective enough to increase the retention of SOA, BA, DHA and SA, TBA and TTA were ineffective at any concentration in the mobile phase. Therefore, CTA was used as the ion-pair reagent in subsequent work.

The influence of the concentration of CTA was examined. As shown in Fig. 5, a satisfactory separation was obtained when the concentration of CTA was 2.5 m*M*, and this concentration was therefore used in the mobile phase.

Proportion of methanol and acetonitrile

Because a satisfactory separation could not be obtained when we used methanol or acetonitrile alone as an organic solvent in the mobile phase, we tried mixtures of them and the effect of the proportions of the components was investigated. As shown in Fig. 6, the relative k' values of SOA, BA, DHA and SA with respect to that of PHBAs decreased with increasing proportion of acetonitrile. Because the efficiency of the ion-pair reagent decreased with increasing proportion of acetonitrile, we concluded that acetonitrile acts as a kind of inhibitor for the ion-pair reagent in this instance. As SA showed the greatest effect with acetonitrile, a ratio of methanol to acetonitrile of 1.5:1 was adopted based mainly on the separation of SA.

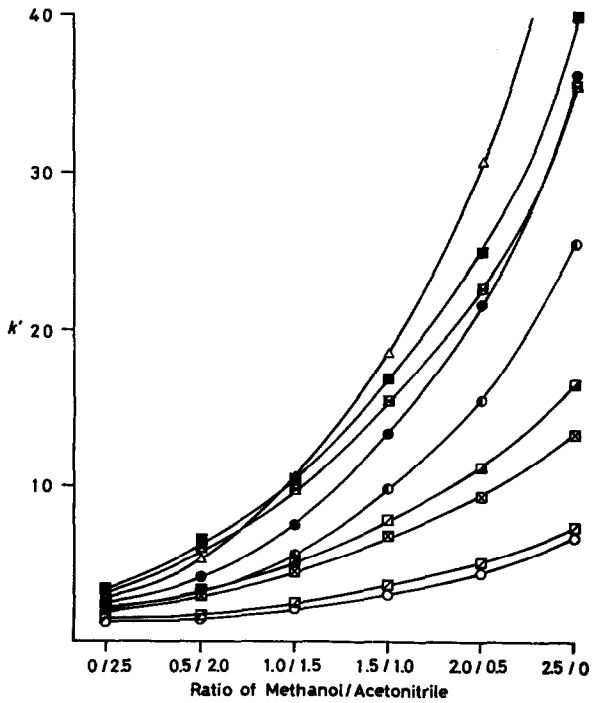

Fig. 6. Influence of ratio of methanol to acetonitrile on k' values of food additives. Symbols as in Fig. 3. Mobile phase, (methanol–acetonitrile)–0.05 *M* aqueous acetonic acid solution (pH 4.5) (2.5:3.1) containing 2.5 m*M* CTA.

Proportions of organic solvents and aqueous solution

The effect of the proportions of organic solvents and aqueous solution was investigated (Fig. 7). We chose a 3.1 ratio of aqueous solution in the mobile phase, because a baseline separation of the nine food additives was achieved.

Application to practical analysis

Using the above HPLC system, the time required for the analysis of the nine food additives was only 18 min. The linearity of the calibration grpahs was tested by peak-height and -area methods, and both calibration graphs were linear between 2 and 200 ng of each food additive. The retention times of other food additives were investigated under the optimal HPLC conditions. As shown in Table II, *p*-hydroxybenzoic acid, methyl-PHBA and *sec.*-butyl-PHBA could also be determined. Some compounds appeared as interfering peaks on the chromatogram, but their influence on the analysis of the food additives was considered to be very slight because they are used in only a limited range of foods at very low concentrations.

In order to clean up food additives present in various foods, steam distillation[8], dialysis[9] and solvent extraction[10] methods are frequently used. We considered applying these clean-up methods to the present HPLC system. The steam distillation and dialysis methods were applied very easily without complicated additional treatments such as extraction, concentration and derivatization, but the steam distillation meth-

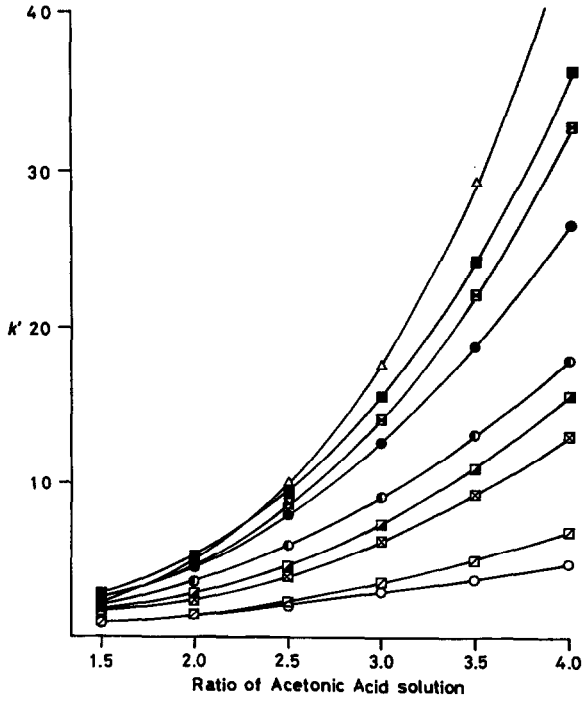

Fig. 7. Influence of ratio of organic solvent to aqueous solution on k' values of food additives. Symbols as in Fig. 3. Mobile phase, methanol–acetonitrile–0.05 *M* aqueous acetonic acid solution (pH 4.5) (1.5:1:*x*) containing 2.5 m*M* CTA.

TABLE II

RETENTION TIMES OF VARIOUS FOOD ADDITIVES

For HPLC conditions, see Fig. 1.

Compound	*Retention time (min)*	*Compound*	*Retention time (min)*
Nicotinamide	0.8	Isopropyl-PHBA	6.3
Acetic acid	1.3	*n*-Propyl-PHBA	7.3
Propionic acid	1.3	SOA	8.8
Citric acid	1.3	*sec.*-Butyl-PHBA	11.5
Tartaric acid	1.3	BA	12.0
Adipic acid	1.3	Isobutyl-PHBA	13.5
Ascorbic acid	2.1	OPP	14.6
TBZ	2.1	BHA	14.6
Erithorbic acid	2.2	*n*-Butyl-PHBA	14.7
Methyl-PHBA	2.3	SA	16.5
p-Hydroxybenzoic acid	2.8	Salicylic acid	35.3
DHA	3.2	DP	38.2
Ethyl-PHBA	3.8	BHT	>60.0
Nicotinic acid	3.8		

od was inapplicable to SA and the dialysis method to PHBAs. Using the following solvent extraction method, the recoveries of nine food additives from some foods (soy sauce, orange juice and yoghurt) were investigated at the level of 100 ppm. After addition of 10 ml of saturated sodium chloride solution and 2 ml of 10% sulphuric acid to 2 g of sample, the food additives were extracted twice with 100 and 50 ml of diethyl ether. The extracts were combined, washed twice with 10 and 10 ml of saturated sodium chloride solution, dried with anhydrous sodium sulphate, evaporated to 1–2 ml and dried by blowing with air. The food additives in the residue were dissolved in 10 ml of the mobile phase and determined by the above HPLC system.

TABLE III

RECOVERIES OF NINE FOOD ADDITIVES FROM SOME FOODS BY THE SOLVENT EXTRACTION METHOD

Results are averages of three replicate determination at the revel of 100 ppm.

Food additive	*Recovery (%)**					
	Soy sauce		*Orange juice*		*Yoghurt*	
DHA	91.0	(0.4)	94.5	(0.2)	92.2	(0.2)
Ethyl-PHBA	91.1	(0.9)	96.5	(3.1)	91.4	(0.8)
Isopropyl-PHBA	93.1	(1.3)	93.7	(4.4)	89.6	(1.3)
n-Propyl-PHBA	91.8	(1.6)	91.4	(3.9)	88.1	(1.6)
SOA	91.7	(0.4)	93.2	(2.2)	90.5	(1.0)
BA	89.8	(2.1)	95.1	(2.6)	90.8	(1.0)
Isobutyl-PHBA	85.7	(3.6)	88.5	(3.9)	88.0	(1.4)
n-Butyl-PHBA	90.1	(1.8)	87.1	(3.7)	88.5	(2.6)
SA	92.6	(1.7)	91.4	(3.9)	92.5	(2.4)

* Coefficients of variation in parentheses.

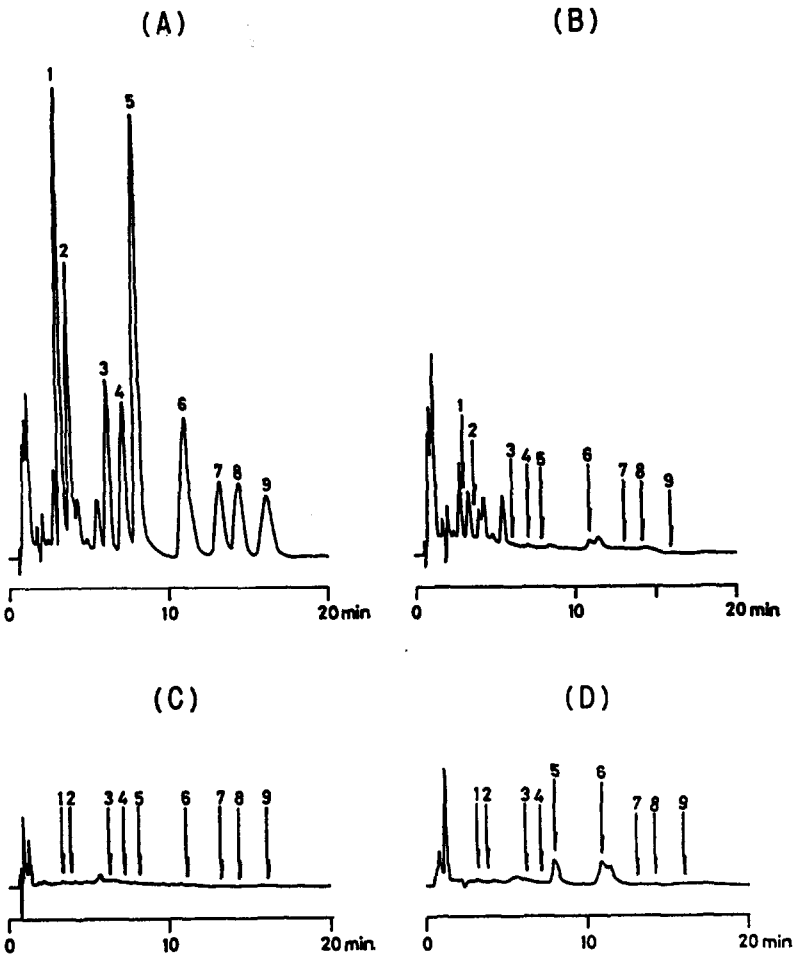

Fig. 8. Typical chromatograms for some foods. HPLC conditions and peak numbers as in Fig. 1. (A) Fortified soy sauce (100 ppm); (B) soy sauce; (C) orange juice; (D) yoghurt.

As shown in Table III, the nine food additives could be simultaneously determined with good recoveries and coefficients of variation (C.V.). However, some interfering peaks appeared on the chromatograms, as shown in Fig. 8, and this method is not applicable to fatty foods such as butter and margarine. We consider that it is the best to consider the use of all three clean-up methods, depending on the particular samples involved. We are currently planning to establish a universal clean-up method and this study will be reported elsewhere in the near future.

CONCLUSION

An ion-pair RP-HPLC system for the simultaneous determination of SAO, BA, DHA, ethyl-, isopropyl-, propyl-, isobutyl- and butyl-PHBA and SA was established. The separation of the nine food additives was performed on a Nucleosil $3C_{18}$ (3 μm)

column (75 × 4.6 mm I.D.) using methanol–acetonitrile–0.05 *M* aqueous acetonic acid solution (pH 4.5) (1.5:1:3.1) containing 2.5 m*M* CTA as the mobile phase, with detection at 233 nm. In this system, acetonic acid was effective in controlling the tailing of DHA and CTA was successfully utilized as an ion-pair reagent for baseline separation. Using this system, the nine food additives were successfully separated within 18 min, and their calibration graphs were linear between 2 and 200 ng. Therefore, we recommend this HPLC system for the routine analysis of food additives.

ACKNOWLEDGEMENTS

We are sincerely grateful to Professors M. Suzuki and K.-I. Harada, Meijo University, for useful suggestions and reading the manuscript. We are also indebted to Dr. S. Isomura, Director of the Aichi Prefectural Institute of Public Health, for his encouragement.

REFERENCES

1 Y. Kitada, K. Tamase, M. Sasaki, Y. Nishikawa and K. Tanigawa, *J. Food Hyg. Soc. Jpn.*, 21 (1980) 480.
2 H. Terada, K. Hisada, Y. Maruyama and Y. Sakabe, *Eisei Kagaku*, 29 (1983) 297.
3 A. Matsunaga, A. Yamamoto and M. Makino, *Eisei Kagaku*, 31 (1985) 269.
4 H. Terada and Y. Sakabe, *J. Chromatogr.*, 346 (1985) 333.
5 M. Sher Ali, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 488.
6 J. T. Hann and I. S. Gilkison, *J. Chromatogr.*, 395 (1987) 317.
7 H. Oka, K. Uno, K.-I. Harada, K. Yasaka and M. Suzuki, *J. Chromatogr.*, 298 (1984) 435.
8 *Standard Methods of Analysis for Hygienic Chemists – with Commentary*, Pharmaceutical Society of Japan, Kinbara, Tokyo, 1980, p. 300.
9 *Standard Methods of Analysis for Hygienic Chemists – with Commentary*, Pharmaceutical Society of Japan, Kinbara, Tokyo, 1980, p. 338.
10 *Standard Methods of Analysis for Hygienic Chemists – with Commentary*, Pharmaceutical Society of Japan, Kinbara, Tokyo, 1980, p. 305.