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Post-column adjustment of conditions for peroxyoxalate chemiluminescence detection for high-performance liquid chromatography

NOBUAKI HANAOKA
Shimadzu-Kansas Research Laboratory, 2095 Constant Avenue, Lawrence, KS 66046 (U.S.A.)
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SUMMARY

Post-column adjustment of the conditions for high-performance liquid chromatography (HPLC) with a peroxyoxalate chemiluminescence (PO-CL) detector was examined using fatty acids, derivatized with 9-anthryldiazomethane, as analytes. Out of fourteen parameters that affect the response of a PO-CL detector, solvent, catalyst, oxalate, mixing and transport volumes for the PO-CL reagents and the reservoir materials were decided on the basis of previously published studies. For the determination of the other parameter values, data for myristic acid, dansylalanine and diphenylanthracene by flow-injection analysis, the results of HPLC measurements on myristic acid and data from published work were compared. From the results, a working knowledge for determining the optimum conditions for PO-CL detection was obtained.

INTRODUCTION

One of the most important tasks in maximizing the capability of high-performance liquid chromatography (HPLC) is optimizing the conditions for the detection of separated analytes. This is especially true for a peroxyoxalate chemiluminescence (PO-CL) detector as there are many parameters that can affect the detector response. To obtain the highest sensitivity and stability with a PO-CL detector, many researchers have investigated the influence of the variables of PO-CL detection[1–7].

In this paper, we report an examination of the post-column adjustment of conditions for HPLC with a PO-CL detector. Fatty acids (FAs) derivatized with 9-anthryldiazomethane (ADAM) were chosen as analytes. These materials have a long excitation wavelength (365 nm)[8] and were considered suitable for PO-CL detection[9–11]. The premise for this study was to modify the eluate for PO-CL detection but not to change the separation conditions, as good separations have already been achieved. ADAM-derivatized FAs were separated with the same column and the same mobile phase as in previous work[12]. The eluate was modified with a condition-

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ing solution to regulate pH, concentration of catalyst and water content for PO-CL detection. A bis(2,4,6-trichlorophenyl)oxalate (TCPO)–H_2O_2 solution was delivered to the mixture of mobile phase and conditioning solution to excite ADAM-FA molecules to exhibit chemiluminescence. Recently, it was found that a TCPO–H_2O_2 mixture was stable when prepared in acetonitrile and stored in a borosilicate glass bottle[13]. This allowed the premixing of TCPO and H_2O_2 and the construction of a three-pump system for the delivery of the mobile phase, the conditioning solution and the TCPO–H_2O_2 mixture.

Initially, five parameters (type of oxalate, solvent, catalyst, and mixing and transport volumes for the PO-CL reagents, and reservoir material) were adopted from published data[1–5,7,13,14]. For the determination of the other variables (pH, temperature, water content, cell volume of the detector, concentrations of imidazole, TCPO and H_2O_2 and flow-rates of the conditioning solution and TCPO–H_2O_2 mixture), the results of flow-injection analysis (FIA) of dansylalanine (Dns-Ala), diphenylanthracene (DPA) and myristic acid (C_{14}) and HPLC measurements of C_{14} are discussed with reference to the results from published work[1–7,15–22]. As a result, useful information concerning the conditioning of the eluate was obtained.

EXPERIMENTAL

Chemicals

ADAM was purchased from Research Organic and used without further purification. FAs [lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}) and stearic (C_{18}) acids] and DPA were purchased from Sigma. All other reagents were the same as those in the previous study[7].

Apparatus

The HPLC system is shown in Fig. 1. The column (C) was a 25 cm × 4.6 mm I.D. Zorbax ODS (Du Pont) and the column oven (V) was a Shimadzu CTO-6A. The waterbath (W) was a Brinkman RH-3 with both heating and cooling functions. A_1 and A_2 were stainless-steel pipes (1.1 mm × 0.5 mm I.D.). B_1 and B_2 were stainless-steel pipes, 1.1 m in length and of 0.5 mm I.D. B_1 and B_2 were stainless-steel pipes of

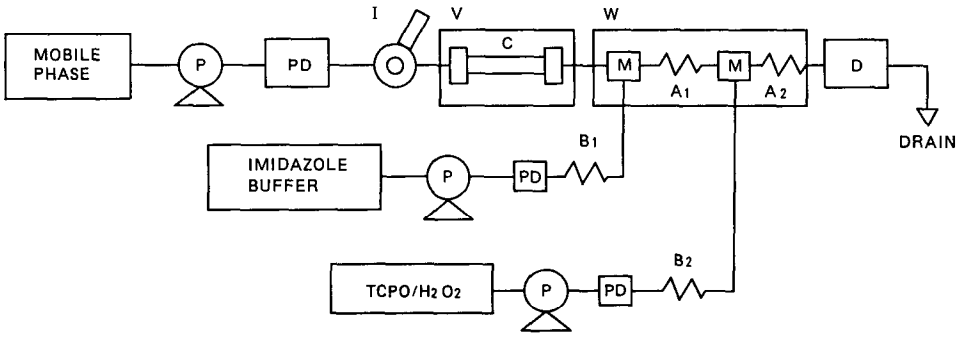

Fig. 1. Schematic diagram of HPLC system: P, pump; PD, pulse damper; I, injector; C, column; D, detector; V, column oven; W, waterbath; B_1, B_2, 0.1 mm I.D. stainless-steel tubes, each of length adjusted according to the flow-rates to apply pressures between 70 and 100 kg/cm^2; A_1, A_2, 1.1 m × 0.5 mm I.D. stainless-steel tubes.

0.1 mm I.D. and their lengths were adjusted according to the flow-rate to apply a pressure of between 70 and 100 kg/cm^2 to the solutions. For example, the length was *ca.* 5 m for an aqueous imidazole buffer at a flow-rate of 0.2 ml/min for an applied pressure of 75 kg/cm^2. PD was a Shimadzu high-performance damper unit to decrease the pulsation from the pumps. The reservoir for the TCPO–H_2O_2 mixture was a Pyrex borosilicate glass bottle purchased from American Scientific Products. For FIA measurements, the column was replaced by a stainless-steel pipe (2 m × 0.5 mm I.D.). All other units including the detector were the same as those in the previous work[7].

Procedure

HPLC measurements. The mobile phase [acetonitrile–water (96:4, v/v)] and the flow-rate (1.2 ml/min) were the same as in ref. 12. The sample was C_{14} derivatized with ADAM according to the procedure of Nimura and Kinoshita[8]; 100 pmol in 20 μl of methanol was injected. A standard set of measuring conditions as outlined below was applied to all the measurements.

The conditioning solution was 100 m*M* aqueous imidazole buffer (pH 7.55; with this buffer, the pH of the final solution was 6.8) and the flow-rate was 0.5 ml/min. The oxalate–H_2O_2 solution was a mixture of equal volumes of 0.3 m*M* TCPO and 1.5 m*M* H_2O_2 in acetonitrile and delivered at 1.0 ml/min. The temperatures of the column oven and the waterbath were set at 40°C and room temperature (*ca.* 22°C), respectively. The cell volume of the detector was 30 μl. When one parameter was under examination, the others were kept unchanged.

FIA measurements. The standard set of conditions including the mobile phase were adopted except for the column temperature (see below). A 4-pmol amount of either Dns-Ala or DPA in 20 μl of mobile phase was measured. The C_{14} analytical sample was prepared by evaporating the methanol after derivatization and dissolving of the residue in mobile phase. This sample concentration was also 4 pmol in 20 μl.

Measurements of pH effects. The pH of the conditioning solution was varied from 5.5 to 8.5 with nitric acid. The pH of the final solutions was also measured.

Water content. The water content of the imidazole solution varied from 20 to 100% for the tests. The pH of each solution was adjusted to 7.7 with nitric acid. The other measuring conditions were the same as above.

Temperature. In the HPLC measurements, the waterbath was kept at room temperature (*ca.* 22°C) and the temperature of the column oven was varied from 22 to 50°C. Next, the temperatures of both the waterbath and the column oven were changed simultaneously from room temperature to 50°C. In FIA measurements, the column oven was not used and the temperature of the waterbath was varied from room temperature to 50°C.

Concentration of reagents. The measured range of imidazole concentration in the conditioning solution was 20–200 m*M*. The change in the pH of final solution caused by varying the concentration of imidazole was countered by regulating the pH of the buffer solution. The pH of the buffer was 7.7 at 20 m*M*, 7.65 at 50 m*M* and 7.55 at 100 m*M* imidazole to achieve pH 6.8 for the final solution. The concentration ranges of TCPO and H_2O_2 in the TCPO–H_2O_2 mixture were 0.1–1.0 and 0.5–5.0 m*M*, respectively.

Cell volume. The cell volume of the detector was varied from 30 to 280 μl by

changing the I.D. of the flow cell. The cells used here were straight glass tubes 40 mm in length.

Flow-rate. The flow-rates of the imidazole buffer and the TCPO–H_2O_2 solution were varied independently from 0.2 to 0.6 ml/min in 0.2 ml/min steps and from 0.6 to 1.8 ml/min in 0.6 ml/min steps, respectively.

Measurements of FAs. A 125-nmol amount of each C_{12}, C_{14}, C_{16} and C_{18} were dissolved together in 100 ml of methanol and derivatized with ADAM according to the method described above. A 20-μl volume of this solution was subjected to HPLC under the decided conditions (see below).

RESULTS AND DISCUSSION

Adoption of parameter values from published work

The fourteen factors that affect the response of a PO-CL detector are temperature, pH, water content, solvent, catalyst, oxalate, concentration of oxalate, concentration of H_2O_2, concentration of catalyst, volume of mixing tube A_2, cell volume of detector, flow-rate of imidazole buffer, flow-rate of oxalate–H_2O_2 mixture and reservoir material[1–7,13,14]. If we obtain three data points for each variable, the total number would be 3^{14} = 4 783 969, making it highly impractical to acquire all the data; hence the number of data points must be reduced by determining some parameter values and/or defining their ranges in advance. Results from the literature were then used to fix certain parameters.

The following parameters were decided upon prior to the measurements: the oxalate was TCPO, which has been shown to be stable in a mixture with H_2O_2[13,14]; acetonitrile was used as a solvent because in it the PO-CL intensities are the highest[1] and the TCPO–H_2O_2 mixture is stable[13,14] (ethyl acetate is widely used as a solvent for TCPO[1–4,15,17–22], but a TCPO–H_2O_2 mixture is unstable in this solvent[13,14]); imidazole was the best catalyst[7]; the reservoirs were Pyrex borosilicate glass bottles in which TCPO–H_2O_2 solution is stable for more than 6 h[13,14]; and the mixing tube A_2 was 1.1 m × 0.5 mm I.D. and the total volume of this tube and the mixer (M, 26 μl) was 242 μl. The time required for the fluorescent species to reach the detector after the start of the PO-CL reactions (t_1) was *ca.* 5 s when the total flow-rate was 3 ml/min. The PO-CL reaction reaches maximum intensity in *ca.* 5 s under normal conditions[7].

The examining ranges for the other parameters could also be established with the help of the published results. The values decided on were as follows: the temperature should be from room temperature to 50°C[7]; the optimum pH is between 6.0 and 8.0[2–5,7,15–22] and the measured pH range is 5.5–8.5; a rather hydrophobic final solution is preferable for stable measurements[1], and the flow-rate of the aqueous conditioning solution should be less than 0.6 ml/min; the concentration of TCPO must be more than 0.1 m*M* to avoid decomposition; the concentration ratio of TCPO–H_2O_2 was 1:5 to obtain simultaneously a stable solution and the highest signal intensity[13]; the cell volume was varied from 30 to 300 μl and the "chemical band narrowing effect"[3] was examined; in addition, the flow-rate of the TCPO–H_2O_2 mixture was fixed at less than 2 ml/min to avoid too rapid consumption of reagents.

Optimum pH

Many optimum pH values for PO-CL measurements using TCPO have been

reported[2–5,7,15–22]. Among these values, those for the aqueous buffer or mobile phase are between 7.0 and 8.0[3–5,15–22]. The optimum pH of the final mixture of aqueous buffer and the acetonitrile solution of TCPO–H_2O_2 was determined to be 6.7 by Hanaoka *et al.*[7]. In this work, the optimum pH values for imidazole buffer and the final solution were obtained by HPLC measurements of C_{14} and were 7.7 and 6.8, respectively. At the same time, the three fluorescent species described under Experimental were measured by FIA, and the optimum values obtained were the same as those for C_{14} by HPLC. From these results and the published values, it may be concluded that the optimum pH for PO-CL reactions with TCPO is independent of the nature of the fluorescent species and is between 7.0 and 8.0 in buffer or mobile phase and *ca.* 6.8 in the final solution.

It was also found that the background and noise levels changed according to the change in signal intensity and, consequently, the signal-to-noise ratio of the measurements was almost constant in the pH range 6.3–7.3 in the final solution. This finding shows that very strict attention to the optimum pH value that gives the highest signal intensity is not necessary for actual HPLC measurements as the signal-to-noise ratio is fairly constant over the pH range adopted.

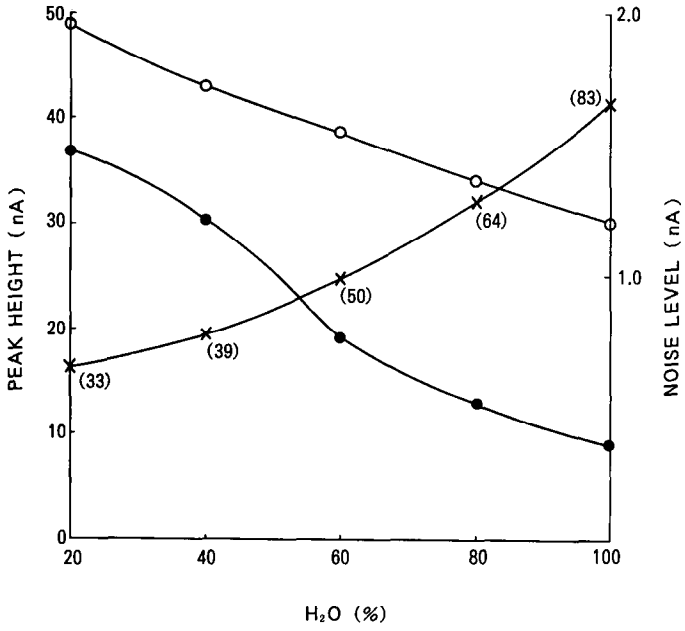

Fig. 2. Effect of water content on the peak height of myristic acid (C_{14}). Values in parentheses are signal-to-noise ratios for 100 m*M* C_{14}. LC column, 25 cm × 4.6 mm I.D. Zorbax ODS; mobile phase, mixture of 960 ml of acetonitrile and 40 ml of water; flow-rate, 1.2 ml/min; column temperature, 40°C; 100 pmol of C_{14} derivatized with ADAM were injected. The eluate conditions were adjusted with 100 m*M* aqueous imidazole buffer (pH 7.55) and C_{14} was excited with an admixture of 0.3 m*M* TCPO and 1.5 m*M* H_2O_2. The flow-rate of the buffer and TCPO–H_2O_2 solution were 0.5 and 1.0 ml/min, respectively. The water content of the imidazole buffer was varied from 20 to 100%. ○, Peak height; ●, noise level; ×, signal-to-noise ratio.

Effect of water content

The effect of water content as measured by HPLC of C_{14} is shown in Fig. 2. Peak height was inversely proportional to the water content with a rate of decrease of height of *ca.* 1.2% per 1% increase in water content. In spite of the fact that the ADAM-derivatized C_{14} sample contained considerable impurities, the response curve for the sample obtained by FIA over a range of water content agreed very well with the results from HPLC. Nevertheless, the responses to Dns-Ala and DPA were slightly different from that for the C_{14} sample, and the rates of decrease of height were -0.7% and -1.8%, respectively, for each 1% increase in water content. These results indicate that the effect of water content on peak height depends on the analyte itself. The background and the noise levels are also dependent on the water content and, as shown in Fig. 2, the best signal-to-noise ratio was observed at 100%. Hence it was decided to make the buffer solution 100% aqueous.

Temperature effects

First, the temperature effects on HPLC measurements of C_{14} were examined. When the waterbath was kept at room temperature (*ca.* 22°C), the noise level was independent of the change in temperature of the column oven. Therefore, the temperature of the oven was set at 40°C, the same as in the published separation conditions[12]. However, when the temperature of the waterbath was elevated from 22 to 40°C, the noise level increased by *ca.* 50%. The increase in peak height caused by this change was only 20%, and it was decided to maintain the waterbath at room temperature. Increased responses for the other analytes were measured in the same waterbath temperature range by FIA and were also *ca.* 20%, indicating that the temperature of the PO-CL reaction is relatively independent of the species of the analyte.

Cell volume of the detector

De Jong *et al.*[3] examined the influence of cell volume on the heights and widths of HPLC peaks. They found that the influence of the cell volume of a PO-CL detector on peak broadening was much smaller than that of the other detectors, and named this phenomenon the "chemical band narrowing effect". In our study, the cell volume was varied from 30 to 280 μl, and the increase in peak width of C_{14} was measured by HPLC. The results are shown in Table I. De Jong *et al.*[3] observed no band broadening with a 70-μl cell, but we observed a *ca.* 6% increase in the peak width at

TABLE I

INFLUENCE OF FLOW CELL VOLUME ON PEAK HEIGHT AND PEAK WIDTH

C_{14} was measured by HPLC. Experimental conditions as in Fig. 2.

Cell volume (μl)	*Half peak width (μl)*	*Peak height (nA)*
30	1485	12.6
70	1570	24.1
125	1710	32.8
200	1850	37.4
280	1950	39.5

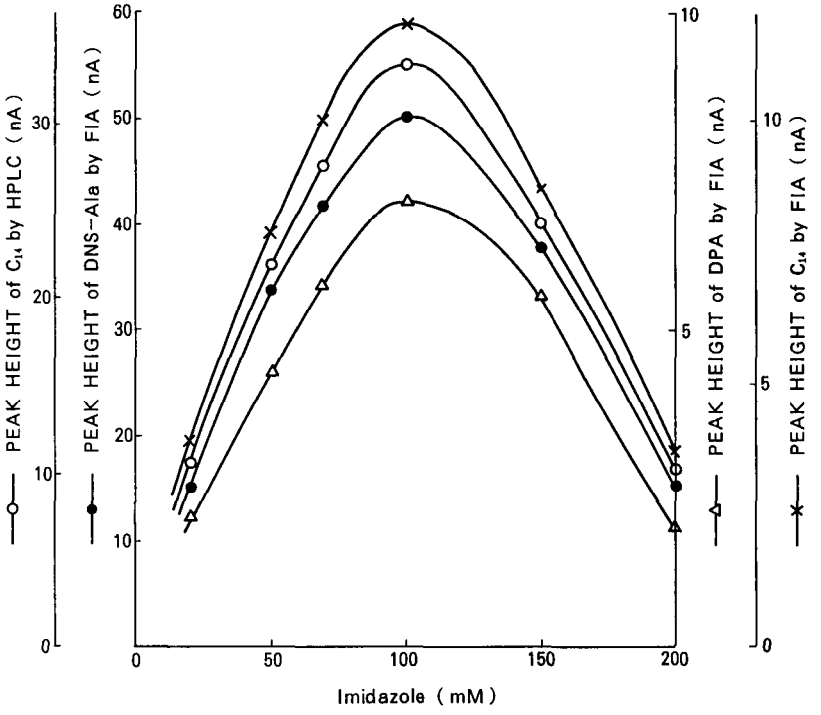

Fig. 3. Effect of imidazole concentration on peak height. Measurement conditions as in Fig. 2. The imidazole concentration in the conditioning solution was varied from 20 to 200 m*M*.

half-height here with a 70-μl flow cell. The different observations might be due to the differences in the total flow-rate or in the composition of the final solution, or both. The cell volume decided on for this study was 30 μl.

Effects of concentrations of imidazole, TCPO and hydrogen peroxide

The concentration ranges of TCPO and H_2O_2 and the concentration ratio were optimized according to the published results (see above). At the same time, it was assumed that the signal intensity would be proportional to the concentration of each reagent[7]. Both HPLC measurements of C_{14} and FIA measurements of the other substances confirmed this supposition. Moreover, the background and noise levels changed according to the change in TCPO-H_2O_2 concentration; the signal-to-noise ratio was constant when the concentrations of TCPO and H_2O_2 were in the range 0.3–1.0 and 1.5–5.0 mM, respectively, while maintaining the 1:5 concentration ratio as previously established.

The influence of imidazole concentration on peak height is shown in Fig. 3. As is clearly seen, the influence was the same for all the analytes, and the largest signal was obtained at 100 m*M* (18.5 m*M* in the final solution). Also, the signal-to-noise ratio remained constant over the imidazole concentration range 20–100 m*M*, but the noise level increased and the signal-to-noise ratio deteriorated when the imidazole concentration exceeded 150 m*M*.

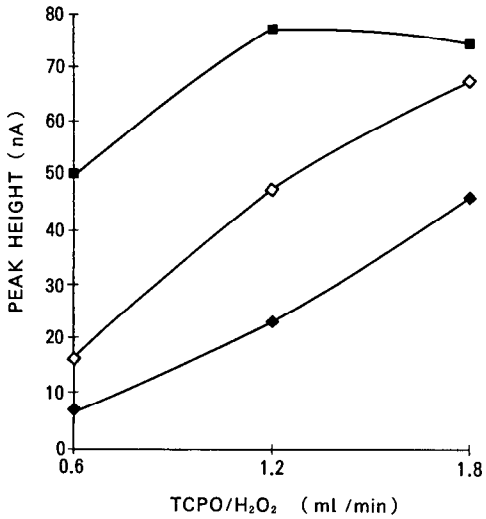

Fig. 4. Effect of flow-rate on the peak height of C_{14} measured by HPLC. Measurement conditions as above. The flow-rates of the imidazole solution and TCPO–H_2O_2 mixture were changed from 0.2 to 0.6 and 0.6 to 1.8 ml/min, respectively. Flow-rates of imidazole solution: ◆, 0.6; ◇, 0.4; and ■, 0.2 ml/min.

Flow-rates of buffer solution and TCPO–H_2O_2 mixture

The parameters evaluated so far could be varied independently, and changes in their individual values did not affect the other parameters. However, changes in the flow-rates of the solutions can vary many of the controlling parameters of the final solution: pH, water content, concentration of TCPO, concentration of H_2O_2, concentration of imidazole, t_1 and t_2. In addition to these parameters, the ratios of sample

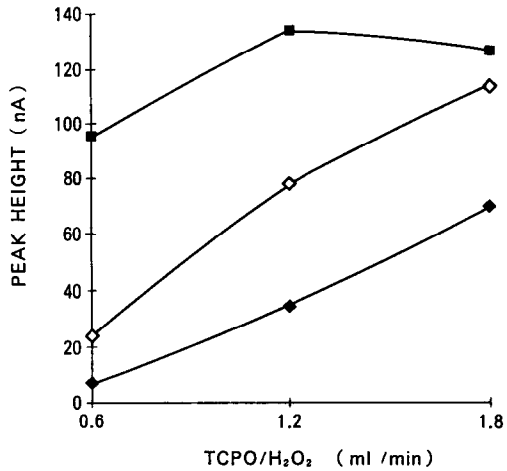

Fig. 5. Peak heights of Dns-Ala measured by FIA. A 2 m × 0.5 mm I.D. stainless-steel pipe was used instead of an LC column. 4 pmol of Dns-Ala dissolved in 20 μl mobile phase were injected. Measurements were made at room temperature. Other conditions and symbols as in Fig. 4.

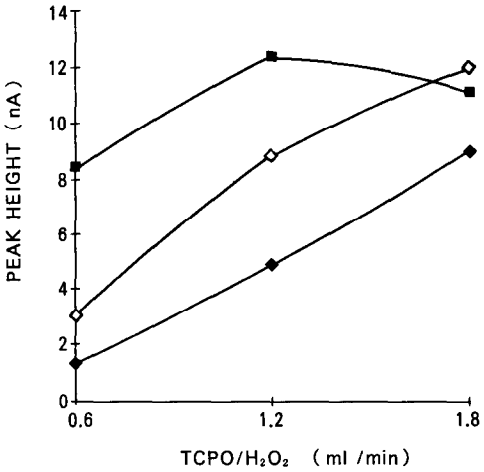

Fig. 6. Peak heights of DPA measured by FIA. Measurement conditions and symbols as in Fig. 5.

dilution by buffer and TCPO–H_2O_2 solutions are also dependent on each flow-rate, and affect the peak heights. Owing to these many factors, it is almost impossible to predict the effects of change in flow-rates from the previously published results, so the optimum values have to be determined empirically. The results of the HPLC assay of

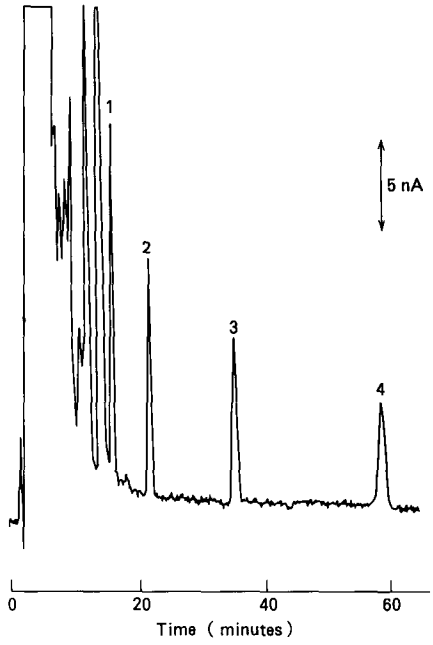

Fig. 7. Chromatogram of 25 pmol each of fatty acids: (1) C_{12}; (2) C_{14}; (3) C_{16}; (4) C_{18}. Separation conditions as in Fig. 2. The imidazole solution was 100 m*M* in 1000 ml of water (pH 7.55) and the flow-rate was 0.2 ml/min. The TCPO–H_2O_2 solution was a mixture of 0.3 m*M* TCPO and 1.5 m*M* H_2O_2 and the flow-rate was 0.6 ml/min. The waterbath was maintained at room temperature.

C_{14} and FIA of Dns-Ala and DPA are shown in Figs. 4, 5 and 6, respectively. The influence of flow-rate on the peak height of C_{14} is similar to that for Dns-Ala, but slightly different from that for DPA. These results suggests that the influence of flow-rate is slightly different for each analyte. Within the measured flow-rate range, no large differences in signal-to-noise ratio were observed for the analytes examined.

Determination of conditions for measurement of FAs

From examination of the results above, the measuring conditions for ADAM-derivatized FAs were established as follows. The conditioning solution was a 100-m*M* aqueous solution of imidazole (with which the best signal-to-noise ratio was obtained). The pH was adjusted to 7.55 with nitric acid to make the pH of final solution 6.8. The flow-rate was 0.2 ml/min. TCPO and H_2O_2 were dissolved in pure acetonitrile at concentrations of 0.3 and 1.5 m*M*, respectively, and the flow-rate was 0.6 ml/min. The adopted concentrations of TCPO and H_2O_2 are the lowest values with which a good signal-to-noise ratio was acquired. The flow-rate of each solution was decided for the same reason. The temperatures of the column oven and the waterbath were set at 40°C and ambient, respectively. A chromatogram of FAs under these conditions is shown in Fig. 7. The reproducibility of peak heights was within $\pm 2\%$ and the detection limit for C_{14} was less than 1 pmol, which is slightly better than reported previously[8,23].

CONCLUSION

Of fourteen affecting factors, five were decided from previously published results: the oxalate was TCPO; the solvent was acetonitrile; the catalyst was imidazole; the material of the reservoir for the TCPO–H_2O_2 mixture was Pyrex borosilicate glass; and the length of the stainless-steel tube was adjusted so that t_1 was 5 s. The other important information also derived from those papers was that the concentration of TCPO must be more than 0.1 m*M*, the flow-rate of the conditioning solution should be less than 0.6 ml/min and the concentration ratio of TCPO to H_2O_2 should be 1:5. The remaining factors were examined in this study, and the following results were obtained.

The optimum pH of PO-CL with TCPO is independent of the nature of the analytes, and should be *ca.* 6.8 in the final solution. The signal-to-noise ratio is fairly constant in the pH range *ca.* 6.3–7.3, and very strict attention to some optimum value is not necessary for actual HPLC assay.

The effects of water content on peak heights are different for each analyte.

The temperature of the eluent affects the noise level considerably. The eluent should be kept at room temperature.

"Chemical band narrowing effects" as observed by de Jong *et al.*[3] are not very large with TCPO and a total flow-rate of 2.7 ml/min. The cell volume should be 30 μl or less to avoid the opposite effect, *i.e.*, band broadening.

The signal-to-noise ratio remained constant in concentration ranges of TCPO, H_2O_2 and imidazole of 0.3–1.0, 1.5–5.0 and 10–100 mM, respectively.

The signal-to-noise ratio was also unchanged for flow-rates of the conditioning solution and TCPO–H_2O_2 mixture of 0.2–0.6 and 0.6–1.8 ml/min, respectively.

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