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A HIGH-SPEED LIQUID CHROMATOGRAPH WITH A FLOW-SPECTROFLUORIMETRIC DETECTOR AND THE ULTRAMICRO-DETERMINATION OF AROMATIC COMPOUNDS

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SUMMARY

A high-speed liquid chromatograph has been used with a new spectrofluorimetric detector, which is more sensitive than a normal ultraviolet absorption or spectrophotometric detector for fluorescent compounds. The spectrofluorimeter is equipped with double-beam optics and with a flow-cell, 3 μl in volume, and is used to record the emission and excitation spectra of the separated components during the chromatographic separation by stopping the elution at peak maxima. This technique enables both qualitative identification and quantitative determination of the separated components. Selective recording of chromatograms is possible by varying the wavelengths for emission and excitation.

The technique is demonstrated using mixtures of vitamins B_2, B_3 and B_6, and of naphthalene, anthracene, pyrene, benz(*a*)anthracene, benz(*a*)pyrene and benz(*e*)pyrene have been separated and identified.

INTRODUCTION

Since the development of the first automatic liquid chromatograph in 1961[1], which permitted the separation of many compounds on various types of columns and the detection of the compounds in eluents with a detector selective in the range 220–700 nm (after colour development if necessary) in a manner similar to the automatic fractionation method, the progressive development of automatic liquid chromatographs in Japan has been continued, including applications in high-performance and high-speed liquid chromatography[2–9].

In the development of modern analytical liquid chromatography, a detection system with highly efficient column packing materials, high-pressure delivery pumps and ultramicro-sensitive detectors is important. Spectrofluorimetry permits both the qualitative and the quantitative detection of separated components of fluorescent compounds in ultramicro-amounts.

This paper deals with recent progress with a high-speed liquid chromatograph equipped with a new high-pressure delivery pump of a screw-driven syringe type with a pulse motor, and with a new spectrofluorimetric detector that is more sensitive than an ordinary ultraviolet absorption detector for fluorescent compounds, with characteristic excitation and emission spectra in the ultraviolet and visible region ranging from 220 to 700 nm. The successful application of the new chromatograph to the analysis of vitamins and aromatic hydrocarbons, and to the ultramicro-determination of fluorescent compounds in nanogram or picogram amounts, is described.

INSTRUMENTATION

A high-speed liquid chromatograph equipped with a new high-pressure delivery pump[10] and a flow-spectrofluorimetric detector was designed and constructed*. The effluent delivery system consists of a high-pressure pump, which can deliver 0–200 ml of the effluent liquid at a flow-rate of 0–3 ml/min or 0–0.3 ml/min under a pressure of 0–350 kg/cm^2 from a carrier eluent reservoir of volume 500 ml. The high-pressure pump is of a motor-driven syringe type with a capacity of 500 ml, which can be filled rapidly from the reservoir within 5 min.

The column system consists of a stainless-steel column tube of I.D. 2.3 mm (alternatively 1.6–6.0 mm) and length 50 cm (alternatively 10–100 cm), and a sample injector, which can be operated under the high pressure.

For the detection system, either an ultraviolet absorption photometer, a differential refractometer or a spectrofluorimeter can be used. The spectrofluorimeter is equipped with double-beam optics and a flow cell of volume 3 μl, as shown by the schematic diagram of the optical system in Fig. 1.

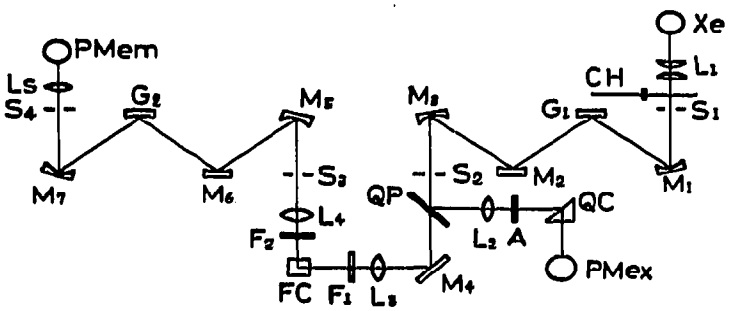

Fig. 1. Schematic diagram of the optical system of the spectrofluorimetric detector for the high-speed liquid chromatograph. Xe, light source (xenon lamp); PMem, photomultiplier for emission light; PMex, photomultiplier for excitation light; QP, quartz plate; QC, quantum counter; FC, flow cell, 3 μl in volume; CH, chopper; G_1 and G_2, grating monochromators; M_1–M_7, mirrors; L_1–L_5, lenses; F_1 and F_2, filters; S_1–S_4, slits.

EXPERIMENTAL

Materials

Jascopack CV-02-500 cation-exchange column packing material and Jasco-

* Available commercially as the JASCO FLC-350-FP-4 fast liquid chromatograph from the Japan Spectroscopy Company Ltd., Tokyo, Japan.

pack SV-02 partition (chemical bonded) column packing material were used in the reversed phase on this high-speed column liquid chromatograph.

A 30:70 mixture of water and methanol was used as the eluent for the reversed-phase partition chromatography and a 0.5 *M* KH_2PO_4 solution, pH 7.0, for the cation-exchange chromatography. Vitamins B_2, B_3 and B_6, products of Wako Pure Chemicals Co. Ltd., Osaka, Japan, and pyrene, benz(*a*)pyrene, benz(*e*)pyrene, anthracene, benz(*a*)anthracene and naphthalene from Tokyo Kasei Co. Ltd, Japan, were used.

Methods

The operating procedures of the high-speed liquid chromatograph were the usual ones except for the operation of the flow-spectrofluorimeter. For the ultramicro-determination of the separated components with the spectrofluorimeter, it is necessary to select a wavelength at which maximum excitation and emission spectra either over-

TABLE I

SELECTION OF SUITABLE WAVELENGTHS FOR QUALITATIVE AND QUANTITATIVE DETECTION OR THE USUAL AND SPECIFIC DETECTION

A, B, and C, represent the most sensitive detection of the respective components, and a, b, and c represent these components detectable at the wavelengths selected.

Wavelength of emission	*Wavelength of exitation*		
Qualitative			
	Ex_1	Ex_2	Ex_3
Em_1	a b c	b c	c
Em_2	b c	b c	c
Em_3	c	c	c
Quantitative			
	Ex_a	Ex_b	Ex_c
Em_a	Ab	a b	b
Em_b	ab	a B	b
Em_c	—	b c	b C

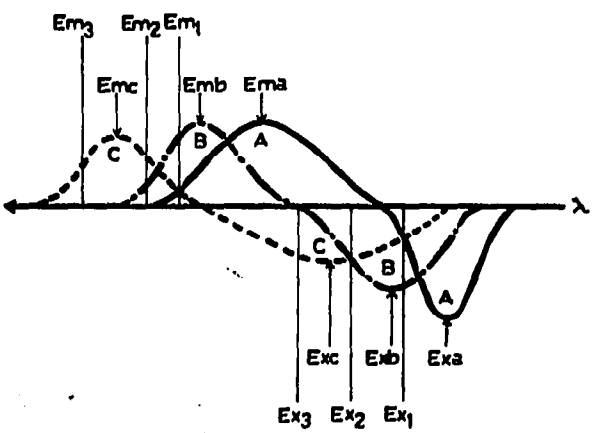

Fig. 2. Schematic diagram of the procedures for spectrofluorimetric detection and measurements of fluorescence excitation and emission spectra. For selection of wavelengths, see text and Table I.

lap or do not overlap. The schematic spectra for the measurements are shown in Fig. 2, and the selection of suitable wavelengths for the detection is presented in Table I.

The usual detections on a chromatogram were carried out at a wavelength (*e.g.*, Em_1 in Fig. 2) in common with the emission spectra of all of the fluorescent components, when all of the components were excited at a wavelength (*e.g.*, Ex_1 in Fig. 2) in common with all of the excitation spectra, as shown in Fig. 2 and Table I. For the specific detection of a characteristic component on the chromatogram, the wavelength (*e.g.*, Em_c in Fig. 2) of the maximum emission of the specific component was used, when the component was excited at the wavelength (*e.g.*, Ex_c in Fig. 2) of its maximum excitation, as shown in Fig. 2 and Table I. This procedure enables one to determine the specific component in ultramicro-amounts both qualitatively and quantitatively. Table I also gives examples of Em_1, Em_2 and Em_3 suitable for qualitative detection and Em_a, Em_b and Em_c suitable for quantitative detection. Fluorescence excitation and emission spectra were obtained by scanning the chromatographic peak of the component when the elution of the component from the chromatographic column was stopped and the component was in the flow cell.

RESULTS

Spectrofluorimetric detection of vitamins B_2, B_3 and B_6 on chromatograms

Cation-exchange chromatograms of a mixture of vitamins B_2, B_3 and B_6 are shown in Fig. 3. When excitation and emission wavelengths of 330 nm and 505 nm, respectively, were used, three peaks were detected and peaks 1, 2 and 3 were identified

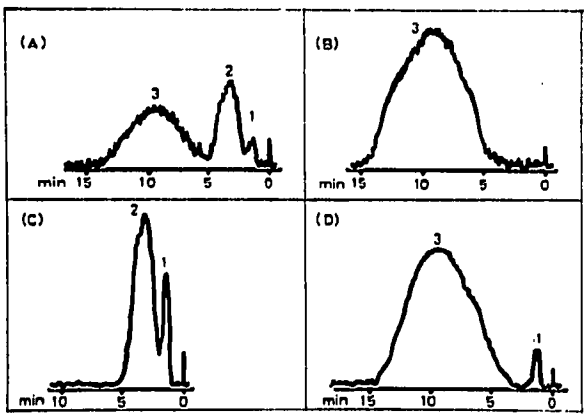

Fig. 3. Cation-exchange chromatograms of a mixture of vitamins B_2, B_3 and B_6. Identifications: peak 1, vitamin B_3; peak 2, vitamin B_2; peak 3, vitamin B_6. (A) Detected at Em 505 nm after excitation at Ex 330 nm. (B) Ex 285 nm and Em 380 nm. (C) Ex 330 nm and Em 425 nm. (D) Ex 345 nm and Em 525 nm.

Fig. 4. Fluorescence excitation (below, solid line) and emission (above) spectra measured on the chromatogram peaks of the aromatic hydrocarbons, and compared with ordinary absorption spectra (below, broken line). Identifications: peak 1, naphthalene excited (Ex) at 275 nm and emitted (Em) at 325 nm; peak 2, anthracene, Ex 320 nm and Em 410 nm; peak 3, pyrene, Ex 320 nm and Em 380 nm; peak 4, benz(*a*)anthracene, Ex 280 nm and Em 395 nm; peak 5, benz(*a*)pyrene, Ex 280 nm and Em 430 nm; peak 6, benz(*e*)pyrene, Ex 285 nm and Em 390 nm.

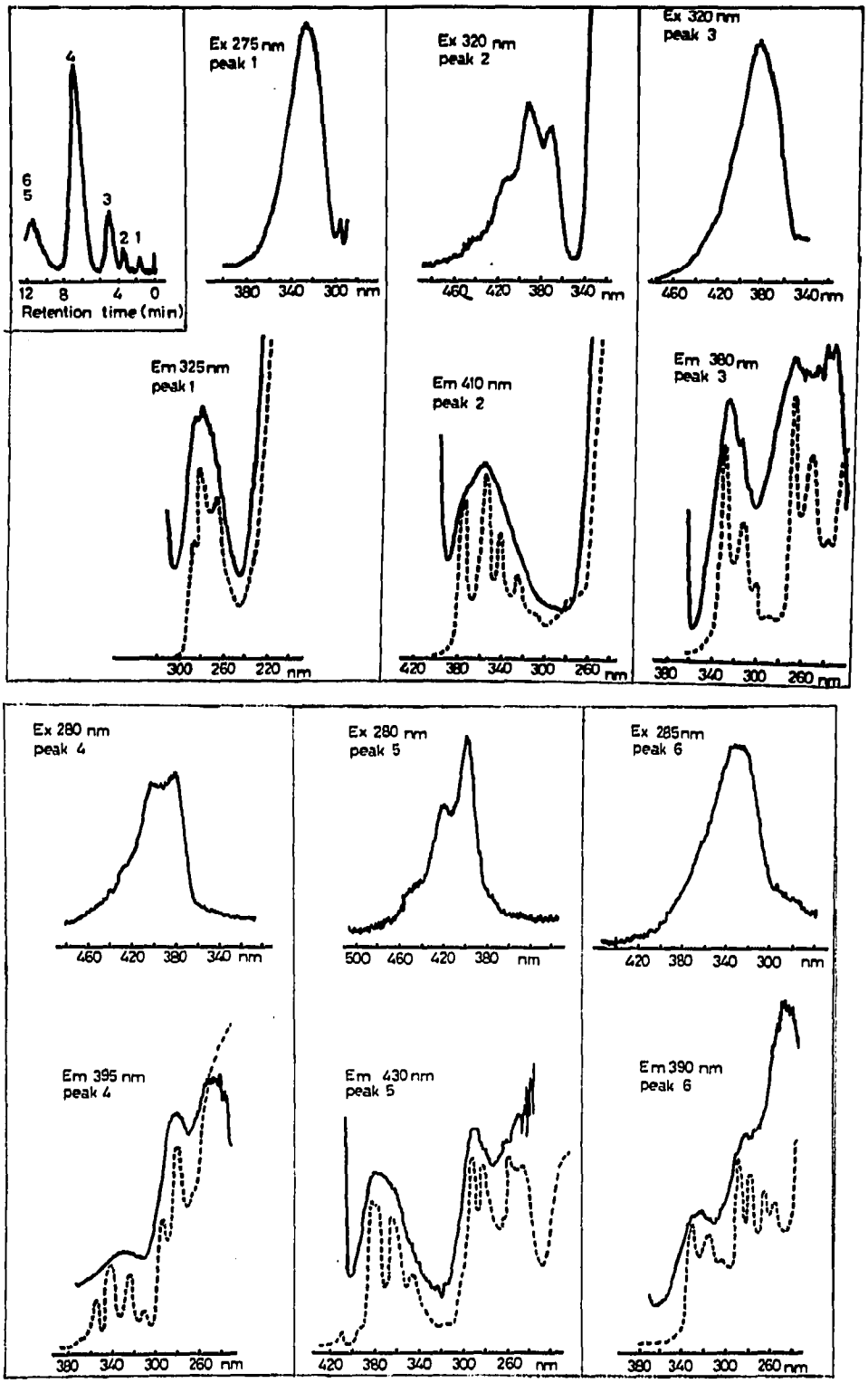
4
6
5
3
2 1
12 8 4 0
Retention time (min)
Ex 275 nm
peak 1
380 340 300 nm
Ex 320 nm
peak 2
460 420 380 340 nm
Ex 320 nm
peak 3
460 420 380 340 nm
Em 325 nm
peak 1
300 260 220 nm
Em 410 nm
peak 2
420 380 340 300 260 nm
Em 380 nm
peak 3
380 340 300 260 nm
Ex 280 nm
peak 4
460 420 380 340 nm
Ex 280 nm
peak 5
500 460 420 380 nm
Ex 285 nm
peak 6
420 380 340 300 nm
Em 395 nm
peak 4
380 340 300 260 nm
Em 430 nm
peak 5
420 380 340 300 260 nm
Em 390 nm
peak 6
380 340 300 260 nm

Fig. 4.

as vitamins B_3, B_2 and B_6, respectively, as shown in Fig. 3A. However, when an excitation wavelength of 285 nm and an emission wavelength of 380 nm were used, only the single peak of vitamin B_6 was observed, as shown in Fig. 3B. This effect occurs because vitamin B_3 was not excited at 285 nm although the two peaks of vitamins B_3 and B_6 were detectable at 380 nm. Similarly, when an excitation wavelength of 330 nm and an emission wavelength of 425 nm were used, two peaks of vitamins B_3 and B_6 appeared, as shown in Fig. 3D because there was no emission from vitamin B_2 at 425 nm. When an excitation wavelength of 345 nm and an emission wavelength of 525 nm were used, two peaks of vitamins B_3 and B_2 appeared, as shown in Fig. 3C, because of the negligible emission from vitamin B_6 at 525 nm.

Spectrofluorimetric detection of naphthalene, anthracene, pyrene, benz(a)anthracene, benz(e)pyrene and benz(a)pyrene on chromatograms

The reversed-phase partition chromatogram of a mixture of naphthalene, anthracene, pyrene, benz(*a*)anthracene, benz(*e*)pyrene and benz(*a*)pyrene, and the fluorescence excitation and emission spectra of the separated peaks on the chromatogram, are given in Fig. 4. The fluorescence spectra of naphthalene excited at 275 nm and emitted at 325 nm were measured on the chromatographic peak which appeared first at 2 min retention time on the reversed-phase partition chromatogram, as shown in Fig. 4, peak 1. The fluorescence excitation spectrum of peak 1 was compared with the absorption spectrum of naphthalene, as shown in Fig. 4, peak 1. Similar results were obtained on peak 2 of anthracene, which appeared second at 3 min retention time, excited at 320 nm and emitted at 410 nm; on peak 3 of pyrene, which appeared third at 5 min retention time, excited at 320 nm and emitted at 380 nm; on peak 4 of benz(*a*)anthracene, which appeared fourth at 8 min retention time, excited at 280 nm and emitted at 395 nm; on peak 5 of benz(*e*)pyrene, which appeared fifth at 13 min retention time, excited at 280 nm and emitted at 430 nm; and on peak 6 of benz(*a*)pyrene, which appeared sixth at 14 min retention time, excited at 285 nm and emitted a 390 nm, as shown in Fig. 4. Good coincidence of the fluorescence excitation spectra of the peaks with the absorption spectra of authentic specimens was observed.

Ultramicro-determination of benz(a)pyrene and benz(e)pyrene by spectrofluorimetric detection

Fluorescence emission spectra of benz(*a*)pyrene and benz(*e*)pyrene, and the fluorescence excitation spectra which were compared with the absorption spectra, are presented in Figs. 5 and 6. While the absorption spectra of benz(*a*)pyrene and benz(*e*)pyrene were recorded on the solutions with a concentration of 10^{-4} g/ml, a fluorescence excitation spectrum of benz(*a*)pyrene could be recorded on a solution with a concentration as low as 10^{-9} g/ml, and similarly benz(*e*)pyrene was observed at a concentration as low as 10^{-13} g/ml.

DISCUSSION

The spectrofluorimetric detector of the high-speed chromatograph has several advantages for modern analytical liquid chromatography. Sensitive detection can be achieved and the determination of nanogram and picogram amounts of fluorescent compounds separated on the chromatograph is carried out by measuring both the

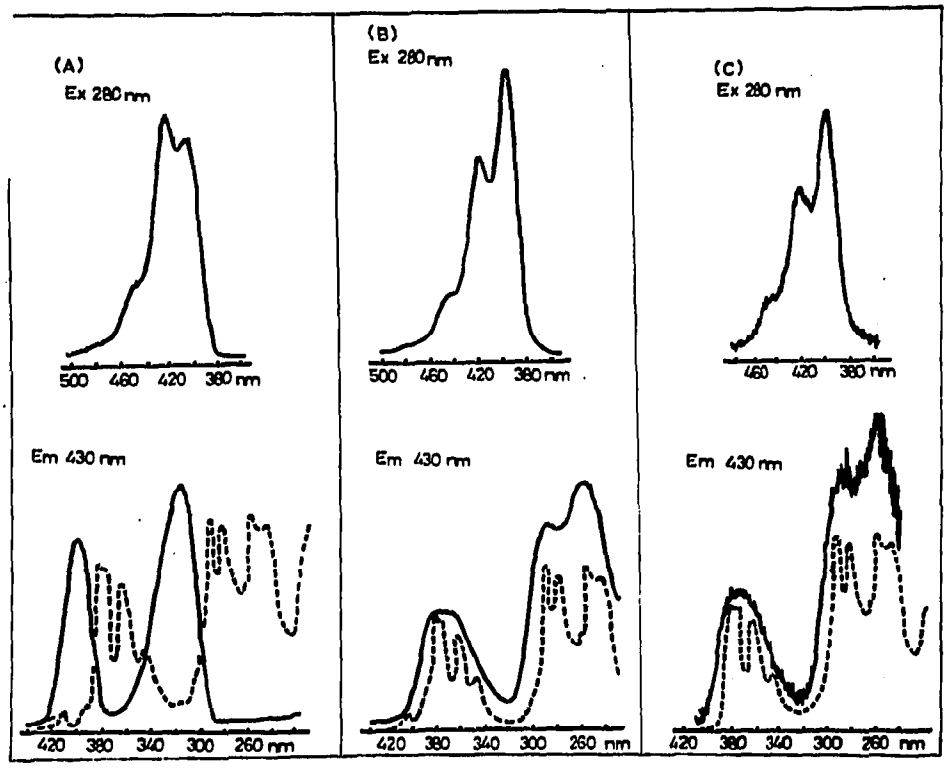

Fig. 5. Fluorescence excitation (below) and emission (above, solid line) spectra of the eluents from the column and compared with ordinary absorption spectra of benz(*a*)pyrene (below, broken line, 10^{-4} g/ml) for the ultramicro-determination of the chromatogram peaks. (A) Ex 280 nm, Em 430 nm, both 10^{-4} g/ml. (B) 10^{-6} g/ml; absorption 10^{-4} g/ml. (C) 10^{-8} g/ml; absorption 10^{-4} g/ml.

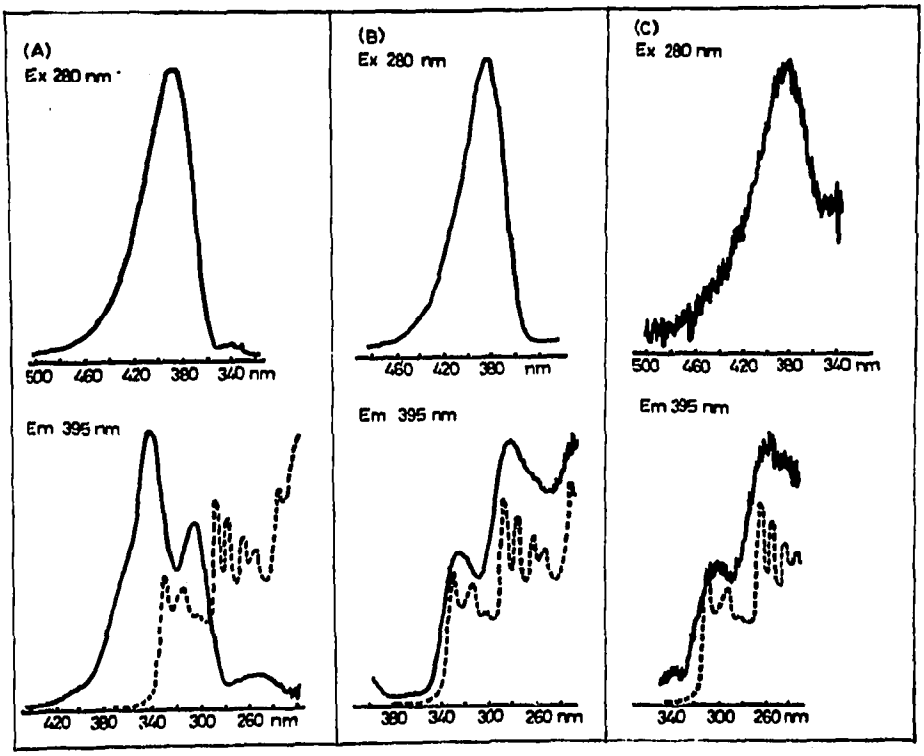

Fig. 6. Fluorescence excitation (below) and emission (above, solid line) spectra of the eluents from the column and compared with ordinary absorption spectra of benz(*e*)pyrene (below, broken line,

fluorescence emission and excitation spectra of the components at the maximum elution point of the peaks. Selective detection of the peaks of fluorescent compounds on the chromatograms is possible.

Excellent high sensitivity for measuring the fluorescence excitation or emission spectra of minute amounts of the samples was obtained at the wavelength of the maximum emission. The fluorescence excitation spectra were about 10^3 times more sensitive than ordinary absorption spectra. The fluorescence excitation and emission spectra were useful for the qualitative identification of the components. While the gradient elution technique cannot be used for differential refractometric detection on the chromatograph, it is possible to use a gradient elution system with a spectrofluorimetric-detector.

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