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NEW DOUBLE-STAGE SEPARATION ANALYSIS METHOD

DIRECTLY COUPLED LABORATORY-SCALE SUPERCRITICAL FLUID EXTRACTION–SUPERCRITICAL FLUID CHROMATOGRAPHY, MONITORED WITH A MULTIWAVELENGTH ULTRAVIOLET DETECTOR

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SUMMARY

Instrumentation and applications of a new double-stage separation analysis method are described. The new method incorporates supercritical fluid extraction as the first separation step and supercritical fluid chromatography as the second separation step. The extraction section of the instrument was tested by caffeine extraction from roasted coffee beans with carbon dioxide, and the effects of extraction parameters on the extracted amounts of caffeine were examined by high-performance liquid chromatography. Then, directly coupled supercritical fluid extraction–supercritical fluid chromatography, monitored with a highly sensitive multiwavelength detector, was performed on the powdered coffee beans, and separation was successfully carried out without any special pretreatment. The obtained data were graphically presented by a data processor, as three-dimensional plots of supercritical fluid chromatograms, at 250 and 270 nm, and a spectrum at 9.60 min, which showed clear characteristics of the caffeine spectrum.

INTRODUCTION

Although the fundamental principles have been known for more than 100 years, supercritical fluid extraction (SFE) was introduced by Zosel *et al.*[1] only about two decades ago. Since then, the method seems to have developed mainly as an industrial-scale extraction technique[2–12], independently of the development of high-performance liquid chromatography (HPLC), which is a separation analysis method not only contemporary with SFE, but also with a similar history of development. In addition to their use in separation, these two techniques have many things in common from the instrumentation aspect. Both use high-pressure pumps, sample introduction devices, packed or hollow separation columns, etc. Since the late 1960s, numerous

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reports on HPLC and SFE have been published. However, they have little to do with each other. Stahl and Schiltz developed an extraction system which was combined with thin-layer chromatography[13,14]. Nieass *et al.* examined the solubility of organic substances in liquefied carbon dioxide by using a high-pressure cylinder, connected to an HPLC system[15,16]. Recently, Unger and Roumeliotis reported a coupling device that allows on-line HPLC analysis of extracts[17]. They aimed primarily to investigate optimal conditions for SFE.

Supercritical fluid chromatography (SFC), which uses supercritical fluid as the mobile phase, also originated, in the 1960s, from high-pressure gas chromatography. It was developed by several research groups[18–27]. In the early 1980s, advances in micro HPLC renewed the interests in SFC. Rapid mass transfer in supercritical mobile phases attracted researchers as it offers high speed separation with high resolution on an open tubular capillary column and also on a packed capillary column. The low consumption of the fluid encouraged chromatographers to use flammable and even toxic fluids under high pressures and at high temperatures. Thus, extensive research has been performed by a number of groups[28–37]. In addition to an HPLC UV detector with a high-pressure cell, other detectors have been used in SFC: mass spectrometry[38–40], Fourier transform infrared spectroscopy[41–43], flame ionization detection[44–46]. Sophisticated SFC systems have also reported by Gere *et al.*[47], and Greibrokk *et al.*[48].

Although SFC seems to be closer to SFE than other types of chromatography, they have little in common[49] and direct coupling of SFE with SFC has not yet been attempted.

Recent advances in HPLC instrumentation technology readily permit SFE to be directly combined with an SFC system. In this paper, the instrumentation of the directly coupled SFE–SFC system and its application to caffeine extraction from roasted coffee beans are described.

INSTRUMENTATION OF THE SFE–SFC SYSTEM

We investigated the direct coupling of SFE to SFC and developed a double-stage separation analysis method[50,51], which consists of extraction with supercritical fluid followed by supercritical fluid chromatography for on-line analysis of the SFE extracts. In this new method, SFE is used as the first separation step in a similar way to a sample pretreatment in HPLC, SFC is used as the second separation step. This configuration allows an analyst to place a raw and/or solid sample in the system in order to obtain a chromatogram of the sample extract. We also used a multiwavelength UV detector, equipped with a high-pressure cell, as an extraction and/or chromatographic monitor. Three-dimensional spectrometric data, namely absorbance, wavelength and time, graphically presented in various fashions by computer-aided techniques, are very effective in the detailed examination of components in the SFE extract. Furthermore, application of peak deconvolution[52–54] allows further investigation of chromatographic peak components of the extract.

In SFE, carbon dioxide is generally the preferred supercritical extraction medium and is widely used[2,3,6–17,22,24,25,31–34,39–48], because it is non-toxic, non-flammable, non-polluting and inexpensive. In addition, it has a relatively low critical pressure, 73 bar, and a low critical temperature, 31.3°C, so that a supercritical phase

is easily obtained. Our system is also primarily designed to use carbon dioxide as both the extraction medium and the chromatographic mobile phase.

In order to operate the SFE–SFC system successfully:

(1) the volume of the extraction chamber should be appropriate for the sample size for SFC;

(2) the pressure decrease of the supercritical carbon dioxide should be kept to a minimum during the transfer of the extract from the extraction cartridge to the sample loop of the SFC system.

(3) the SFC system should be pre-pressurized and equilibrated at the SFC analysis pressure before the extract is introduced.

The hydraulics of the SFE–SFC system we designed are shown in Fig. 1. The system allows several modes of operation:

(1) directly coupled SFE–SFC, *i.e.*, batch SFE with a trap loop, which is followed by SFC analysis with direct sample introduction;

(2) continuous flow SFE with an extract trap column, which can be followed by off-line analysis by gas chromatography (GC), HPLC, etc.;

(3) continuous flow SFE with an extract trap column in a recycle operation, which can also be followed by off-line analysis, by GC, HPLC, etc.

Liquefied carbon dioxide from the cylinder (1) is fed to the pump (2) whose pump heads are cooled with dry ice at 0–5°C (TRI ROTAR-II modifed for liquefied carbon dioxide delivery; JASCO, Tokyo, Japan). An entrainer or modifier solvent is

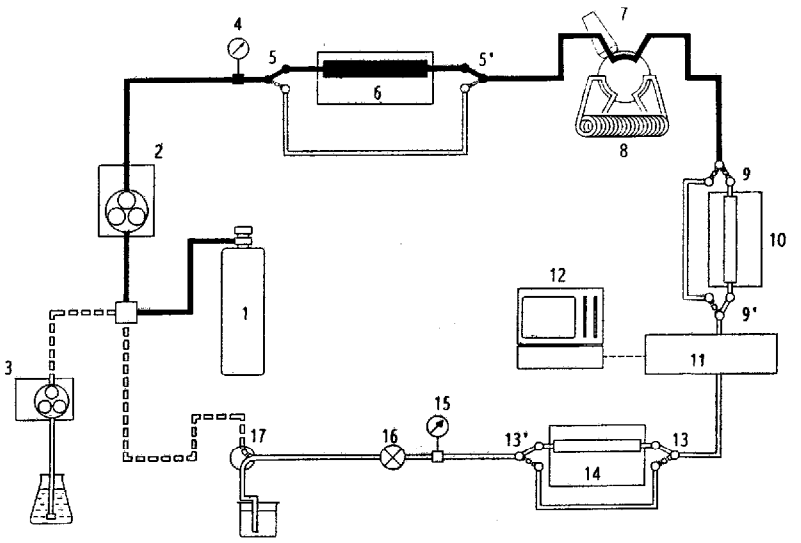

Fig. 1. Hydraulics of directly coupled SFE–SFC for extraction. Components: 1 = carbon dioxide cylinder; 2 = pump for delivering liquefied carbon dioxide; 3 = pump for delivering modifier solvent; 4 = pressure gauge; 5/5′ = six-way valve; 6 = extraction cartridge, thermostatted in oven; 7 = injector valve; 8 = extract trap loop; 9/9′ = six-way valve; 10 = chromatographic separation column in oven; 11 = highly sensitive multiwavelength UV detector; 12 = data processor for 11; 13/13′ = six-way valve; 14 = extract trap column in oven; 15 = pressure gauge for monitoring back-pressure; 16 = pressure regulator; 17 = three-way valve. After SFE, the injector (7) is switched to load the extract trap loop (8) with the extract. The injector is then switched back to by-pass the loop for pre-pressurization and equilibration of the separation column (10), while the loop holds the extract.

delivered by the pump (3) (JASCO TRI ROTAR-V) and pre-mixed with liquefied carbon dioxide before entering the pump (2). The pump (2) is operated generally in the constant-pressure mode up to 300 bar, while the pump (3) is in the constant-flow mode. The switching valves (5)/(5'), (9)/(9'), and (13)/(13') (three JASCO HV-614 high-pressure six-way valves) are switched in accordance with the desired mode.

Hydraulics of directly coupled SFE–SFC

The flow-line for the directly coupled SFE–SFC mode is indicated by the solid line in Fig. 1. Carbon dioxide is delivered to the extraction cartridge (6), where extraction takes place, then to the injector valve (7) (JASCO VL-614) with the extract trap loop (8), which is purged with carbon dioxide gas at atmospheric pressure prior to the extraction. The valve (9)/(9') is set in the non-connecting position to make a dead-end for the extraction line and, at the same time, the valve (9)/(9') maintains the pressure of the column, which has been pre-pressurized and is to be equilibrated at the SFC pressure. The extraction cartridge (6), the separation column (10) and the extract trap column (14) are thermostatted in an oven (JASCO TU-300). When SFC is performed at a different temperature, a separate oven is used.

At the beginning of the extraction, the pump delivers liquefied carbon dioxide at its maximum flow-rate to pressurize the extraction cartridge (6) quickly. As the pressure approaches the preset extraction pressure, the flow-rate is gradually decreased and, finally, the flow is automatically stopped when the pressure reaches the preset value. Then, the pressure will be maintained throughout extraction period. On completion of the extraction, the injector valve (7) is switched into the position shown in Fig. 2, to load the trap loop (8) with the extract, and the pump (2) automatically starts operating to compensate for the pressure decrease caused by the transfer of carbon dioxide and the extract in the extraction cartridge (6) to the trap loop (8), which has been purged with carbon dioxide gas at atmospheric pressure. When the transfer is completed and the pressure is restored, the pump (2) is stopped. Then, the injector valve (7) is switched back to the position shown in Fig. 1, so that the loop (8) is by-passed, and the extract dissolved in the supercritical carbon dioxide is retained in the loop (8) until the injection is made. The valves (5)/(5') and (9)/(9') are then switched to the SFC separation line, as indicated by the solid line in Fig. 2. The system is now operated in the chromatography mode for equilibration of the separation column (10).

Finally, the injector (7) is switched to the position shown in Fig. 2, to inject the extract into the separation column (10). The chromatography mode can be easily converted from SFC to ordinary HPLC by using an ordinary solvent without any hardware modification. A highly sensitive multiwavelength UV detector (11) (JASCO MULTI-320, modified for high-pressure application) together with its dedicated data processor (12) (JASCO DS-L800) are used as an extraction and/or chromatographic monitor. The flow-cell, whose volume is 4 μl, is modified to withstand 300 bar pressure to meet pressure requirements in SFE and SFC. The flow detector cell is kept at the necessary pressure for SFE and SFC by a pressure regulator (16) (TESCOM, Minneapolis, MN, U.S.A.) where the main pressure drop takes place. The back-pressure is monitored by the pressure gauge (15), and the column effluent is vented into water through the three-way valve (17).

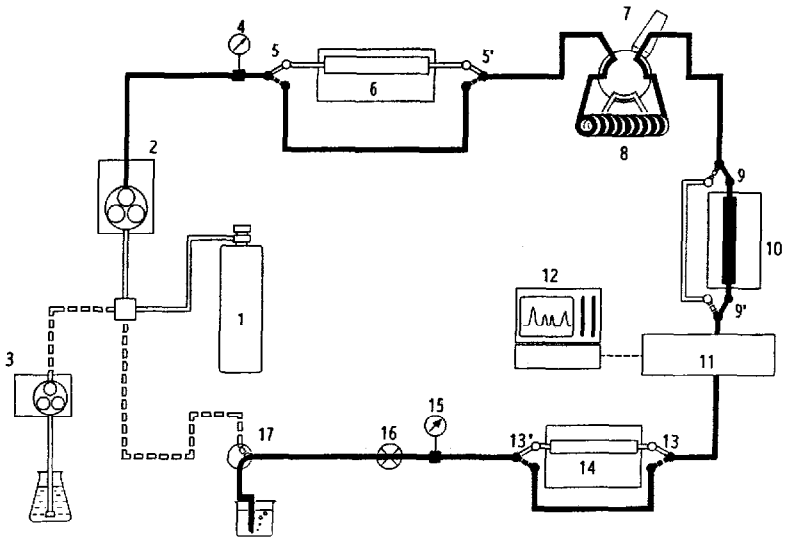

Fig. 2. Hydraulics of directly coupled SFE–SFC for chromatography. After pre-pressurization and equilibration of the separation column, the injector is switched to inject the extract held in the trap loop into the column. The injector valve in this figure is shown in the position for injection.

Hydraulics of SFE with extract trap column

In SFE in the extract trap column mode, the fluid flows through the extraction cartridge (6), the detector (11), by-passing the separation column (10), via the extract trap column (14), the pressure gauge (15), and the pressure regulator (16), and then to the waste or back to the pump (2) via the valve (17), when recycle is selected. After SFE, the extract trap column (14) is disconnected from the system, and the extract is eluted with solvent. The extract is then applied to other analytical instruments, such as GC or HPLC systems.

RESULTS AND DISCUSSION

For our preliminary work, we started with caffeine extraction from coffee beans, which is one of the classical applications of SFE, using continuous flow SFE in the extract trap column mode. The coffee extract, which was eluted from the extract trap column, has been applied to an HPLC system (off-line SFE–HPLC). SFE was performed under various conditions, and the contribution of each extraction parameter to the extracted amount of caffeine was examined.

After the extraction conditions had been examined, directly coupled SFE–SFC was performed successfully, and three-dimensional SFC data of the coffee extract were obtained by placing the coffee powder in the system. The data were represented, by the data processor, as three-dimensional plots, chromatograms at 250 and 270 nm and a spectrum at 9.60 min, which showed clear characteristics of the caffeine spectrum.

SFE–HPLC analysis of coffee beans

In the food industry, decaffeination or caffeine extraction is usually performed

on green coffee beans with a particular water content[1]. In our experiment, roasted coffee beans were used instead of green beans. Roasted coffee beans, obtained from a grocery store, were ground and sieved to 30–60 mesh. They contained 1–2% water, significantly less than in green beans. In order to vary the water content, different amounts of water were added to *ca.* 20 g of coffee powder kept in a glass vessel (100-ml capacity) with an air-tight stopper, mixed by shaking, then equilibrated for at least 24 h. Then, *ca.* 350 mg of the moistened powder were packed by tapping into an extraction cartridge (50 × 4.6 mm I.D.). Extraction was performed with continuous flow in a recycle operation with a trap column of the same dimensions, packed with activated carbon (30–60 mesh; Gasukuro Kogyo, Tokyo, Japan). After SFE, the column was disconnected from the system, and the extract was eluted with 25 ml of methanol–water (55:45). Then, 20 μl of the solution was injected into the HPLC system, consisting of a TRI ROTAR-V pump, a VL-614 injector, a Fine Pak SIL C_{18} column and UVIDEC-100-V UV detector (all from JASCO). Carbon dioxide (Toyoko Kagaku, Kawasaki, Japan) was used as the extraction medium.

Fig. 3 shows an HPLC chromatogram of the coffee extract, obtained by the procedure described above. The coffee powder contained added water (20% of the coffee weight), besides the original water content. The extraction pressure was 200 bar, the temperature was 48°C, and the time was 60 min.

Amount of caffeine extracted from coffee beans under various conditions

The amount of caffeine extracted from coffee beans was examined under var-

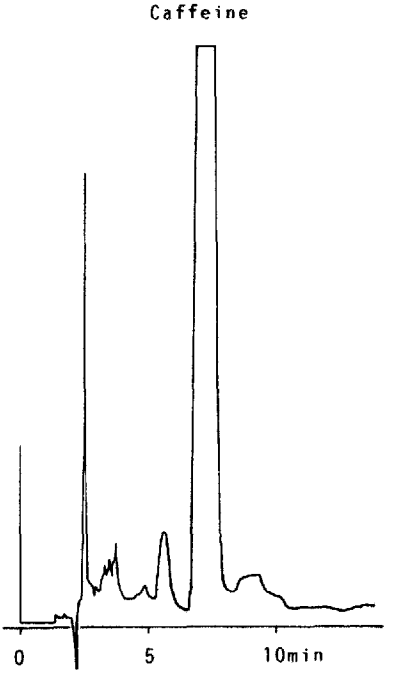

Fig. 3. HPLC chromatogram of coffee extract by SFE. SFE conditions: pressure, 200 bar; temperature, 48°C; added water, 20%; time, 60 min. HPLC conditions: column, JASCO Fine Pak SIL C_{18}; eluent, methanol–water (55:45); flow-rate, 1.2 ml/min; UV monitored at 272 nm and 0.64 a.u.f.s.

ious conditions of pressure, extraction time, added water and temperature, by the procedure described above. In Fig. 4 the amounts of caffeine extracted are represented as percentages of the amount extracted with hot water, *i.e.*, as percentages of the caffeine level in ordinary drinking coffee. The amounts increased with increasing extraction pressure and time, as shown by the heavy lines. However, the amounts rapidly decreased with increasing temperature, and above 60°C, caffeine was hardly extracted. This decrease is considered to be due to the decrease of caffeine solubility in carbon dioxide, resulting from the density reduction. As the amount of added water decreased, the amount of caffeine extracted also decreased. This suggests that the water content of coffee plays the role of an entrainer solvent in extraction. Therefore, in order to extract caffeine from the roasted coffee beans efficiently, the extraction temperature should be below 50°C and an amount, at least 15% of the coffee weight, of water should be added.

The variation of the extracted amounts of caffeine in five successive experi-

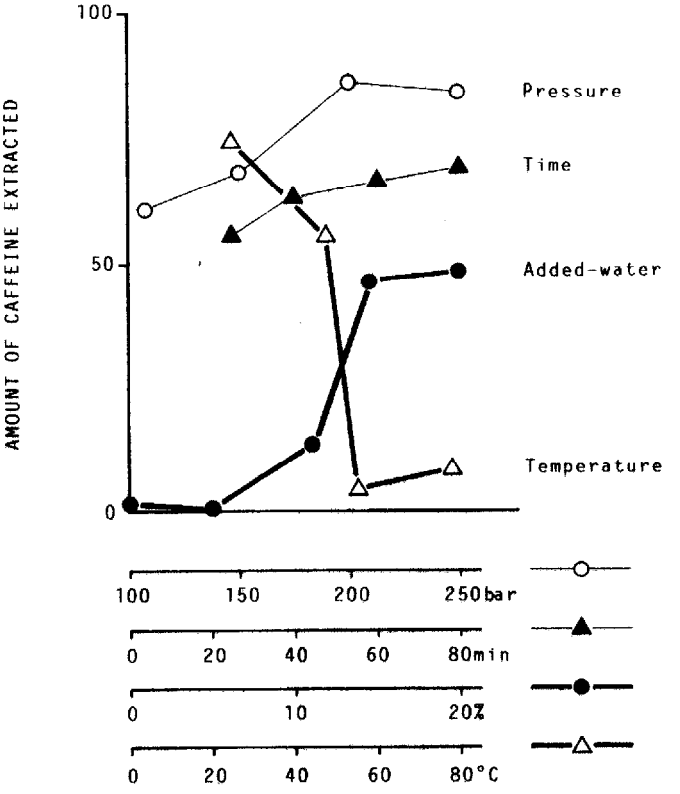

Fig. 4. Amounts (percentages) of caffeine extracted from roasted coffee beans under various conditions, with hot water. Curves: —○—, various pressures with the added water, temperature, and extraction time constant at 20%, 48°C, and 60 min, respectively; —▲—, various extraction times with other parameters constant, at 150 bar, 20% and 48°C; —●—, various amounts of water added to coffee powder with other parameters constant, at 150 bar, 48°C and 60 min; —△—, various temperatures with other parameters constant, at 150 bar, 60 min and 20%. The temperature and the amount of added water have significant effects on the extraction, as shown by heavy lines.

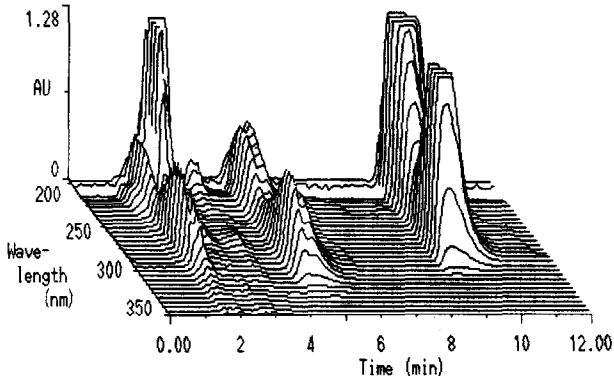

Fig. 5. Three-dimensional SFC chromatogram obtained by directly coupled SFE–SFC. SFE conditions: pressure, 200 bar; temperature, 40°C; added water, 20%; time, 15 min. SFC conditions: mobile phase, supercritical carbon dioxide–methanol (100 μl/min); total flow-rate, *ca.* 5 ml/min as liquid; pressure, 150 bar; column, JASCO Fine Pak SIL C_{18} (150 × 6 mm I.D.); temperature, 40°C.

ments was calculated to be ±8% under the following conditions: pressure, 200 bar; temperature, 48°C;added water, 20%; time, 60 min.

Directly coupled SFE–SFC analysis of coffee beans

About 100 mg of the same coffee powder was placed in the extraction cartridge by the same procedure in SFE–HPLC. Batch SFE was then performed with a 500-μl trap loop instead of the extract trap column. After SFE, the carbon dioxide containing the extract was transferred to the trap loop, and introduced directly into the separation column by switching the injector valve, as described in *Hydraulics of directly coupled SFE–SFC.*

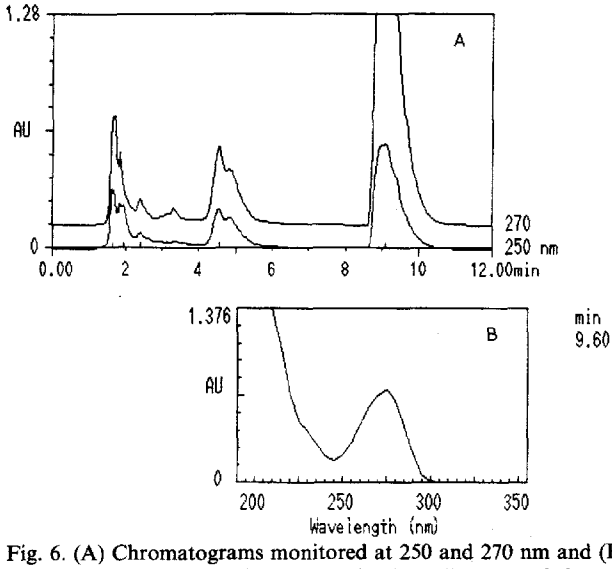

Fig. 6. (A) Chromatograms monitored at 250 and 270 nm and (B) UV spectrum taken at 9.60 min. The chromatograms were produced from the three-dimensional chromatogram shown in Fig. 5. The UV spectrum shows that the chromatographic peak eluted at 9.2 min is that of caffeine.

The three-dimensional chromatogram obtained by the SFE–SFC method is shown in Fig. 5 (the conditions are listed in the caption). A large caffeine peak is clearly seen at 9.2 min in the chromatogram. Ordinary chromatograms monitored at 250 and 270 nm are shown in Fig. 6A; these are not very informative without spectral data. In order to identify the caffeine peak chromatographically, one might subject the caffeine standard to SFC. However, the solvent in which caffeine is dissolved influences the retention behaviour significantly, resulting in identification difficulties. Therefore, spectral data are necessary for efficient identification of peak components in SFC. Fig. 6B shows the spectrum taken at 9.60 min. The curve shows the clear characteristics of the caffeine spectrum.

So far, we have discussed the directly coupled SFE–SFC method from the viewpoint of qualitative analysis. The quantitative accuracy of the method has not yet been closely examined, partly because the amount of coffee powder was so large that the chromatographic peak gave absorbances too high for quantitation, and partly because the volume of the extraction cartridge did not properly match the volume of the trap loop for quantitative analysis. A study of quantitative analysis by this method is currently underway.

CONCLUSION

We have demonstrated that the directly coupled SFE–SFC system allows the analyst to apply raw and/or solid samples to the system to obtain chromatograms of sample extracts. This could be a powerful technique for extending the application of chromatography to natural products, biological compounds, petrochemical products, etc., where extraction is necessary before analysis.

In addition, a highly sensitive multiwavelength detector permits on-line UV spectrum monitoring of the extraction process, which has not previously been possible in a large-scale extraction system. Therefore, one can easily investigate optimal extraction parameters at low cost without operating a pilot-plant extraction system, which requires large amounts of sample and extraction medium.

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