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High-resolution analysis of polyprenols by supercritical fluid chromatography ☆

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Abstract

A high-resolution analysis of polyprenol mixtures was achieved by supercritical fluid chromatography (SFC). The separation of polyprenols was examined on an octadecylsilane-packed column with liquid carbon dioxide as the mobile phase and ethanol as modifier. Using this chromatography system, the resolution of separation (R_s) between octadecaprenol (prenol 18) and nonadecaprenol (prenol 19) was two times higher than that using conventional reversed-phase high-performance liquid chromatography. Our SFC technique allows the advantage of baseline separation of polyprenol samples containing hydrophobic components such as terpenes or fatty acids that are unfavorable for good separation. This method is very useful for the analysis of structurally close polyprenol analogues of rubber plant metabolites.

Keywords: Polyprenols

1. Introduction

Polyprenols are linear polymers of C_5 isoprenoid units. A number of polyprenols with different polymerization degrees are present in plants, animals and microorganisms [1,2]. Among the polyprenols, dolichol plays an important role as a sugar-carrying intermediate in the biosynthesis of glycoproteins [3].

The polymerization degrees of isoprene units and the structural features of polyprenols are specific to plant species, growth stages, and growth conditions. Thereby the detailed analyses about polyprenol are physiologically and chemotaxonomically interesting.

High-performance analysis of polyprenol components is needed for the biosynthetic work on a variety of intermediates in dolichol fractions and rubber latices. For polyprenol analyses, reversed-phase high-performance liquid chromatography (HPLC) using an octadecylsilane (ODS) packed-column has been widely used [4–6]. However, this system fails to give satisfactory baseline separation for authentic polyprenols. The reversed-phase HPLC accepted as a conventional analysis of polyprenols is

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compromised in that the co-existing terpenes and fatty acids in natural polyprenol samples deteriorate the resolution performance of the column with respect to polyprenols. In ODS column chromatography using ordinary eluents, such interfering factors make the separation of geometric isomers difficult because the hydrophobic contaminants often possess stronger affinity for the stationary phase than polyprenols, and decline the resolution potential of the column packing.

In the course of an analytical study of waxy compounds, we investigated the separation potential of supercritical fluid chromatography (SFC) for hydrophobic agrochemicals. SFC has several unique features [7,8]. Firstly, the viscosity of a supercritical fluid is smaller by one or more orders of magnitude than that of typical HPLC solvents. In a supercritical fluid, the diffusivity of solute molecules is high, allowing homogenous diffusion of the solute molecules into the column packing. Thus, high-resolution potentials can be obtained even for high-molecular-mass components, and rapid and precise separation can be achieved. Secondly, the elution power of a supercritical fluid used as a mobile phase can be varied greatly by changing the temperature, pressure, and fluid composition of the mobile phase by adding a polar solvent. Besides the modulation of such separation factors, modification of the elution mode, i.e., isocratic or gradient elution, can enhance separation potentials for close-structural analogues of hydrophobic compounds.

Moreover, using supercritical fluids of carbon dioxide is advantageous for practical use. Unlike HPLC, SFC does not require handling of a large amount of organic solvents. Furthermore, carbon dioxide as an elution medium would be easily removed after chromatography. Therefore, fraction collection is easy.

On the other hand, SFC has disadvantages because it requires high pressure. So, available detectors have been restricted for SFC, however recently, study efforts have been made to use special detectors for SFC and the hyphenated analyses became possible such as SFC–mass spectrometry (MS), SFC–Fourier transform infra red (FT-IR) spectroscopy, SFC–nuclear magnetic resonance (NMR) spectroscopy and so on [9–11]. SFC with such excellent advantages has been used in various fields. In natural product research, there are many reports on the separation of wax, lipid, drug, etc. [12–14]. The advantages were shown in the separation of a hydrophobic compound and an optical isomer.

In this paper, we report the successful analysis of polyprenols by the SFC method.

2. Experimental

2.1. Chemicals and materials

Prenol C80-110 was purchased from Sigma, and carbon dioxide (99.9%) from Daiwa Youzai (Osaka, Japan). Modifiers for SFC (ethanol, methanol, 2-propanol and acetonitrile) were obtained from Wako (Osaka, Japan) and were of purity>99.5%. For HPLC analyses, HPLC-grade methanol, 2-propanol and *n*-hexane were used (Wako). Water was purified with a Millipore Milli-Q system.

Tochu (*Eucommia ulmoides*) leaves were collected in September at the Hitachi Zosen Corporation experimental station (Habu 2264-1 Innoshima, Hiroshima, Japan).

2.2. Analysis by HPLC

HPLC of polyprenols was performed on a reversed-phase column, Inertsil ODS-3 (250×4.6 mm I.D.; particle size, 5 μm; pore size, 100 Å; surface area, 450 m^2/g; GL Sciences) using a dual pump apparatus (Hitachi L7100), a UV detector (set at 210 nm, Hitachi L7420), a column oven (set at 40°C, Hitachi L7300), a degasser (Hitachi L7610), and a data station (SIC-480II data station) were used. For elution, a gradient was applied from an initial 2-propanol–methanol–water (60:40:5, v/v) mix in pump A to 100% *n*-hexane–2-propanol (70:30, v/v) in pump B. The solvent flow-rate was 1 ml/min and the end of the gradient was reached after 40 min.

2.3. Analysis by SFC

SFC analyses were performed on an Inertsil ODS-3 column (250×4.6 mm I.D.; particle size, 5 μm; pore size, 100 Å; surface area, 450 m^2/g; GL Sciences) using a Super-201 chromatograph (Jasco). The system consisted of two pumps, one for delivery

of liquid CO_2 as a mobile phase (flow-rate=3.0 ml/min) and the other for delivering ethanol as a modifier. The ethanol flow-rate (0.8 ml/min) increased to 2.0 ml/min within the experimental time scale of 30 min. The fluid pressure was controlled at 19.6 MPa by back-pressure regulator. The column temperature was set at 130°C. Chromatograms were recorded using a UV detector (950-UV, Jasco) operating at a wavelength of 210 nm.

2.4. Evaluation of analysis

The resolution (R_s) between octadecaprenol (prenol 18) and nonadecaprenol (prenol 19) was calculated using SIC-480II (System Instruments).

2.5. Extraction and isolation of polyprenols from Eucommia ulmoides

Tochu leaves were heated in an oven at 95°C for 3 h and then ground for 1 min using a blender. The ground leaves (2 g) were then treated with alkali (40 ml of 11.2% ethanolic potassium hydroxide containing 2% pyrogallol) for 2.5 h at 100°C. The saponified lipid was extracted with *n*-hexane and weighed. The saponified lipid portion was loaded onto a silica gel Sep-Pak column (Waters). The column was washed thoroughly with 30 ml of *n*-hexane–ethyl ether (98:2, v/v) and non-polar lipids containing free polyprenols were eluted with 50 ml of *n*-hexane–ethyl ether (85:15, v/v). Unfortunately, HPLC analysis of the sample treated with only silica Sep-Pak indicated unsatisfying separation and the serious depletion of packed-column, which should be caused by hydrophobic impurities. So we also employed C_{18} Sep-Pak treatment of samples for HPLC. SFC showed acceptable baseline separation without C_{18} Sep-Pak pretreatment of samples. Polyprenols were eluted with *n*-hexane–2-propanol (7:3, v/v).

3. Results and discussion

The SFC analytical conditions suitable for the separation of natural polyprenols were examined using an authentic polyprenol sample, prenol C80-110. In this experiment an ordinary ODS-packed column was employed and CO_2 modified with ethanol was used as a mobile phase. The optimum column oven temperature was determined to be 130°C after repeated runs at different temperatures. In the SFC system the mobile phase was delivered into the column by two independently driven high-performance pumps. Satisfactory separation of each of the polyprenol components was achieved with flow-rates of CO_2: 3.0 ml/min (pump A) and ethanol: 0.8–2.0 ml/min (pump B) within 30 min. Each peak was collected by preparative SFC and subjected to field desorption (FD)–MS analyses (data not shown). It was revealed that authentic polyprenols with molecular sizes over prenol 16 were eluted in order of the degree of polymerization (Fig. 1).

Under the same separation conditions, common water-soluble solvents (methanol, 2-propanol and acetonitrile) were investigated as the modifier. The two alcohols gave similar separation profiles to that of ethanol, but acetonitrile proved inferior to the alcohols. 2-Propanol was not a suitable modifier for this experiment because its viscosity elevated the column pressure. Overall separation with methanol as modifier was almost same as that of ethanol, but the minor peaks shown as arrows in Fig. 2 were not found when methanol was used as a modifier. Therefore ethanol is the recommended solvent modifier for polyprenol separation by the SFC system.

With an eye to improving relative resolution of the individual peaks and time efficiency (the time span

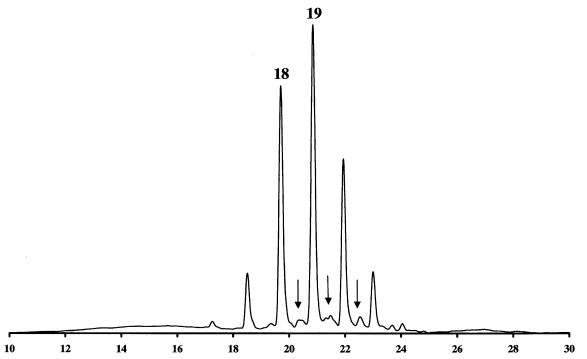

Fig. 1. Chromatogram of authentic polyprenol mixtures separated by SFC with ODS column. Conditions: CO_2 flow 3.0 ml/min, modifier (ethanol) 0.8→2.0 ml/min within 30 min, 19.6 MPa, 130°C, UV detection at 210 nm. **18**: Octadecaprenol (prenol 18), **19**: nonadecaprenol (prenol 19). Arrow peaks undetected with methanol as modifier in place of ethanol.

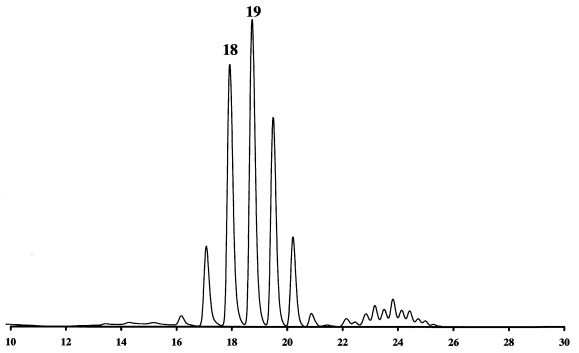

Fig. 2. Chromatogram of authentic polyprenol mixtures separated by HPLC with ODS column. Conditions: methanol–isopropanol–water (60:40:5)→*n*-hexane–isopropanol (70:30) within 40 min, 1.0 ml/min, 40°C, UV detection at 210 nm. **18**: Octadecaprenol (prenol 18), **19**: nonadecaprenol (prenol 19).

for a single run), separation profiles were examined at various temperatures ranging from 50 to 140°C. The better separations were made in the range of 120–140°C. Finally, 130°C was chosen as a holding temperature since this temperature setting afforded the most reliable results over numerous trial and error experiments.

Silica gel columns were also tested for separation efficiency and durability. The eluted peaks however, gradually broadened as the analytical time course was prolonged, and the reproducibility of peak separation was not satisfactory and silica gel columns were found unsuitable for the polyprenol analysis. So, the ODS column was employed for this purpose.

The separation of polyprenols by SFC was then compared with that of reversed-phase HPLC using the conditions conventionally employed for polyprenol analysis. The resolution of separation (R_s) between prenols 18 and 19 was found to be 2.09 using reversed-phase HPLC (Fig. 2). On the other hand, SFC showed an R_s value of 4.28 for these polyprenols (Fig. 1), indicating that SFC had two times higher resolution than reversed-phase HPLC.

The separation modes for authentic polyprenols established by SFC and reversed-phase HPLC were then applied to samples from a woody plant, Tochu (*Eucommia ulmoides*), which was found to contain *trans*-polyisoprenes as a major terpene together with monoterpenes and fatty acids [15]. A C_{18} reversed-phase column gave overlapping peaks, and it is hard to estimate the number of prenol components present in the sample (data not shown). Co-existing hydrophobic components such as monoterpenes and fatty acids significantly affected the separation potential. The content of the objective targets, polyprenols, is relatively low compared to those of the other hydrophobic components. Therefore, such interfering components were removed by a pretreatment. The improvement in separation was provided by a simple pretreatment using silica and C_{18} Sep-Pak (Waters) (Fig. 3). However, several peaks at 15–20 min overlapped and could not be separated well. In the reversed-phase HPLC system, overloading and repeated sample injections soon deteriorated the separation, and time consuming washing had to be continued to recover the original separation quality of the column. In contrast, using SFC, the same samples used for the HPLC were successfully separated into a series of sharp peaks over the entire separation range (Fig. 4), and satisfactory baseline separation allowed us to collect the peak of each component.

Thus, using the SFC system for polyprenol separations should provide considerable benefits when conducting analytical studies on polyprenol geometric *cis*- or *trans*-isomers. We further suggest that the SFC technique should have a wide range of application in the analysis of many hydrophobic plant samples. Moreover, the SFC technique will be very useful to identify polyprenol intermediates in rubber

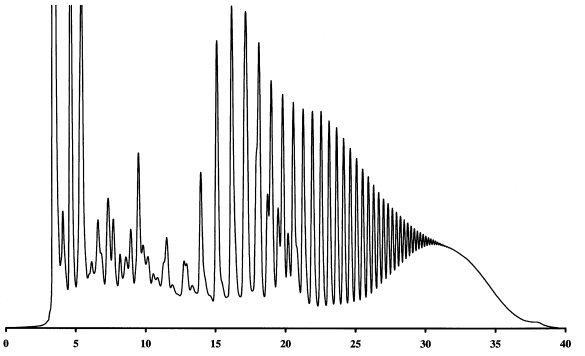

Fig. 3. Separation of the polyprenols from *E. ulmoides* leaves by HPLC. Conditions: solvent system, methanol–isopropanol–water (60:40:5)→*n*-hexane–isopropanol (70:30) within 40 min; flow-rate, 1.0 ml/min; column temperature, 40°C; detection, UV at 210 nm.

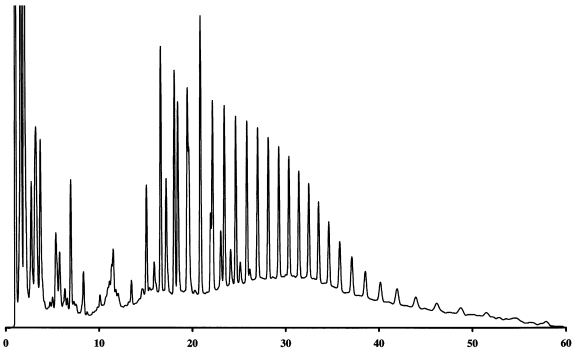

Fig. 4. Separation of the polyprenols from *E. ulmoides* leaves by SFC. Conditions: mobile phase (CO_2) flow, 3.0 ml/min; modifier (ethanol) flow, 0.8→2.0 ml/min within 30 min; top pressure, 19.6 MPa; column temperature, 130°C; detection, UV at 210 nm.

biosynthetic pathways. The method holds promise of further development by further fine-tuning of the separation factors, e.g., mobile phase, pressure and temperature.

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