Preparative High-Performance Liquid Chromatography for the Purification of Natural Acylated Anthocyanins from Red Radish (*Raphanus sativus L.*)

Yonghong Liu[1,*], Nobutoshi Murakami[2], Lishu Wang[3], and **Si Zhang[1]**

[1]Key Laboratory of Marine Bio-resources Sustainable Utilization, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510-301, China; [2]Graduate School of Pharmaceutical Science, Osaka University, 1-6 Yamada, Suita, Osaka 565-0871, Japan; [3]College of Pharmacy, Changchun University of Chinese Medicine, Changchun 130117, China

Abstract

Preparative high-performance liquid chromatography (HPLC) is applied to the purification of anthocyanins from raw extracts of red radish (*Raphanus sativus L*). For each separation, the chromatographic conditions are optimized to achieve an efficient purification in the shortest time. In addition, UV–vis characterization is carried out on all purified anthocyanins. The current work shows that analytical chromatographic experiments alone are useful for the prediction of scale-up conditions of preparative HPLC separations. Ten known acylated anthocyanins (See the Appendix for compounds 1–10) are isolated from the red radish by isocratic HPLC. The structures are established on the basis of nuclear magnetic resonance and mass spectrometric analysis. The acylated anthocyanins are all based on pelargonidin 3-sophoroside-5-glucoside, acylated with caffeoyl, feruloyl, or *p*-coumaroyl moieties.

Introduction

Anthocyanins comprise a diverse group of intensely colored pigments responsible for the appealing and often spectacular orange and red. They are water-soluble, which facilitates their incorporation into aqueous food systems, and have been consumed for centuries without adverse effects. Interest in anthocyanin-rich foods and extracts has intensified because of their possible health benefits. Heath benefits associated with anthocyanin extracts include enhancement of sight acuteness, antioxidant capacity, treatment of various blood circulation disorders resulting from capillary fragility, vaso-protective, and anti-inflammatory properties, inhibition of platelet aggregation, maintenance of normal vascular permeability, controlling diabetes, anti-neoplstic agents, and possibly others, due to their diverse action on various enzymes and metabolic processes. These qualities make anthocyanins attractive alternatives to synthetic dyes. Findings of acylated anthocyanins with increased stability have shown that these pigments may impart desirable color and stability for commercial food products. Examples of suitable acylated anthocyanin sources may be radishes, red potatoes, red cabbage, black carrots, and purple sweet potatoes. Among these, radishes stand out as potential applications for acylated anthocyanins may include other challenging systems such as dairy products. The increased stability of these pigments, together with their added value due to potential beneficial effects, opens a new window of opportunities for use of these extracts in a variety of food applications (1).

Preparative liquid chromatography is becoming increasingly popular; the use of preparative chromatography is widely accepted. The development of preparative purification processes can be achieved very rapidly, and most important is that procedures can operate quickly to produce pure compounds. Information about the cycle time and the separation factor can be obtained from analytical-scale chromatographic data. Optimization of a preparative high-performance liquid chromatography (HPLC) separation is a complex endeavor. This paper presents an HPLC method development procedure where only analytical-scale data are used to predict optimal preparative operation conditions for the separation. For the studied separation, the production rate, which is the time that is necessary to separate a specified amount of pure compounds, was the most critical parameter. Furthermore, the separation should yield very pure compounds and have a high product recovery as well, so that there is no need to re-inject mixed fractions. For such a situation, the following operating procedure is most useful; there is little or no overlapping of the chromatographic bands.

The structure of the anthocyanins were elucidated by comparing with the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectral data of those reported (2,3), and confirming by the aid of high-resolution fast atom bombardment-mass spectrometry (FAB-MS).

* Author to whom correspondence should be addressed: email yonghongliu@scsio.ac.cn.

Experimental

Plant material

The red radish from Yunnan (China) was obtained from San-ei-gen, FFI, Ltd. (Osaka, Japan).

General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on JEOL NMR spectrometers, JNM-GX-500. Chemical shifts were reported with reference to the respective residual solvent peaks (d_H 2.49 and d_C 39.0 for DMSO-d_6). FAB-MS data were obtained on a JMS SX-102 (JEOL, Tokyo, Japan) instrument using m-nitrobenzyl alcohol as the matrix. Thin-layer chromatography (TLC) analysis was done over precoated reversed-phase HPTLC (RP-18 WF_{254}, Merck, Darmstadt, Germany). Reversed-phase HPLC was performed with a Cosmosil column (150 mm × 4.6 mm i.d.; particle size 5 µm, Nacalai Tesque Inc., Kyoto, Japan), a Cosmosil column (C18-AR-II, preparative, 250 × 20 mm, particle size 5 µm, Nacalai Tesque Inc., Kyoto, Japan), and a Develosil C-30-UG-5 column (semipreparative, 250 × 10 mm, particle size 5 µm, Nomura Chemical, Tokyo, Japan) using a Shimadzu SPD-10A vp UV–vis detector, with monitoring at 520 nm. All reagents were of HPLC or special grade and purchased from Wako Chemical Ind. (Osaka, Japan), and were used without further purification.

Extraction and isolation

The pigments in the raw red radish (10 KG) were extracted with 0.05% H2SO4 and applied to a polymeric resin, and eluted with 70% MeOH containing 1% citric acid, then parts of the extract (600 mg) were subjected to preparative reversed-phase HPLC (Cosmosil C18-AR-II, 20 mm × 250 mm, MeOH–H_2O = 40:60 containing 0.1% TFA; flow rate = 7.0 mL/min; UV-vis detector; monitoring on 520 nm; 4 injections [300 µL]; and provided 10 fractions). The fractions were collected and freeze-dried, to get F1 (24.5 mg), F2 (24.9 mg), F3 (18.5 mg), F4 (18.5 mg), F5 (29.0 mg), F6 (16.8 mg), F7 (44.1 mg), F8 (26.9mg), F9 (16.5 mg), and F10 (20.7 mg). Compounds **1** (2.9 mg), **2** (6.5 mg), **3** (3.1 mg), **4** (4.0 mg), **5** (5.3 mg), **6** (2.1 mg), **7** (2.6 mg), 8 (6.7 mg), **9** (3.5 mg), and **10** (1.5 mg) (for more information on the compounds, see the Appendix) were obtained by purification of fraction F1–F10 by semi-preparative reversed-phase HPLC (Develosil C30-UG-5, 10 mm × 250 mm, MeCN–H_2O = 18 → 22: 82 → 78, containing 0.1 % TFA, flow-rate = 4.0–5.0 mL/min, UV-VIS detector, 520 nm). Compounds are described in the Identification of anthocyanins section.

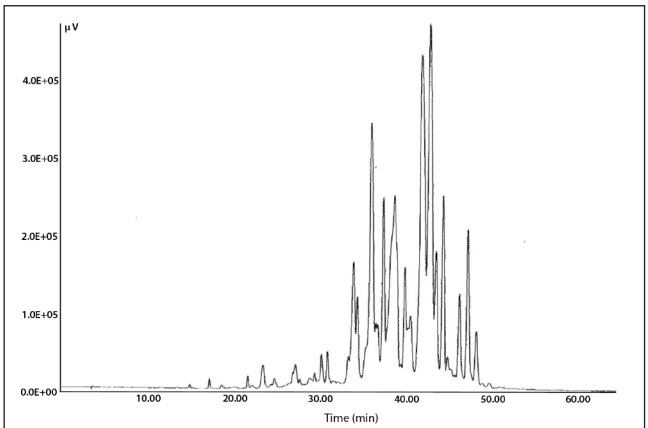


Figure 1. Analytical HPLC profile.

Chromatographic conditions

Analytical HPLC

Analytical HPLC was performed with a Cosmosil C-18 column (150 mm × 4.6 mm, 5 µm) using a Gilson Model 576 pump. The mobile phase was MeOH–H_2O = 40:60 containing 0.1% TFA. Before delivering into the system, it was filtered through 0.45 µm, and degassed using a vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min at room temperature (25ºC). Chromatograms were recorded using a Shimadzu SPD-10A vp UV-VIS detector, with monitoring at 520 nm. The injection volume was 5 µL for analytical-size injections. The chromatogram is shown in Figure 1. Separation conditions, in particular the amount of sample load, were identical for all varieties to ensure comparability of the separations.

Preparative HPLC

Preparative HPLC was performed with a Gilson Model 576 pump and column (Cosmosil C18-AR-II, 20 mm × 250 mm, 5 µm) using MeOH–H_2O = 40:60 containing 0.1% TFA as mobile phase at a flow rate of 7 mL/min; UV-vis detector, monitoring on 520 nm. Chromatograms of the 10 fractions found are shown in Figure 2. The pooled fractions were concentrated by rotary evaporator and each fraction was analyzed by analytical HPLC to check the purity prior to characterization. Separation conditions, in particular the amount of sample load, were identical for all varieties to ensure comparability of the separations.

Semi-preparative HPLC for purification of each fraction

Semi-preparative reversed-phase HPLC was performed (Develosil C30-UG-5 column, 10 mm × 250 mm, 5 µm, Nomura

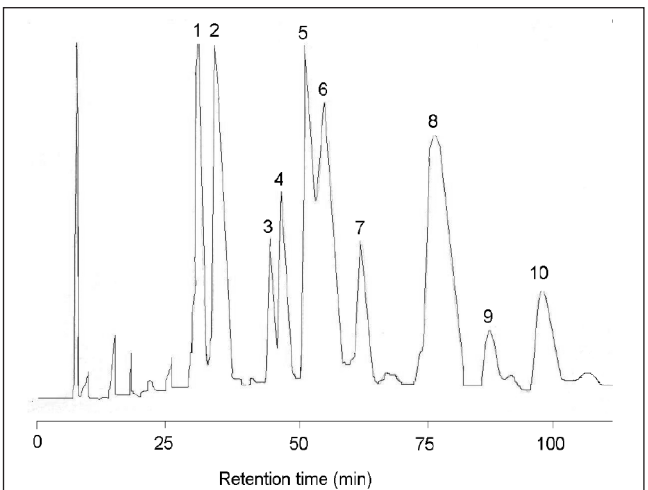


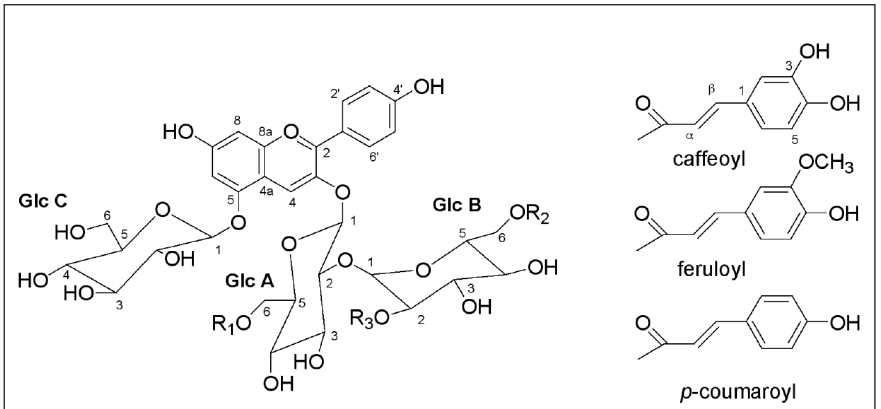
Figure 2. Preparative HPLC profile, the numbers showed Compounds 1–10 were isolated from these fractions.

Chemical), using mobile phase MeCN–H_2O = 18:82, containing 0.1% TFA, at flow rate = 4.0 mL/min for purification of compounds **1** and **2**; mobile phase MeCN–H_2O = 18:82, containing 0.1% TFA, at flow rate = 4.5 mL/min for purification of compounds **3** and **4**; mobile phase MeCN–H_2O = 20:80, containing 0.1% TFA, at flow rate = 5.0 mL/min for purification of compounds **5** and **6**; mobile phase MeCN–H_2O = 22:78, containing 0.1% TFA, at flow rate = 5.0 mL/min for purification of compounds **7–10**, UV–vis detector, 520 nm), respectively.

Results and Discussion

Optimization of HPLC conditions

Several elution systems were tested in the HPLC separation of the crude sample, such as isocratic elution of methanol–water (30 → 40:70 → 60), THF–water (20 → 30:80 → 70), acetone–water (25 → 35:75 → 65), acetonitrile–water (20 → 30:80 → 70), methanol–acetonitrile–water (10 → 20:20 → 10:70), etc. The results indicated that when methanol–water was used as the mobile phase (40:60), relatively good separation results could be obtained, and the process was economical. Changing the organic solvent did not result in any higher selectivity. Increasing the percentage of water increased the resolution at the expense of time (more than 100 min in the flow rate at 7 mL/min). The crude sample and peak fractions separated by preparative HPLC were analyzed by analytical HPLC under the optimum conditions. Analytical HPLC was used initially to develop the conditions for the separation of anthocyanins. Once the stationary phase and mobile phase are selected, the usual optimization procedure is to change the solvent strength of the mobile phase. Figure 1 shows the effect of mobile phase composition on the chromatographic separation. The separation improves with decreasing solvent strength. The retention times of components decrease with increasing methanol concentration (i.e., increasing solvent strength). The dependence of the column efficiency is calculated from the peak width at half height. The efficiency increases with increasing temperature, but is not significantly affected by a change in the solvent composition. Decreasing the solvent strength improves the separation factor. The resolution plot can be used for the optimization of the analytical method development. The approach is the optimization of the cycle time by changing the solvent strength. The time-saving approach presented here can be very useful and vital for a continuous study, especially considering its potential savings in resources and finances. The optimization procedure used in this study, based solely on analytical chromatographic data, predicts an improvement. It is quite possible that a better production rate can be achieved by maximizing the separation factor. The advantage of looking for the shortest cycle time as suggested in this study is clearly that an estimation can be made of how much the production rate improves when changing operating conditions. If a preparative separation is in operation, it is possible to predict the possible time savings based on limited analytical data using the approach presented in this paper. There are some other points to consider for the preparative separation. The solubility is important: a good solubility is necessary to achieve a high sample loading with a reasonably small injection size, thus preventing volume overloading that will limit the productivity. TFA was tried with MeOH in different compositions. When the content of MeOH was 40%, the separation was found to be good, but the solubility of anthocyanins was poor in the mobile solvent. Small quantities of TFA were added to different compositions of MeOH–water mixtures to study its effect on the retention of anthocyanins. The mobile phase containing MeOH–water–TFA (40:60:0.1, v/v/v) resulted in optimum separation of anthocyanins, increased the solubility, and did not change the retention time. The developed conditions were transported onto a preparative chromatograph and optimized for injecting large amounts of sample for the isolation of anthocyanins in sufficient quantities for further characterization. The flow rate, the sample loading capacity, and concentration were optimized to collect the maximum amount of anthocyanins of highest purity in a single run. The maximum flow rate used in this system was 7 mL/min. The TFA content was slightly increased relative to the analytical conditions. This increased the solubility of anthocyanins, as well as reduced the total run time to less than 2 h.



Compound	R_1	R_2	R_3
1	caffeoyl	H	H
2	caffeoyl	caffeoyl	H
3	caffeoy	feruloyl	H
4	*p*-coumaroyl	H	H
5	*p*-coumaroyl	caffeoyl	H
6	feruloyl	H	H
7	feruloyl	caffeoyl	H
8	*p*-coumaroyl	feruloyl	H
9	feruloyl	feruloyl	H
10	feruloyl	H	feruloyl

Figure 3. Compounds 1–10. See the Appendix for details.

Under the preparative conditions, anthocyanins eluted at 110.0 min and good separations were still achieved (Figure 2). Once the stationary phase and mobile phase are selected, the usual optimization procedure is to change the solvent strength of the mobile phase. This approach was followed in this study. From analytical chromatograms, many useful parameters can be extracted (e.g., retention time, peak efficiency, separation factor, resolution, and the cycle time). In this study, we attempted to find the best possible preparative separation conditions based on analytical experiments only.

Identification of anthocyanins

The present work is directed towards preparative isolation of the anthocyanins and their characterization by spectroscopic techniques like NMR and MS. After the collection of each fraction, the solvent is evaporated under vacuum and the purity checked by analytical HPLC. Later, the fractions were purified by semipreparative HPLC, and then analyzed by ^{1}H and ^{13}C NMR spectrometry to result in known compounds **1–10**. The spectral data was used for characterization of anthocyanins collected by semi-preparative HPLC. The FAB-MS data of the fractions provided evidence of molecular masses. The ^{1}H and ^{13}C NMR data of these anthocyanins has been compared with the values reported in the literature and found to be in good agreement with the anthocyanins. The ten known compounds were identified by spectral analyses and comparison with reported data (2,3).

Conclusion

From analytical chromatograms as seen in Figure 1, many useful parameters can be extracted (e.g., retention time, peak efficiency, separation factor, resolution, and the cycle time). Scale-up optimizations are usually conducted by using analytical data; however, in this study, we attempted to find the best possible preparative separation conditions based on analytical experiments only. This work shows that analytical chromatographic experiments alone are useful for the prediction of scale-up conditions of preparative HPLC separations.

Acknowledgments

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Appendix

Compound **1**: Red amorphous powder, isolated at retention time 32.8 min, identified as Pelargonidin 3-O-[6-O-(E)-caffeoyl-2-O-β-D-glucopyranosyl]-(1 → 2)-β-D-glucopyranoside)-5-O-(β-D-glucopyranoside).

Compound **2**: Red amorphous powder, isolated at retention time 36.3 min, identified as Pelargonidin 3-O-[6-O-(E)-caffeoyl-2-O-(6-(E)-caffeoyl-β-D-glucopyranosyl]-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **3**: Red amorphous powder, isolated at retention time 46.8 min, identified as Pelargonidin 3-O-[6-O-(E)-caffeoyl-2-O-(6-{E}-feruloyl-β-D-glucopyranosyl]-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **4**: Red amorphous powder isolated at retention time 49.4 min, identified as Pelargonidin 3-O-[6-O-(E)-p-coumaroyl-2-O-β-D-glucopyranosyl]-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **5**: Red amorphous powder, isolated at retention time 54.1 min, identified as Pelargonidin 3-O-[6-O-(E)-p-coumaroyl -2-O-(6-{E}-caffeoyl-β-D-glucopyranosyl)-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **6**: Red amorphous powder, isolated at retention time 57.5 min, identified as Pelargonidin 3-O-[6-O-(E)-feruloyl-2-O-β-D-glucopyranosyl]-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **7**: Red amorphous powder, isolated at retention time 64.6 min, identified as Pelargonidin 3-O-[6-O-(E)-feruloyl-2-O-(6-{E}-caffeoyl-β-D-glucopyranosyl)-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **8**: Red amorphous powder, isolated at retention time 79.1 min, identified as Pelargonidin 3-O-[6-O-(E)-p-coumaroyl-2-O-(6-{E}-feruloyl-β-D-glucopyranosyl)-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **9**: Red amorphous powder, isolated at retention time 89.9 min, identified as Pelargonidin 3-O-[6-O-(E)-feruloyl-2-O-(6-{E}-feruloyl-β-D-glucopyranosyl)-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).

Compound **10**: Red amorphous powder, isolated at retention time 100.2 min, identified as Pelargonidin 3-O-[6-O-(E)-feruloyl -2-O-(2-{E}-feruloyl-β-D-glucopyranosyl)-(1 → 2)-β-D-glucopyranoside]-5-O-(β-D-glucopyranoside).