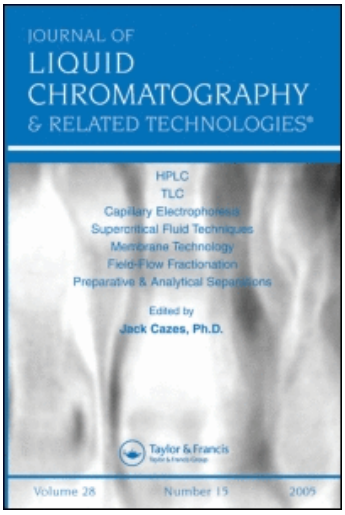

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Separation of Proanthocyanidins into Oligomeric and Polymeric Components Using a Novel Collagen Fiber Adsorbent

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Abstract: A novel collagen fiber adsorbent (CFA) was prepared by modification of natural collagen fiber using glutaraldehyde, and its adsorption behaviors to proanthocyanidins were investigated. Both batch and column adsorption experiments indicated that CFA has high adsorption selectivity to polymeric proanthocyanidins because of the stronger trend of these polymers to combine with collagen fiber through hydrophobic and hydrogen bonds. Based on this character of CFA, the purified oligomeric proanthocyanidins was obtained by using CFA as adsorbent. Meanwhile, it was found that the polymeric proanthocyanidins adsorbed on CFA can be easily desorbed by 50% (v/v) acetone-water solution. Thus, as a cost effective approach, CFA can be used to separate proanthocyanidins extracts into two parts, oligomeric proanthocyanidins and polymeric proanthocyanidins, which would favor the fine uses of proanthocyanidins extracts. Column adsorption studies showed that CFA used can be regenerated by using acetone-water solution as eluant, and its adsorption selectivity to polymeric proanthocyanidins was almost unchanged in 6 cycles.

Keywords: Adsorbent, Collagen fiber, Fine use, Proanthocyanidins, Selective adsorption, Separation

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INTRODUCTION

Proanthocyanidins, the phenolic compounds composed of oligomers and polymers of flavan-3-ols and/or flavan-3,4-diols, are widely distributed in the plant kingdom.[1] In recent years, the biological activities of proanthocyanidins, such as antioxidation and radical scavenging properties, have been revealed and therefore, the utilization of proanthocyanidins become more and more interesting for researchers in food and medicine fields.[2–5]

The commercially available proanthocyanidin extracts are usually a mixture of oligomeric and polymeric proanthocyanidins. The oligomeric proanthocyanidins are commonly clarified as those having polymerization degrees lower than 5.[6] It has been proven that the biological activities of proanthocyanidins are dependent on their degree of polymerization, and oligomeric proanthocyanidins usually exhibit higher activities than polymeric ones. For example, the antioxidative ability of oligomeric proanthocyanidins was found to be much higher than those of polymeric proanthocyanidins and vitamins, due to the fact that they can easily pass through the film of cells.[7,8] On the other hand, polymeric proanthocyanidins were found to be a kind of good raw material for preparing natural pigments because they can be easily derived into anthocyanidins in acidic condition. Anthocyanidins have attractive color and proper water solubility, and are suitable to be used in the food industry.[9–11] It is beyond doubt that proanthocyanidins extracts might be most effectively utilized if separated into pure compounds. In fact, many chromatographic methods, such as size exclusion,[12] countercurrent,[13,14] and gel permeation technologies,[15,16] have been attempted for this purpose. But, it was clarified that these approaches are unsuitable for industrial applications in consideration of the effectiveness and cost. Therefore, an appropriate separation of proanthocyanidins extracts, such as fractionation of proanthocyanidins into oligomeric and polymeric proanthocyanidins, would be of significance in the practice of the fine uses of these kinds of natural products.

It is well known that natural polyphenols are able to react with proteins like collagen through hydrophobic and hydrogen bonds, and that the higher molecular weigh of polyphenols leads to stronger interaction. In fact, the so called vegetable tanning in the leather industry is based on the interaction between collagen fibers and tannins, namely, the polyphenols with molecular weights ranging from 500 to 3000 Dalton. Hence, it is reasonable to deduce that collagen fiber is capable of associating with polymeric proanthocyanidins more easily than with oligomeric proanthocyanidins, and consequently shows binding/adsorption selectivity to the polymers in proanthocyanidins extracts. In fact, our previous study indicated that collagen fiber has high adsorption

selectivity to tannins in the mixture solution of medicinal plant extracts.[17]

In this study, a novel collagen fiber adsorbent (CFA) was prepared from bovine hide. The selective adsorption of CFA to polymeric proanthocyanidins and the desorption behaviors were investigated. Based on the results obtained, an approach of cost effective fine use of proanthocyanidin extracts is suggested.

EXPERIMENTAL

Materials

Collagen fiber was prepared from bovine skin according to the procedures in our previous work.[17] Natural collagen fiber is easily attacked by bacteria and chemicals and has low hydrothermal stability. Therefore, a proper cross linking reaction was carried out to enhance the stability of collagen fiber. Herein, glutaraldehyde was employed as the cross linking agent, due to its reaction activity with the $-NH_2$ groups on collagen fibers. The cross linking reaction was initiated by mixing 15 g collagen fiber and 1.5 g glutaraldehyde into 300 mL distilled water. The mixture was stirred at room temperature for 4 h, followed by a further stirring at 45°C for 4 h. The mixture solution was then filtrated and washed with distilled water. After drying at 50°C in vacuum, the collagen fiber adsorbent (CFA) with increased thermal and chemical stabilities was obtained. The thermal denaturation temperature of CFA was 80–86°C, detected by Differential Scanning Calorimetry (DSC, 200PC, Netzsch, Germany).

The commercially available proanthocyanidins, extracted from grape seed, was purchased from Jianfeng Co. Ltd., Tianjing, China. The standard samples of oligomeric proanthocyanidins, including two monomers (catechin and epicatechin, Mw 290) and two dimmers (procyanidin B1 and B2, Mw 578), were purchased from Sigma Co. Ltd., USA.

Adsorption Procedures

Batch Adsorption Experiments

Two hundred mg proanthocyanidins was dissolved in 100 mL ethanol–water solution (v/v = 30:70). The pH of the solution was 4.0 without adjusting. CFA, 0.5 g, was then suspended in the proanthocyanidin solution and shaken at 30°C for an indicated duration. The CFA was subsequently removed from the solution by centrifugation. The proanthocyanidin solutions before and after adsorption were analyzed by HPLC (Agilent

1100). To attain adsorption equilibrium, the adsorption process lasted for 24 h. The adsorption ratio of proanthocyanidins was calculated based on the peak areas in the HPLC patterns. The adsorption ratio (AR) was expressed as:

$$AR = (A_1 - A_2)/A_1,$$

where A_1 and A_2 are the peak areas of proanthocyanidins in the HPLC patterns of solutions before and after adsorption, respectively.

After adsorption, the loaded CFA was desorbed by suspending it in 100 mL water-acetone (v/v = 50:50) solution and shaking at 30°C for 1 h. The desorption solution was analyzed by HPLC, and the desorption ratio (DR) was expressed as:

$$DR = A_3/(A_1 - A_2)$$

where A_1 and A_2 are the same as indicated above, and A_3 is the peak area of proanthocyanidins in the HPLC pattern of desorption solution.

The influence of pH on the adsorption selectivity of CFA to proanthocyanidins, as well as the re-adsorbing properties of desorbed/recycled CFA, was investigated by the same procedures as described above.

Column Adsorption Experiments

CFA (2 g) was soaked in distilled water for 12 h and then filled into a column with internal diameter 11 mm and height 160 mm. The CFA column was equilibrated with ethanol–water solution (v/v = 30:70, pH 4.0) before loading. Proanthocyanidin solution (2.0 g/L, pH 4.0) was prepared by dissolving proanthocyanidins into ethanol–water solution (v/v = 30:70), and was pumped into the CFA column with a constant velocity of 2.0 BV/h (BV = bed volume). The effluent was collected by an automatic collector and analyzed by HPLC. Once polymeric proanthocyanidins was detected, the CFA column was considered to reach adsorption equilibrium. Subsequently, 200 mL acetone-water solution (v/v = 50:50) was used to elute proanthocyanidins from the column at velocity of 2.0 BV/h, and the eluent was collected for HPLC analysis.

RESULTS AND DISCUSSION

Selective Adsorption of CFA to Polymeric Proanthocyanidins

Figure 1 shows the HPLC patterns of proanthocyanidin solutions before and after adsorption by CFA, where the elutes with retention time

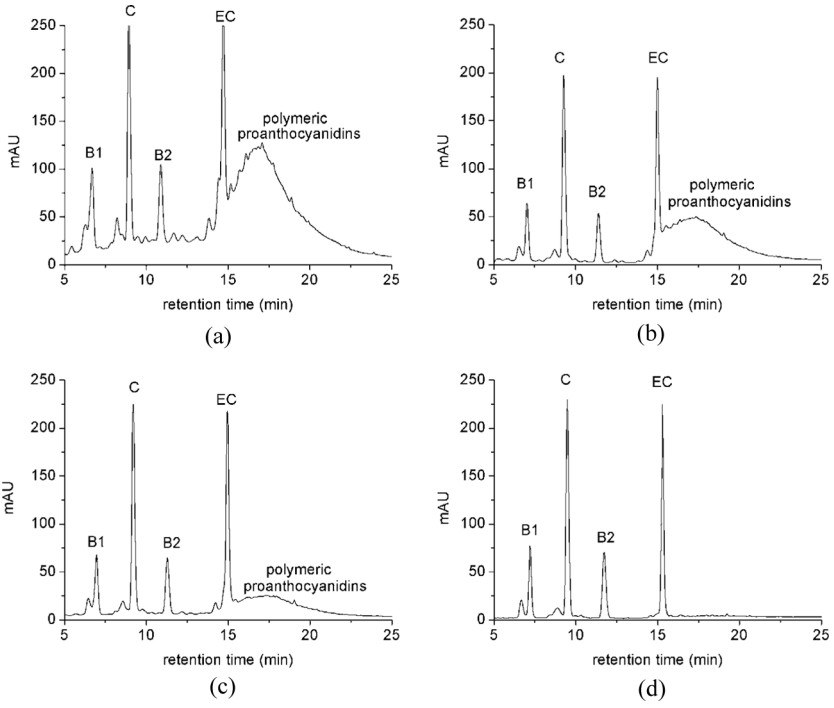

Figure 1. HPLC patterns of proanthocyanidin solutions before and after adsorption by CFA. (a) before adsorption; (b) adsorbed for 1 h; (c) adsorbed for 3 h; (d) adsorbed for 24 h. The pH value of proanthocyanidins solution was 4.0, and adsorption was conducted without pH adjusting. Mobile phase: A = H_2O (0.5% H_3PO_4), B = CH_3OH; column: Hypersil ODS C_{18}, 4.0 × 250 mm, 5 μm, 30 °C; flow rate: 0.8 mL/min; detector: DAD, 280 nm; gradient: 0–10 min with 18–24% B, 10–25 min with 24–70% B, 25–35 min with 70% B; injection volume: 20 μL.

≤15 min are oligomeric proanthocyanidins and those with retention time >15 min are the polymeric ones, as discussed in our previous reports.[17,18] The adsorption of CFA to proanthocyanidins was lasted for 24 h to ensure the attainment of adsorption equilibrium. It is remarkable that the polymeric proanthocyanidins were completely removed from solution (AR = 100%), while the peak areas of oligomeric proanthocyanidins was a little reduced. The ARs of catechin (C), epicatechin (EC), procyanidin B1 (B1), and procyanidin B2 (B2) were 5.3%, 5.8%, 8.2%, and 8.9%, respectively, as shown in Figure 1d and Table 1. These observations indicate that CFA strongly adsorbs polymeric proanthocyanidins, whereas it has low adsorption capacity to oligomers.

The adsorption of CFA to polyphenols is mainly attributed to the formation of hydrophobic and hydrogen bonds, where the pyrogallol

Table 1. The adsorption ratios (AR, %) of proanthocyanidins on CFA during adsorption process

Components of proanthocyanidins	Adsorption duration		
	1 h	3 h	24 h
C	8.1	5.6	5.3
EC	9.2	6.4	5.8
B1	11.9	9.6	8.2
B2	13.0	10.0	8.9
Polymers	52.5	83.6	100

structure in polyphenol molecule plays an important role.[19] The pyrogallol groups facilitate the access of polyphenol to CFA by hydrophobic bonds, and then phenolic hydroxyl groups enhance the polyphenol-CFA combination by multi-hydrogen bonds.[1,19] Polymeric proanthocyanidins contain more pyrogallol and phenolic hydroxyl groups in comparison with oligomeric proanthocyanidins, and therefore, can be firmly adsorbed by CFA through hydrophobic and hydrogen bonds. However, oligomeric proanthocyanidins with less pyrogallol and phenolic hydroxyl groups can only form weaker association with CFA.

An interesting phenomenon was observed during the adsorption process. After 1 h of adsorption, the ARs of C, EC, B1, B2, and polymeric proanthocyanidins were 8.1%, 9.2%, 11.9%, 13.0%, and 52.5%, respectively, as shown in Figure 1 and Table 1. Afterwards, the adsorbed amount of polymeric proanthocyanidins increased while the ARs of oligomers decreased. This observation suggests that the oligomeric proanthocyanidins adsorbed on CFA can be replaced by polymeric proanthocyanidins during the adsorption process because of the weaker association ability of oligomers on CFA.

The desorption of the polymeric proanthocyanidins adsorbed on CFA was tested by using water, methanol, ethanol, acetone, and their mixtures as eluants. It was found that water-acetone (v/v = 50:50) solution performed effectively. The HPLC pattern of the water-acetone desorption solution was shown in Figure 2, accompanied by a desorption ratio (DA) of 95.8%.

All the results above indicate that CFA has remarkable adsorption selectivity to the polymers in proanthocyanidins, and the polymeric proanthocyanidins adsorbed on CFA can be desorbed effectively. The adsorption and desorption properties of proanthocyanidins on CFA facilitates a cost effective separation of oligomeric and polymeric proanthocyanidins, which would favor the fine and value added uses of proanthocyanidin extracts.

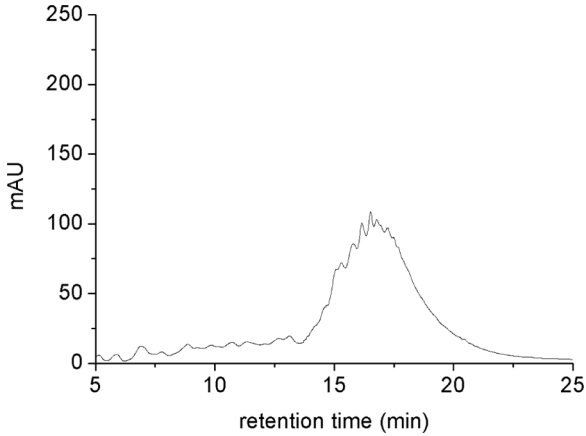

Figure 2. HPLC pattern of proanthocyanidins in desorption solution. (Chromatographic conditions were the same as those in Figure 1.)

Influence of pH on Adsorption of CFA to Proanthocyanidins

All the results above were obtained in the natural pH (pH 4.0) of the proanthocyanidin solution. The adsorption of CFA to proanthocyanidins under acidic (pH 3.0) and neutral (pH 7.0) conditions was further investigated in this section. As shown in Figure 3 and Figure 1c, the HPLC patterns of proanthocyanidin solutions after 3 h of adsorption by CFA at pH 3.0, 4.0, and 7.0 are very similar. This similarity kept up even though the adsorption process lasted for 24 h (data not shown). These observations indicate a negligible effect of pH on the adsorption selectivity of CFA to polymeric proanthocyanidins, implying that the change of pH does not considerably affect the hydrophobic and hydrogen bond interactions between proanthocyanidins and CFA. Considering the fact that auto-oxidation of proanthocyanidins increases with the increase of pH, a relatively lower pH is more suitable for the adsorption process. Therefore, the adsorption of CFA to proanthocyanidins was carried out without adjusting the pH in the following experiments.

Adsorption Property of Regenerated CFA to Proanthocyanidins

As mentioned above, CFA can be regenerated by eluating with 50% (v/v) water-acetone solution. The adsorption property of regenerated CFA was studied, which is economically crucial for the practical application of CFA. As presented in Figure 4 and Table 2, CFA kept an

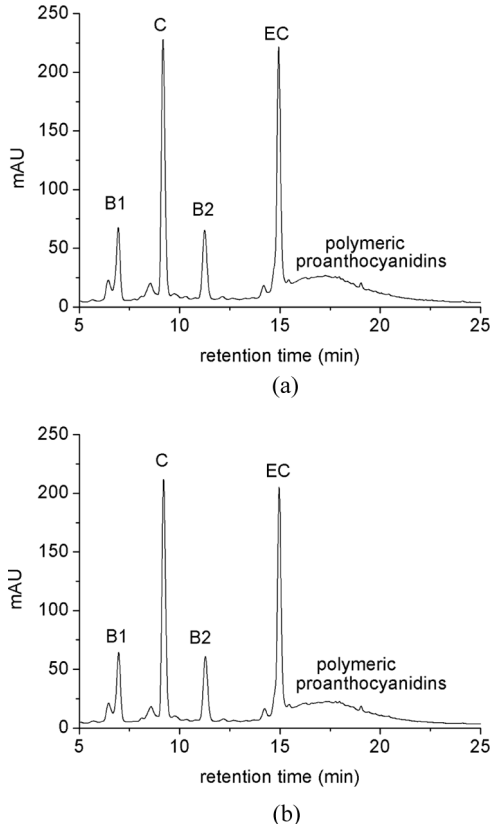

Figure 3. HPLC patterns of proanthocyanidin solutions after adsorption by CFA at different pH values. (a) pH = 7.0; (b) pH = 3.0. (Initial Conc. 2.0 g/L; adsorbed for 3 h; chromatographic conditions were the same as those in Figure 1.)

adsorption ratio of 100% to polymeric proanthocyanidins in the first three cycles, meanwhile the adsorption ratios of oligomeric proanthocyanidins were all lower than 10%. Afterwards, the regenerated CFA still exhibited much higher adsorption to polymeric proanthocyanidins than to oligomeric proanthocyanidins, though the AR values of oligomers slightly increased. This adsorption selectivity of CFA was still remarkable, even in the sixth cycle, where the adsorption ratios of C, EC, B1, B2, and polymeric proanthocyanidins were 8.5%, 8.8%, 11.4%, 11.7%, and 86.8%, respectively. These results indicate that CFA can be repeatedly used for the separation of oligomeric and polymeric proanthocyanidins, promising a cost effective application of CFA in practice.

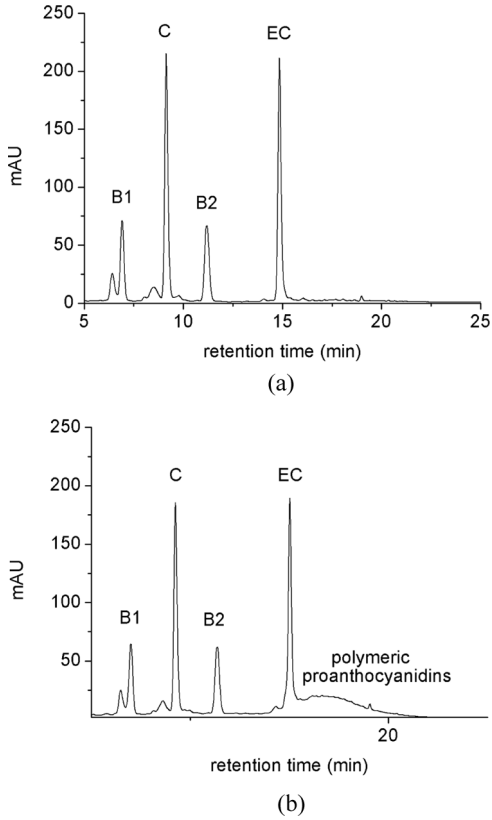

Figure 4. HPLC patterns of proanthocyanidin solutions after adsorption by regenerated CFA. (a) cycle 3; (b) cycle 6. (Initial Conc. 2.0 g/L; adsorbed for 24 h; chromatographic conditions were the same as those in Figure 1.)

Table 2. The adsorption ratio (AR, %) of proanthocyanidins on regenerated CFA

Components of proanthocyanidins	Cycles					
	1	2	3	4	5	6
C	5.3	5.8	6.2	6.0	7.2	8.5
EC	5.8	6.2	6.3	7.5	8.7	8.8
B1	8.2	8.0	8.5	8.9	9.3	11.4
B2	8.9	9.1	9.4	10.2	11.5	11.7
Polymers	100	100	100	96.7	92.3	86.8

The Adsorption of Proanthocyanidins on CFA Column

Every 10 mL of effluent from CFA column was collected as a sample for HPLC analysis. As shown in Figure 5, oligomeric and polymeric proanthocyanidins passed through the column at different retention times because of their difference in combination intensity with collagen fibers. Nothing was found in the first 100 mL of effluent, meaning that all the proanthocyanidins were adsorbed by the CFA column (Figure 5a). C and EC were detected when the volume of effluent was increased to 200 mL (Figure 5b), and C, EC, B1, and B2 were all detected in the effluent sample amounting to 400 mL (Figure 5c). Polymeric proanthocyanidins were detected when the total volume of effluent reached 800 mL (Figure 5d).

Many physical factors might affect column adsorption/separation process, such as surface property of adsorbent and the molecular size of adsorbates. However, the retainment of proanthocyanidins on the

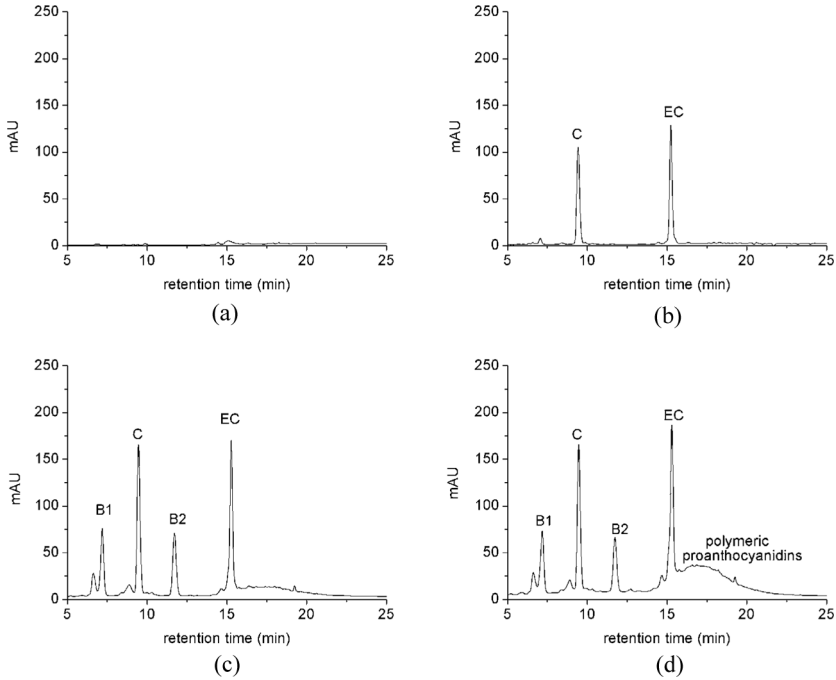

Figure 5. HPLC patterns of effluent samples from CFA column. (a) at 100 mL; (b) at 200 mL; (c) at 400 mL; (d) at 800 mL. (Conc. of proanthocyanidins in input solution was at 2.0 g/L. Chromatographic conditions were the same as those in Figure 1.)

CFA column is mainly dependent on the formation of hydrophobic and hydrogen bonds between proanthocyanidins and CFA. Catechin and epicatechin are monomer proanthocyanidins, which have the weakest combination with CFA through limited hydrophobic and hydrogen bonds. So, C and EC were detected prior to B1 and B2 in the effluent, and polymeric proanthocyanidins exhibited a longer retainment on the CFA column than the oligomers did. These observations indicate that oligomeric and polymeric proanthocyanidins can be separated by the CFA column. In addition, it can be also suggested that the CFA column might be suitable to be used for separating monomer and dimmer proanthocyanidins from proanthocyanidins extracts.

The CFA column reached a saturated adsorption when polymeric proanthocyanidins were detected in the effluent. Then, the column was eluted using 200 mL of acetone-water solution (v/v = 50:50). As shown in Figure 6, the HPLC pattern of eluent is similar to that of the desorption solution in batch experiments (Figure 2), which indicates that the polymeric proanthocyanidins in proanthocyanidins extracts can be isolated by CFA columns through adsorption-elution operation.

At the first stage of column separation (Figure 5a), proanthocyanidins were all adsorbed on the column, whereas no oligomeric proanthocyanidins (C, EC, B1, or B2) except the polymers was detected in the eluent (Figure 6). These facts indicate that the oligomers adsorbed at the beginning were subsequently replaced by polymers, leading to a selective adsorption of polymeric proanthocyanidins on CFA column, which is consistent with the results discussed in the Batch Adsorption Experiments Section. These adsorption and desorption behaviors of

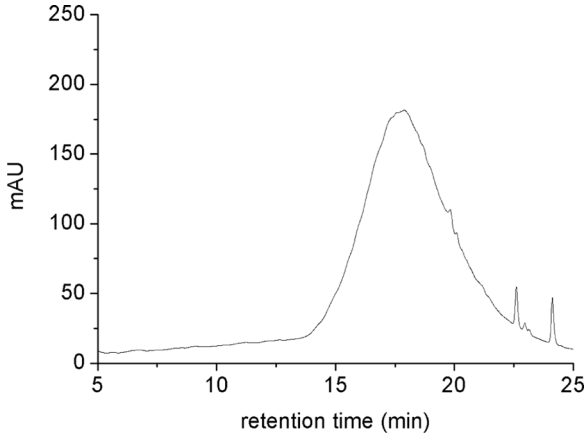

Figure 6. HPLC pattern of proanthocyanidins in the eluent from CFA column. (Chromatographic conditions were the same as those in Figure 1.)

proanthocyanidins on CFA column promise an effective separation of oligomeric and polymeric proanthocyanidins in practice.

CONCLUSION

The collagen fiber adsorbent (CFA) prepared in this study can effectively fractionate proanthocyanidins into oligomeric and polymeric parts due to its high adsorption selectivity to polymeric proanthocyanidins. This kind of separation of proanthocyanidins might favor the fine uses of proanthocyanidins based on the characteristics of different fractions. This adsorbent is easy to prepare and convenient to regenerate, and therefore, its industrial application in the fractionation of proanthocyanidins can be expected.

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