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Journal of Chromatography A, 1063 (2005) 161–169
JOURNAL OF CHROMATOGRAPHY A
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Isolation and purification of acteoside and isoacteoside from *Plantago psyllium* L. by high-speed counter-current chromatography

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Received 1 September 2004; received in revised form 29 October 2004; accepted 9 November 2004
Available online 7 December 2004

Abstract

Two isomeric phenylethanoid glycosides, acteoside and isoacteoside were isolated and purified from the seeds of *Plantago psyllium* L. for the first time by high-speed counter-current chromatography (HSCCC) using a solvent system consisting of ethyl acetate–water (1:1, v:v). By injecting 200 mg of the *n*-butanol extract of *P. psyllium* for five consecutive times, the two-step HSCCC procedure yielded a total of 165 mg of acteoside and 17.5 mg of isoacteoside from 978 mg extract. The recovery rates for acteoside and isoacteoside were 90 and 84%, respectively, and the purities were 98 and 94%, respectively. The HSCCC fractions were analyzed by HPLC and the structures were identified by UV, LC–APCI–MS in negative ion mode, and confirmed by NMR experiments.

Keywords: *Plantago psyllium*; Acteoside; Isoacteoside; Phenylethanoid glycoside; HSCCC; LC–APCI–MS; NMR

1. Introduction

Plantago psyllium L. belongs to the *Plantago* family. Its seeds have been used as demulcents and in the treatment of chronic constipation [1]. The major bioactive constitute in the seeds of *P. psyllium* are phenolic compounds, including acteoside and isoacteoside (Fig. 1). Acteoside and related phenylethanoid glycosides are widely distributed in the plant kingdom [2–7] and have been found to have various biological activities, including anti-hepatotoxic [8], anti-inflammatory, anti-nociceptive [9] and antioxidant [10,11] activities. The preparative separation and purification of acteoside and related phenylethanoid glycosides from many plants by conventional methods are tedious and usually require repeated chromatographic steps on silica gel and Sephadex LH-20 column [3–7]. The overall yields of these methods were poor, because the hydroxyl groups in the phenylethanoid glycosides make these compounds strongly adsorbed onto the solid support during separation [12].

High-speed counter-current chromatography (HSCCC) is a support-free all liquid chromatographic technique that has been successfully applied to separation and isolation of many natural products [12–18]. The HSCCC method is considered as a suitable alternative for the separation of phenolic compounds [19–21]. Lei et al. [12] successfully separated acteoside and 2′-acetyl acteoside from *Cistanches salsa* (C.A. Mey) G. Beck by using HSCCC, however, no report has been published on the use of HSCCC for the separation and purification of acteoside and isoacteoside from the seeds of *P. psyllium*. The identification of acteoside and other phenolic compounds by mass spectrometry also has been limited to electrospray ionization (ESI)–MS, fast-atom bombardment (FAB)–MS and field desorption (FD)–MS [6,22,23]. The objective of this paper was to develop a simple and efficient method for the preparation of acteoside and isoacteoside from

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doi:10.1016/j.chroma.2004.11.024

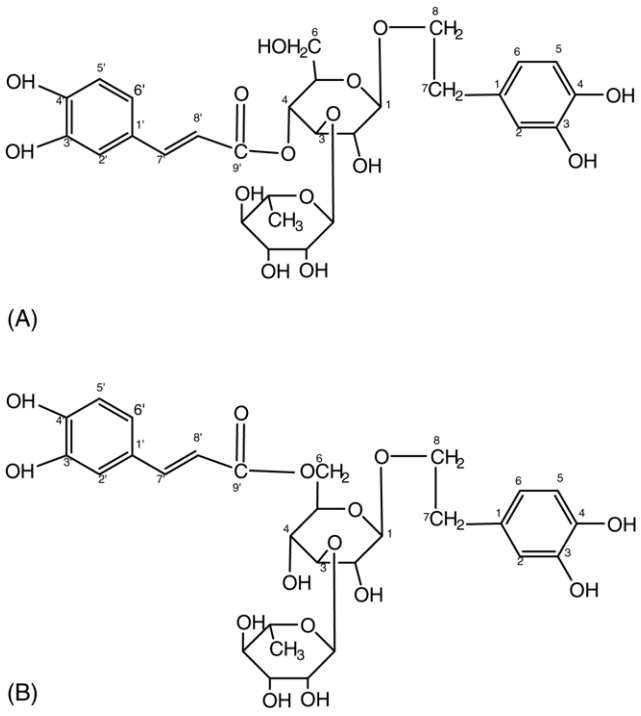

Fig. 1. Structures of acteoside (A) and isoacteoside (B).

P. psyllium seeds by high-speed counter-current chromatography. In our experiment, LC coupled with on-line APCI mass spectrometry and NMR experiments were used for the analysis and identification of these two isomeric phenylethanoid glycosides.

2. Experimental

2.1. Chemicals and reagents

Acteoside, caffeic acid and hydroxytyrosol were purchased from Sigma–Aldrich (Oakville, ON). *P. psyllium* seeds were provided by Prof. Zeyuan Deng (NanChang University, China). All solvents were of HPLC grade and purchased from Caledon Laboratories Ltd. (Georgetown, Canada).

2.2. Sample preparation

P. psyllium seeds (25 g) were milled to powder (ca. 50 mesh) by using a Retsch (MM 2000) machine, soaked in 500 mL of 80% aqueous methanol for 12 h for three times at room temperature. Each time, the extraction mixture was filtered through a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK). All three filtrates were combined and concentrated to 300 mL in vacuo at <40 °C. The resulting aqueous solution was defatted twice, each with 300 mL of hexane and then extracted successively for three times, each with 300 mL *n*-butanol. The *n*-butanol layers were combined and concentrated to dryness in vacuo at <40 °C, which yielded 978 mg of crude extract. The extract was stored at −4 °C before HSCCC separation.

2.3. HSCCC separation procedure

The preparative HSCCC was carried out using a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). This apparatus had three preparative coils, connected in series (total volume, 325 mL). The revolution speed of the apparatus could be regulated between 0 and 2000 rpm. The HSCCC system was equipped with an HPLC pump (Pharma-Tech Research, Baltimore, Maryland, USA), a Model 450 UV detector (Alltech, USA), a Model L 120 E flat-bed recorder (Linseis Inc., Princeton Jct, USA), a fraction collector (Advantec MFS Inc., USA) and a sample injection valve with a 10 mL sample loop.

A mixture of ethyl acetate–water (1:1, v:v) was shaken vigorously in a separatory funnel and let stand at room temperature until there were two clearly separated phases. The two phases were then used in the HSCCC after they reached equilibrium.

The entire coiled column was first filled with the upper layer which serves as the stationary phase. Then, the pump was set at 1045 rpm. The lower layer (mobile phase) was pumped into head end of the column at a flow-rate of 1.5 mL/min. A sample (200 mg) dissolved in 8 mL of the mixture of ethyl acetate–water (1:1, v:v) was loaded into the injection valve after the system reached hydrodynamic equilibrium. The effluent from the outlet of the column was continuously monitored by a UV detector at 254 nm and collected into test tubes with a fraction collector set at 4 min for each tube. Five consecutive injections, each containing ca. 200 mg of the *n*-butanol extract in 8 mL of the two-phase solvent system, were made. Fractions from the HSCCC that had only pure compound as determined by HPLC were combined, and freeze-dried. Those containing a mixture of the compounds were pooled, concentrated and re-injected to the system for further purification.

2.4. LC conditions

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler and a photodiode array detector (DAD), was used for the analysis of phenylethanoid glycosides in the *n*-butanol extract and fractions collected from the HSCCC separation. The analysis was carried out with a Phenomenex ODS-C_{18} column (150 mm × 4.6 mm, 5 μm) and a C_{18} guard column. The binary mobile phase consisted of acetonitrile (solvent A) and water containing 2% acetic acid (solvent B). All solvents were filtered through a 0.45 μm filter prior to use. The flow-rate was kept constant at 1.0 mL/min for a total run time of 30 min. The system was run with a gradient program: 100% B to 75% B in 20 min, 75% B to 0% B in 5 min, and

0% B to 100% B in 5 min. The sample injection volume was 10 μL. Peaks of interest were monitored at 320 nm by a DAD detector.

2.5. LC–APCI–MS for identification

LC–MS experiments were carried out using an LC coupled to a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) which was equipped with an atmospheric pressure chemical ionization (APCI) source. The mass spectrometer conditions were optimized for acteoside prior to sample analysis in order to achieve maximum sensitivity. As a result, the shear gas and auxiliary flow rates were set at 54 and 12 (arbitrary units), respectively. The capillary voltage was fixed at −28 kV and its temperature was controlled at 200 °C. The vaporizer temperature was set at 450 °C and the current of discharge needle was at 4.50 μA. The entrance lens voltage was fixed at 60 V. The tube lens offset was −21 V, the multipole 1 offset was 5 V and the multipole 2 offset was 12.5 V. The lens voltage was 38 V. The electron multiplier voltage was set at 400 V for ion detection. Full scan of ions was performed in the negative ion mode with a molecular mass range set from 50 to 2000. The same separation conditions were used as in the LC experiment.

2.6. Acid hydrolysis and monosaccharide analysis

One milligram of compound 1 or 2 was hydrolyzed with 1 ml of 1 M H_2SO_4 which was heated at 90 °C in a water bath for 1.5 h. The hydrolyzed product was diluted with water and filtered through a 0.45 μm syringe filter before being analyzed by HPLC using the same conditions. Monosaccharides were analyzed in a Dionex (Sunnydale, CA, USA) DX-500 ion chromatograph using a Dionex CarboPac PA1 column (4 mm × 250 mm) and a Dionex PA guard column (4 mm × 25 mm). A pulsed amperometric detector (PAD) was used for detection. A gold electrode was used as the working electrode and silver/silver chloride as the reference electrode. The mobile phase consisted of 100 mM NaOH (solvent A), 30 mM NaOH (solvent B) and water (solvent C). Prior to sample injection, the instrument was run with 100% B for 15 min, and then a combination of 8% A and 92% C for 10 min. After injection, 8% A and 92% C were run for 7 min and then 100% C for 18 min. The column was held at 35 °C with a flow rate of 1 mL/min. The injection volume was 50 μL for both samples.

2.7. NMR for identification

Proton NMR spectra were recorded on a Bruker Avance-600 spectrometer (Bruker BioSpin Ltd., Canada). Compounds 1 and 2 were dissolved in CD_3OD, separately. TMS was an internal standard. In addition, H–H COSY and C–H correlation were also recorded.

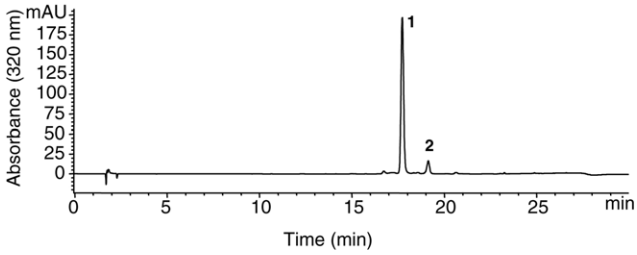

Fig. 2. HPLC profile of the *n*-butanol extract of *P. psyllium* seed. Peak 1, compound 1; peak 2, compound 2. Experimental conditions: column, Phenomenex C_{18}-ODS column (150 mm × 4.6 mm, 5 μm) and a C_{18} guard column; mobile phase: acetonitrile (A) and 2% acetic acid (B), the gradient program: 100% B to 75% B in 20 min, 75% B to 0% B in 5 min, and 0% B to 100% B in 5 min; sample injection volume: 10 μL; flow rate: 1.0 mL/min; detection: 320 nm.

3. Results and discussion

3.1. HSCCC separation

The LC chromatogram of *n*-butanol extract from *P. psyllium* is given in Fig. 2. Two major peaks were separated and detected with retention times at 17.6 and 19.2 min. Compound 1 was identified as acteoside by congruence in its retention time and UV spectra with that of authentic acteoside.

In a HSCCC experiment, selection of the two-phase solvent system is the first and critical step; a good solvent system can provide an ideal partition coefficient (*K*) for the target compounds. The key of solvent optimization is first to find a solvent combination in which the sample is freely soluble, then to adjust this solvent combination to ensure that the *K* value of the target compounds is close to 1 [24–26]. The *K* value of a two-phase solvent system is critical for efficient separation. If it is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in loss of peak resolution; if the *K* valve is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time [13].

In our experiment, we selected five series of solvent systems according to the solubility of the target compounds. LC was used to measure the sample concentration in each phase, from which the *K* values of the target compounds were calculated. The *K* values of compounds 1 and 2 in these systems are given in Table 1. A solvent system containing ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5, v:v:v:v) has been used in HSCCC to separate acteoside and 2′-acetyl acteoside from *Cistanches salsa* (C.A. Mey) G. Beck [12]. However, we found that this system was not suitable for the separation of the two phenylethanoid glycosides from *P. psyllium*; the *K* values of compounds 1 and 2 were greater than 1.5, and the settling time of the two-phase solvent system was rather long. A modified version of this system with solvent ratios at 4:0.6:0.6:4, v:v:v:v was also tried. Although the two phases separated quickly, the *K* value was too high for both com-

Table 1
The *K* (partition coefficient) values of compound 1 and 2 in different solvent systems[a]

Solvent system	Compound 1	Compound 2
Ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5)	1.58	1.94
Ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:4)	2.34	2.76
Ethyl acetate–*n*-butanol–water (0.75:0.25:1)	1.63	1.74
Hexane–ethyl acetate–methanol–water (0.5:0.5:0.25:0.75)	0.68	0.69
Ethyl acetate–water (1:1)	0.82	0.92

[a] Experimental procedure: approximately 1 mg of each sample was weighed in a 10 mL test tube into which 1 mL of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was capped and shaken vigorously for 1 min, and allowed to stand until it separated completely. An aliquot of 100 μL of each layer was taken out and evaporated separately to dryness in vacuo at <40 °C. The residue was dissolved in 100 μL methanol and analyzed by LC for determining the partition coefficient (*K*) of compound 1 and 2. The *K* value was expressed as the peak area of target compound in the upper phase divided by that in the lower phase.

pounds 1 and 2 (Table 1). The *K* values were reduced but still greater than 1 when ethanol was removed in a system containing ethyl acetate–*n*-butanol–water (0.75:0.25:1, v:v:v). A system containing hexane–ethyl acetate–methanol–water (0.5:0.5:0.25:0.75, v:v:v:v) was found to have a short settling time, but poor separation due to the low *K* values (Table 1). The solvent ratios of the these systems produced *K* values very close to the targeted value of 1, and the ratios could still be optimized further, however, due to the number of solvents involved, large number of combinations will have to be tested. To avoid this, we examined several two-solvent systems. Ethyl acetate–water (1:1, v:v) gave the best results. This system is simple, and the two phases reached equilibrium very quickly. The *K* values for compounds 1 and 2 in this system were 0.82 and 0.92, respectively, which produced a good separation (Table 1, Fig. 3). This system was then used in preparative separation and purification of the two main components in the *n*-butanol extract of *P. psyllium*.

Fig. 3A shows the HSCCC separation of one of the five injections containing ca. 200 mg of the *n*-butanol extract of *P. psyllium* using this solvent system. The stationary phase of this system, ethyl acetate–water (1:1, v:v), once filled into the coils, was found to give satisfactory resolution for five consecutive runs of separation. Fractions that were confirmed by HPLC to contain only compound 1 or 2 were combined separately, and those containing both compounds were pooled, freeze-dried and re-subjected to the HSCCC for further separation (Fig. 3B). The two-step HSCCC separation described above yielded a total of 165 mg of compound 1 and 17.5 mg of compound 2 from 978 mg *n*-butanol extract. The recovery rate of compounds 1 and 2 were 89.8 and 84.1%, respectively. The chromatographic purity of the freeze-dried compounds 1 and 2 were 98.4 and 94.2%, respectively, and these samples were directly used for LC–APCI–MS and NMR analyses.

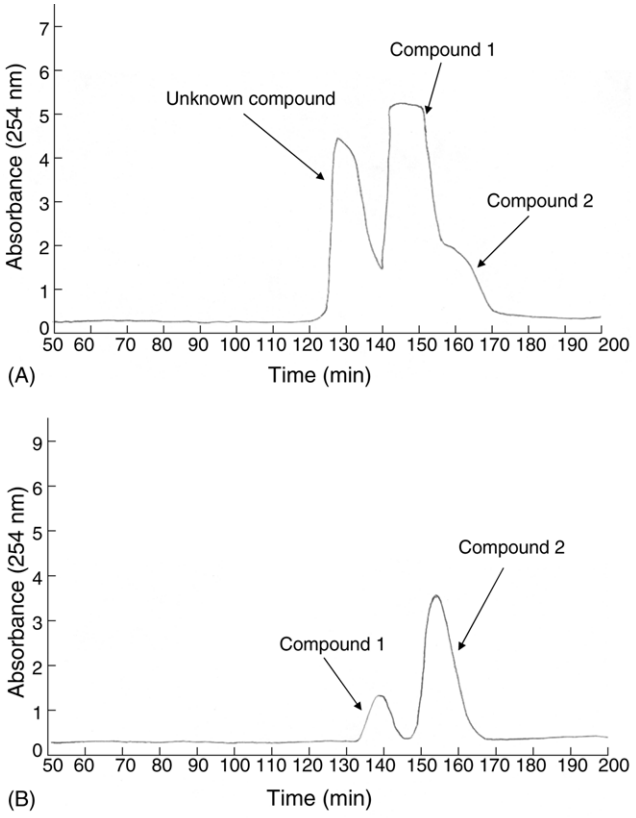

Fig. 3. HSCCC chromatograms of the *n*-butanol extract of *P. psyllium* seed. (A) Crude extract; (B) combined fractions containing both compounds 1 and 2. Experimental conditions: coil volume, 325 mL; rotation speed, 1045 rpm; flow rate, 1.5 mL/min; sample injection volume, 10 mL; sample injected, 200 mg extract dissolved in 8 mL of the mixture of acetate–water (1:1, v:v).

3.2. Identification by LC–APCI–MS and NMR experiments

Tentative identification of the compounds was first achieved by congruent retention times and UV spectra with that of the authentic acteoside standard (Fig. 4). Compound 1 was thus identified as acteoside; compound 2 was an unknown

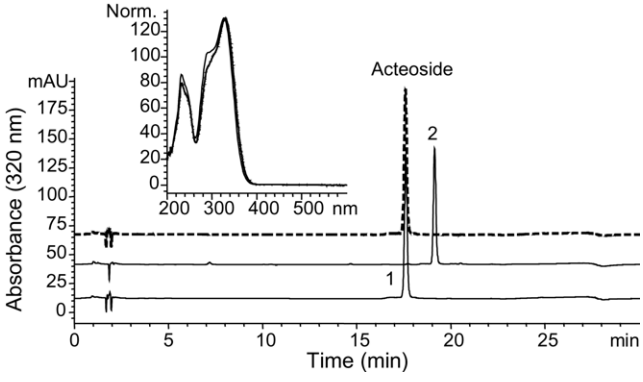

Fig. 4. HPLC chromatograms and UV spectra of standard acteoside, purified compounds 1 and 2. Separation conditions were the same as described in Fig. 2.

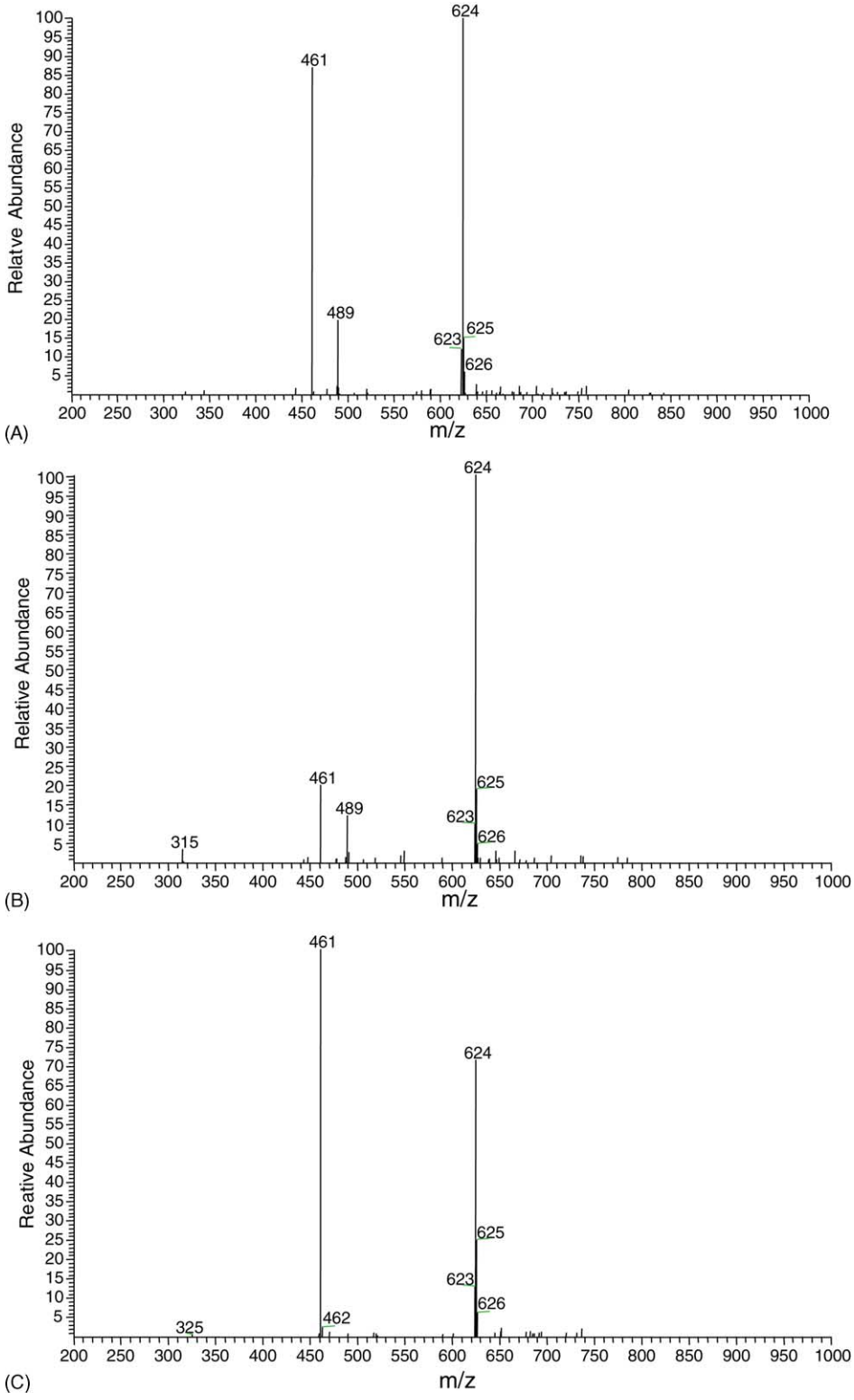

Fig. 5. (A) LC–APCI–MS/MS spectrum of *m*/*z* 623 of compound 1; (B) LC–MS^3 spectrum of the parent ion *m*/*z* 461 of compound 1; (C) LC–MS^3 spectrum of the parent ion *m*/*z* 489 of compound 1; (D) LC–APCI–MS/MS spectrum of *m*/*z* 623 of compound 2; (E) LC–MS^3 spectrum of the parent ion *m*/*z* 461 of compound 2; LC–MS^3 spectrum of the parent ion *m*/*z* 489 of compound 2; (F) LC–MS^3 spectrum of the parent ion *m*/*z* 489 of compound 2.

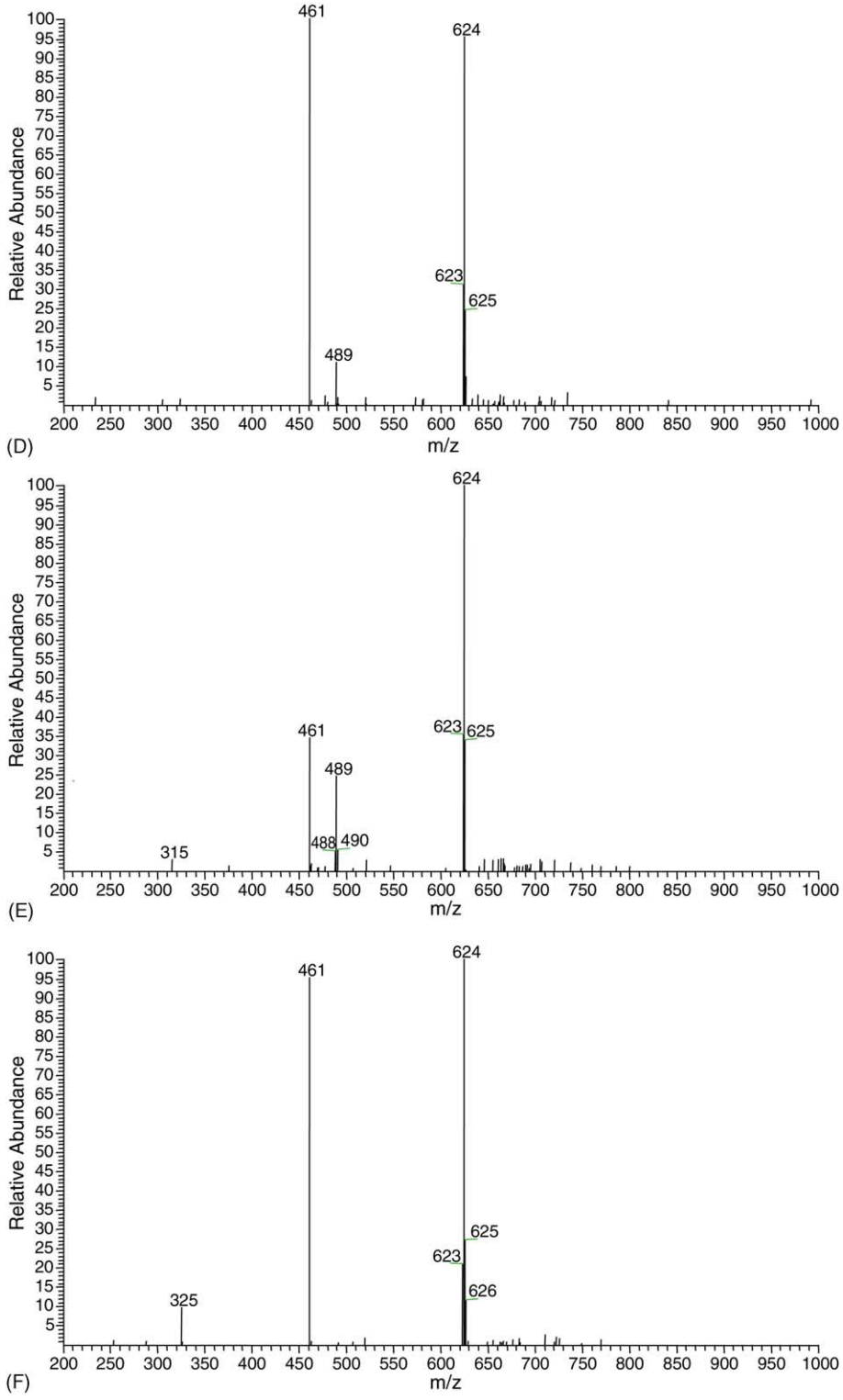

Fig. 5. (*Continued*).

compound. The UV spectrum of compound 1 showed the characteristic of phenylethanoid glycosides with absorption peaks at 232, 246, 289, 332 nm. The UV spectrum of compound 2 was highly similar (232, 246, 286, 328 nm).

Further identification of the structures of these two compounds by LC–APCI–MS, MS^2 and MS^3 are shown in Fig. 5. The LC–APCI–MS of compound 1 in the negative mode gave *m*/*z* 623 as the deprotonated molecular ion $[M–H]^-$, which confirmed the molecular mass as 624, the same as that for acteoside. Further experiments in MS^2 of the *m*/*z* 623 ion ($[M–H]^-$) produced two main fragment ions at *m*/*z* 489 and 461 (Fig. 5A). The ion at *m*/*z* 461 is considered to be from the loss of the caffeoyl moiety $[M–H–162]^-$ from the parent ion *m*/*z* 623, and 489 is produced from the cleavage of the bond between C8′ and C9′ $[M–H–134]^-$ (Figs. 5A and 6). The MS^3 spectrum of the ion at *m*/*z* 461 yielded one very weak ion at *m*/*z* 315 by losing a rhamnose unit (Figs. 5B and 6). The MS^3 spectrum of the ion at *m*/*z* 489 yielded one very weak daughter ion at *m*/*z* 325 by losing a 3,4-dihydroxyphenethyl moiety and a carbonyl group (C9′) of the caffeic acid (Figs. 5C and 6). All MS, MS^2 and MS^3 data for compound 1 were the same as that for the authentic acteoside. Possible fragmentation pathways of acteoside are illustrated in Fig. 6.

For compound 2, the LC–APCI–MS data provided *m*/*z* 623 as the deprotonated molecular ion $[M–H]^-$, which indicates that it has the same molecular mass of 624 as acteoside. The MS^2 spectrum of the ion at *m*/*z* 623 and MS^3 spectra of the ion at *m*/*z* 461 and 489 (Fig. 5D–F) were all the same as those of compound 1 (acteoside) (Fig. 5A–C), suggesting that these two compounds are isomers with similar structures.

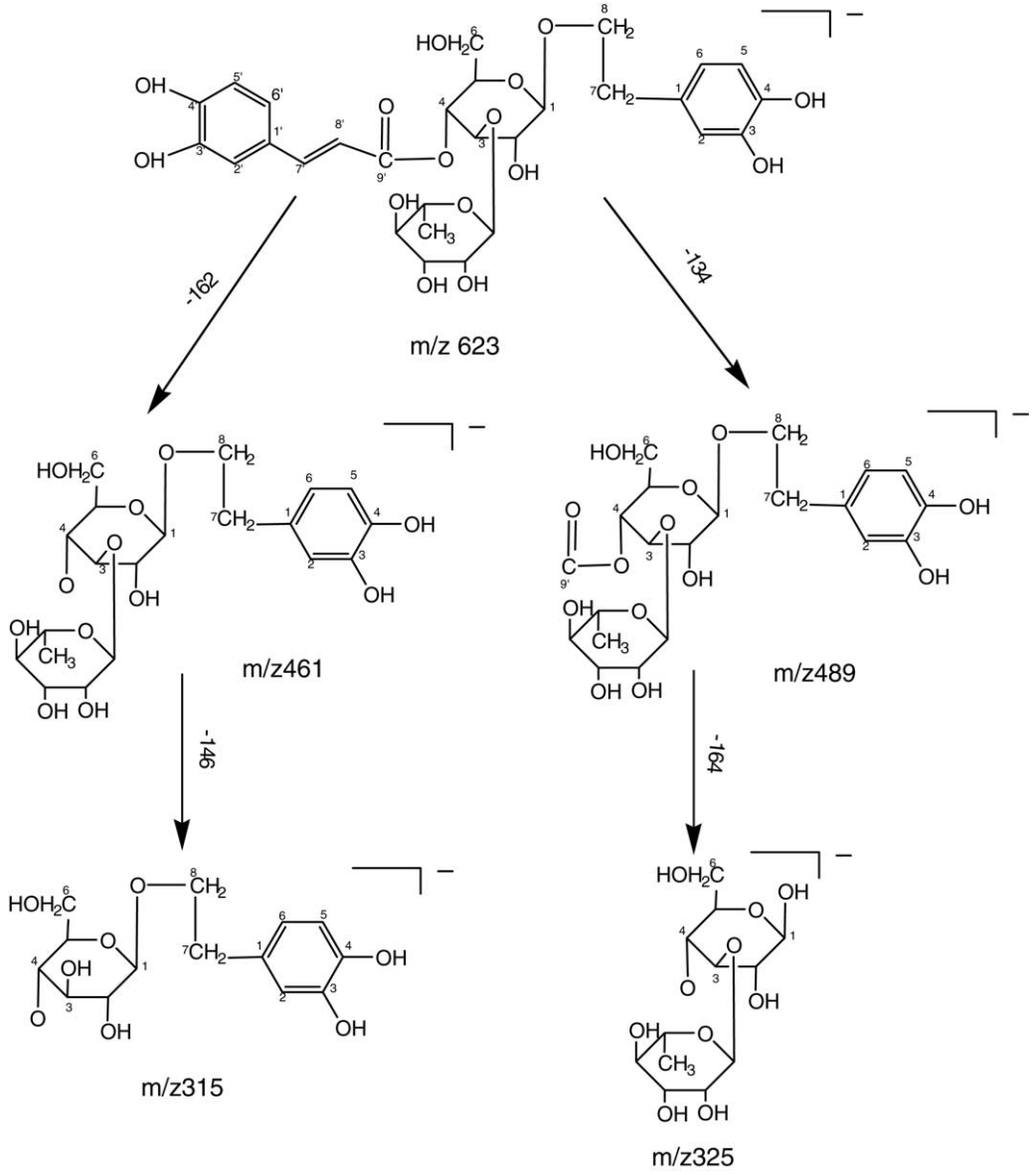

Fig. 6. Proposed fragmentation pathway for acteoside.

Table 2
Proton NMR data of acteoside and isoacteoside[a]

Moiety	C position	δ_H (J)	
		Acteoside	Isoacteoside
Hydroxytyrosol	1		
	2	6.702 (d, 1.8)	6.713 (d, 1.8)
	3		
	4		
	5	6.715 (d, 7.8)	6.674 (d, 7.8)
	6	6.607 (dd, 7.8, 1.8)	6.575 (dd, 7.8, 1.8)
	7	3.760 (dd, 12.6, 7.2), 4.044 (dd, 12.6, 7.2)	3.750 (dd, 9.6, 6.6) 4.025 (dd, 9.6, 6.6)
	8	2.890 (m)	2.821 (m)
Caffeic acid	1		
	2	7.095 (d, 1.8)	7.076 (d, 1.8)
	3		
	4		
	5	6.819 (d, 8.4)	6.809 (d, 7.8)
	6	6.997 (dd, 8.4, 1.8)	6.926 (d, 7.2)
	7	7.634 (d, 15.6)	7.603 (d, 16.2)
	8	6.314 (d, 15.6)	6.328 (d, 15.6)
	9		
Glucose	1	4.417 (d, 8.4)	4.327 (d, 7.8)
	2	3.432 (dd, 9.0, 8.4)	3.350 (dd, 9.0, 6.0)
	3	3.856 (t, 9.0)	3.571 (t, 9.0)
	4	**4.952 (d, 9.6)**	**3.432 (dd, 9.6)**
	5	3.575 (m)	3.598 (m)
	6	**3.664 (dd, 11.7), 3.570 (dd, 11.7)**	**4.536 (dd, 12.0), 4.399 (dd, 12.0)**
Rhamnose	1	5.229 (d, 1.2)	5.220 (s)
	2	3.906 (dd, 3.0, 1.8)	3.986 (d, 3.0)
	3	3.606 (dd, 8.4, 3.2)	3.750 (dd, 9.6, 3.0)
	4	3.354 (dd, 8.4)	3.454 (dd, 9.0)
	5	3.607 (q, 3.6)	4.025 (q, 3.6)
	6	1.133 (d, 6.0)	1.288 (d, 5.4)

[a] δ_H: chemical shift in ppm; splitting patterns and the coupling constant J values (Hz) are in parentheses. The main differences between these two compounds are in bold.

Data collected from our LC–APCI–MS, MS^2 and MS^3 experiment provided much detailed structural information about acteoside and isoacteoside than the MS methods reported in the literature [6,22,23].

Acid hydrolysis of compounds 1 and 2 was performed to determine if the two isomers were only different in sugar content. However, all hydrolysis products were proven to be the same for the two compounds. Caffeic acid and hydroxytyrosol were confirmed by LC–MS, and two sugar moieties were confirmed to be glucose and rhamnose at a molecular ratio of 1:1 by a Dionex (Sunnyvale, CA) DX-500 ion chromatograph.

Further studies in ^{1}H-NMR and 2D-NMR experiments (long-range COSY, ROESY, and CH correlation) of these two compounds, showed that all NMR data of compound 1 matched with the reported NMR data for acteoside, and all NMR data for compound 2 matched with those of isoacteoside [23,27]. ^{1}H-NMR chemical shifts and the coupling constants of the two compounds are shown in Table 2. The major differences in ^{1}H-NMR between compounds 1 and 2 are the protons on C4 and C6 of the glucose moiety. Based on all available data, we therefore conclude that compounds 1 and 2 in *P. psyllium* are acteoside and isoacteoside, respectively.

4. Conclusions

Our study demonstrated that HSCCC could be a highly useful technique for isolating and purifying acteoside and isoacteoside from the seeds of *P. psyllium.* A simple two-phase solvent system containing ethyl acetate:water (1:1, v/v) provided fast and efficient separation, and good purity and recovery rates for the two major phenylethanoid glycosides in *P. psyllium* extract.

Acknowledgements

The authors would like to thank Yolanda Brummer of the Food Research Program, Agriculture & Agri-Food Canada, Guelph (FRP-AAFC) for her assistance in the analysis of sugars, and Valerie Robertson of the Nuclear Magnetic Resonance Centre, University of Guelph for the NMR experiments. This is scientific publication number S197 of the FRP-AAFC, Guelph.

References

[1] M. Stuart, The Encyclopedia of Herbs and Herbalism, Orbis Publishing, London, 1979, 242.
[2] T. Miyase, A. Koizumi, A. Ueno, T. Noro, M. Kuroyanagi, S. Fukushima, Y. Akiyama, T. Takemoto, Chem. Pharm. Bull. 30 (1982) 2732.
[3] H. Ravn, S. Nishibe, M. Sasahara, X. Li, Phytochemistry 29 (1990) 3627.
[4] H. Nishimura, H. Sasaki, N. Inagaki, M. Chin, H. Mitsuhashi, Phytochemistry 30 (1991) 965.
[5] G.-A. Gross, M.F. Lahloub, C. Anklin, H.-R. Schulten, O. Sticher, Phytochemistry 27 (1988) 1459.
[6] Y. Shoyama, M. Matsumoto, I. Nishioka, Phytochemistry 26 (1987) 983.
[7] S. Nishibe, Y. Tamayama, M. Sasahara, C. Andary, Phytochemistry 38 (1995) 741.
[8] Q. Xiong, K. Hase, Y. Tezuka, T. Tani, T. Namba, S. Kadota, Planta Med. 64 (1998) 120.
[9] E.E. Schapoval, M.R. Winter de Vargas, C.G. Chaves, R. Bridi, J.A. Zuanazzi, A.T. Henriques, J. Ethnopharmacol. 60 (1998) 53.
[10] Z. He, K. Lau, H. Xu, P. Li, P. Puihay But, J. Ethnopharmacol. 71 (2000) 483.
[11] Q. Xiong, S. Kadota, T. Tani, T. Namba, Biol. Pharm. Bull. 19 (1996) 1580.
[12] L. Lei, F. Yang, T. Zhang, P. Tu, L. Wu, Y. Ito, J. Chromatogr. A 912 (2001) 181.
[13] L.-J. Chen, D.E. Games, J. Jones, J. Chromatogr. A 988 (2003) 95.
[14] Q. Du, G. Jerz, R. Waibel, P. Winterhalter, J. Chromatogr. A 1008 (2003) 173.
[15] G. Tian, T. Zhang, F. Yang, Y. Ito, J. Chromatogr. A 886 (2000) 309.

[16] Q. Du, M. Xia, Y. Ito, J. Chromatogr. A 962 (2002) 239.
[17] H.-T. Lu, Y. Jiang, F. Chen, J. Chromatogr. A 1017 (2003) 117.
[18] X. Wang, Y. Wang, Y. Geng, F. Li, C. Zheng, J. Chromatogr. A 1036 (2004) 171.
[19] F.Q. Yang, T.-Y. Zhang, B.-X. Mo, L.-J. Yang, Y.-Q. Gao, Y. Ito, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 209.
[20] T.-Y. Zhang, X. Hua, R. Xiao, S. Kong, J. Liq. Chromatogr. 11 (1988) 233.
[21] T.-Y. Zhang, R. Xiao, Z.-Y. Xiao, L.K. Pannell, Y. Ito, J. Chromatogr. 445 (1988) 199.
[22] S. Nishibe, A. Kodama, Y. Noguchi, Y. Han, Nat. Med. 55 (2001) 258.
[23] R.W. Owen, R. Haubner, W. Mier, A. Giacosa, W.E. Hull, B. Spiegelhalder, H. Bartsch, Food Chem. Toxicol. 41 (2003) 703.
[24] A.P. Foucault, L. Chevolot, J. Chromatogr. A 808 (1998) 3.
[25] H. Oka, K. Harada, M. Suzuki, Y. Ito, J. Chromatogr. A 903 (2000) 93.
[26] H. Oka, K. Harada, Y. Ito, J. Chromatogr. A 812 (1998) 32.
[27] J.C. Chun, J.C. Kim, I.T. Hwang, S.E. Kim, Pest. Biochem. Physiol. 72 (2002) 153.