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# CHROMATOGRAPHIC SEPARATION OF TANNIN FRACTIONS FROM A BEARBERRY-LEAF (*ARCTOSTAPHYLOS UVA-URSI* L. SPRENGEL) EXTRACT BY SE-HPLC – A SHORT REPORT

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Phenolic compounds from the leaves of the bearberry plant (*Arctostaphylos uva-ursi* L. Sprengel) were extracted into 95% (v/v) ethanol. Tannin fractions were obtained by Sephadex LH-20 column chromatography using 50% (v/v) acetone as the mobile phase. The tannin fraction was then further characterised by size exclusion high-performance liquid chromatography (SE-HPLC) on a TSK G2000$SW_{XL}$ column with a mobile phase comprising 45% acetonitrile and 0.1% (v/v) TFA. The chromatography revealed the presence of additional phenolic compounds in the tannin fractions which are not proanthocyanidins (*i.e.* condensed tannins).

## INTRODUCTION

Bearberry (*Arctostaphylos uva-ursi* L. Sprengel) is a ubiquitous procumbent evergreen shrub located throughout North America, Asia, and Europe that serves little purpose other than as wildlife forage and an occasional ornamental species, despite its presence as an active ingredient in many commercial products. The name bearberry is derived from the edible fruit, which is said to be greatly enjoyed by bears. For humans, the berries are mealy and almost tasteless when raw, but quite palatable when cooked [Willard, 1992]. The plant grows preferentially on sandy and well-drained soil and is common in woodlands, rocky hills, and eroded slopes throughout the North American Prairies [Simonot, 2000]. Bearberry, which also goes under the names of uva-ursi, kinnikinnik, mealberry, and bear's grape, has official classification as a phytomedicine in parts of Europe. The commercial importance of bearberry is based on its astringent properties and beneficial effects in nephritis, kidney stones, and other diseases of the urinary tract. In chronic inflammation of the bladder and kidneys, bearberry has no equal [Willard, 1992]. The leaves are oval, leathery and evergreen. The main constituents of bearberry leaf are arbutin (5-15%), variable amounts of methylarbutin (up to 4%) and small quantities of the free aglycones. Other constituents include ursolic acid, tannic acid, gallic acid, *p*-coumaric acid, syringic acid, galloylarbutin, and up to 20% gallotannins, as well as some flavonoids, notably glycosides of quercetin, kaempferol and myricetin [Barl, 1996]. In contrast to other species of the family (Ericaceae), bearberry contains only small quantities of proanthocyanidins. This lack of ability to synthesize proanthocyanidins is associated with the plant's capacity to synthesize gallotannins [Hänsel *et al.*, 1992]. The bearberry plant is a tremendously underutilized renewable natural resource.

Due to the presence of phenolic constituents, the bearberry plant is a warehouse of various bioactives. Antioxidant and antimicrobial activities of the extracts of bearberry leaves have been reported by several authors. Using the PHOTOCHEM® device, an ethanolic extract from bearberry leaves exhibited high antioxidative activity at inhibiting the photo-induced chemiluminescence (PCL) of luminol; that is, 5.93 mmol ascorbic acid eq/g extract and 10.4 mmol Trolox eq/g extract for the water- (ACW) and lipid-soluble (ACL) compounds assays, respectively [Pegg *et al.*, 2007]. Amarowicz *et al*. [1999, 2004] demonstrated very strong reducing power, antioxidant properties in a β-carotene-linoleate model system, and antiradical properties investigated using the DPPH radical scavenging assay and an EPR spin-trapping technique. The crude bearberry-leaf extract, as well as its low-molecular-weight phenolics and tannin fractions, inhibited TBARS formation in cooked pork systems after seven days of refrigerated storage by 97, 49, and 100%, respectively, when added at a 200-ppm concentration [Pegg *et al.,* 2005]. The observed retardation in lipid oxidation/autoxidation by the bearberry-leaf extract in cooked pork patties demonstrates thermal stability of the bioactive constituents in the extract which impart antioxidant activity. In the study of Carpenter *et al*. [2007], addition of the bearberry-leaf extract decreased lipid oxidation (TBARS) in raw pork patties on days 9 and 12 of storage, relative to controls.

The inhibitory effects of bearberry-leaf extracts against *Arcobacter butzleri*, *A. cryaerophilus*, and *A. skirrowii* were reported by Cervenka *et al.* [2006]. It was established by Annuk *et al.* [1999] that aqueous extracts of bearberry and cow-

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berry leaves enhance cell aggregation of *Helicobacter pylori* strains tested by the salt aggregation test, and that the extract of bearberry possesses a remarkable bacteriostatic activity. In the investigation of Dykes *et al.* [2003], the bearberry-leaf extract displayed no antimicrobial activity on its own but enhanced the activity of nisin, as determined by minimum inhibitory concentrations against many Gram-positive, but none of the Gram-negative, bacteria.

The bearberry-leaf extract strongly protected against hydrogen peroxide- and *tert*-butylhydroperoxide-induced DNA damage in U937 cells [Carpenter *et al*., 2006]. Application of an extract from bearberry leaves has been reported in cosmetic applications for skin lightening [Marks, 1997].

Up to this point, analysis of bioactive constituents in the bearberry-leaf extract has been limited to thin-layer chromatography and high-performance thin-layer chromatography (HPTLC) [Amarowicz *et al.,* 1999; Slaveska-Raicki *et al.,* 2003]. Therefore it was decided to apply size-exclusion high-performance liquid chromatography (SE-HPLC) on a TSK G-2000 series column as a technique for further separation of tannins in the bearberry-leaf extract.

## MATERIALS AND METHODS

### Chemicals

All solvents used were either ACS or HPLC grade unless otherwise specified. Sephadex LH-20, silicic acid, tannic acid, gallic acid, (+)-catechin, and procyanidin $B_2$ were purchased from the Sigma Chemical Co. (St. Louis, MO). Ethanol, acetone, methanol, hexanes, chloroform, trifluoroacetic acid (TFA), hydrochloric acid, and Whatman No. 1 filter paper were acquired from the Fisher Scientific Co. (Suwanee, GA).

### Plant material

Branches and leaves from bearberry (*Arctostaphylos uva-ursi* L. Sprengel) were collected at various locations throughout Saskatchewan. The plant material was dried in a forced-air convection oven (Precision Instruments, Model DN-43) at 35°C for *ca.* 2 days. The amount of moisture removed was calculated and the sample was then stored in a herb room at 10°C with a relative humidity of less than 50% until used. The remaining moisture content in the bearberry samples after this "drying" process is *ca.* 5-7%.

### Preparation of the bearberry-leaf extract

Dried bearberry leaves were ground in a commercial coffee mill. Prepared material was transferred to dark-colored flasks, mixed with 95% (v/v) ethanol at a material-to-solvent ratio of 15:100 (m/v) and placed in a Gyrotory Water Bath Shaker (Model G76, New Brunswick Scientific Co., Inc., Edison, NJ) at 50°C for 30 min. Afterwards, the slurry was filtered through Whatman No. 1 filter paper and the residue was re-extracted twice more. Combined supernatants were evaporated to dryness under vacuum at <40°C using a Büchi Rotavapor R-210 with a V-700 vacuum pump and V-850 vacuum controller (Büchi Corporation, New Castle, DE). The crude preparation was then dechlorophyllized according to Pegg *et al*. [2005] on a silicic acid column using hexanes and 95% (v/v) ethanol as the mobile phases. The dried extract was stored at 4°C until further analysed.

### Isolation of a tannin fraction using Sephadex LH-20 column chromatography

Approximately 4 g of the crude dechlorophyllized bearberry-leaf extract were suspended in 5 mL of 95% (v/v) ethanol and then applied to a chromatographic column (45 × 180 mm) packed with Sephadex LH-20 that had been equilibrated with 95% (v/v) ethanol. The low-molecular-weight phenolics were exhaustively "washed" from the column with 1200 mL of ethanol. Then, tannin fractions (1-4) were eluted from the column using four portions of 150 mL of 50% (v/v) acetone. Acetone in these fractions was removed under vacuum at <40°C using the Rotavapor. Residual water was then removed from each fraction by lyophilization.

### Thin-layer chromatography (TLC)

The four tannin fractions (1-4) were examined by TLC using silica gel plates (200 $\mu$m layer thickness, Merck), chloroform: methanol:water (65:35:10, v/v/v) as the mobile phase [Amarowicz & Shahidi, 1996], followed by colour development of the separated compounds with a 0.5% (w/v) vanillin solution prepared in 4% (w/v) HCl [Bate-Smith, 1953].

### Separation of tannins using SE-HPLC

Phenolic compounds present in the acetonic fractions recovered from the Sephadex LH-20 column were analysed using a TSK G2000$SW_{XL}$ column (7.8 × 300 mm, 5 $\mu$m; Tosoh Bioscience LLC, Montgomeryville, PA) and an Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser, autosampler, UV/Vis diode array detector (DAD) with standard flow cell, and 2D ChemStation software (Agilent Technologies, Wilmington, DE). The mobile phase, consisting of 45% (v/v) acetonitrile with 0.1% (v/v) TFA, was delivered at a rate of 1.0 mL/min. Samples (20 $\mu$L injection) at concentration of 2 mg/mL were introduced onto the column using an autosampler. The DAD was set to a wavelength of 280 nm.

## RESULTS AND DISCUSSION

The SE-HPLC chromatograms of the four tannin fractions (1-4) recovered from the Sephadex LH-20 column are depicted in Figure 1. The peaks in each chromatogram were characterised by retention times of 6.10 min (peak from fraction 1); 11.56, 12.01, and 12.31 min (peaks from fraction 2); 6.14, 9.09, 11.56, and 11.80 min (peaks from fraction 3); as well as 6.14, 9.84, 11.26, and 11.52 min (peaks from fraction 4). It is worth noting that peaks "b" from fraction 3 and 4 were broad and short whereas other peaks were high and sharp.

When comparing retention times of the aforementioned peaks with those originating from tannic acid, procyanidin $B_2$, and gallic acid (Figure 2), it is evident that the tannins present in the bearberry-leaf extract comprise different molecular masses. For instance, peak 'a' from fraction 1, 3, and 4 as well as peak 'b' from fraction 3 and 4 originates from compounds characterized by molecular weights greater than that of tannic acid (*i.e.* >1200 Da). The compounds highlighted on the SE-HPLC chromatograms with 'c' and 'd' were rather low-mo-

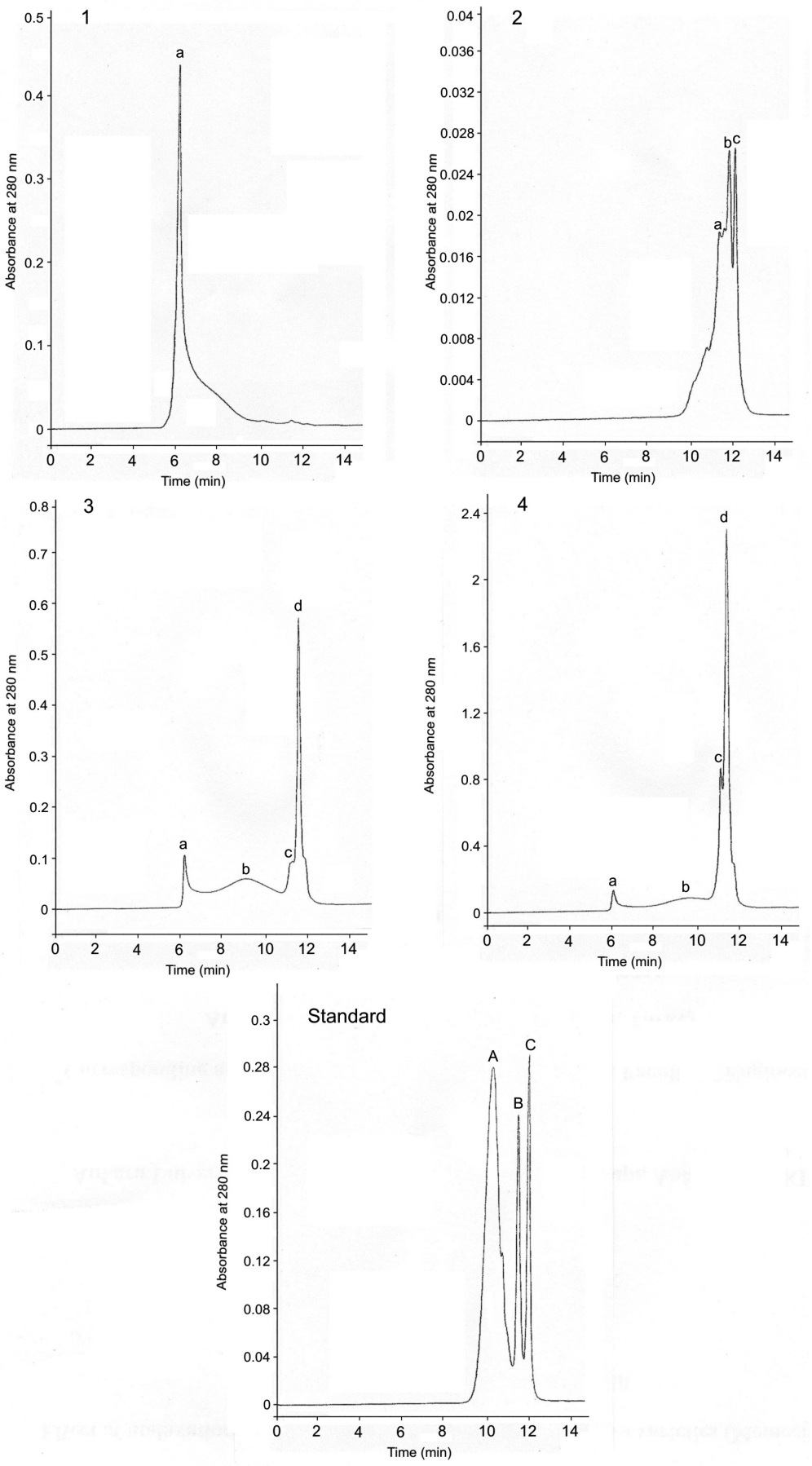
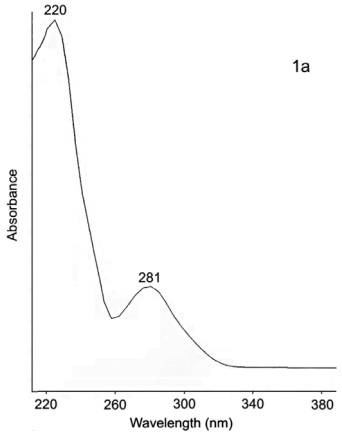
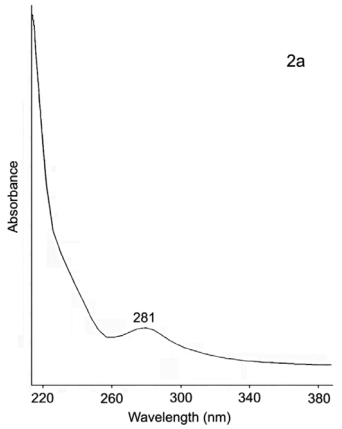
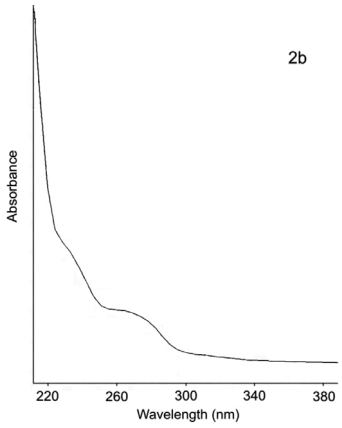



FIGURE 1. SE-HPLC chromatograms of the fractions 1-4 separated from the dechlorophyllized crude extract of bearberry leaf using Sephadex LH-20 column chromatography with comparison to the standards (A – tannic acid; B – procyanidin $B_2$; C – gallic acid).



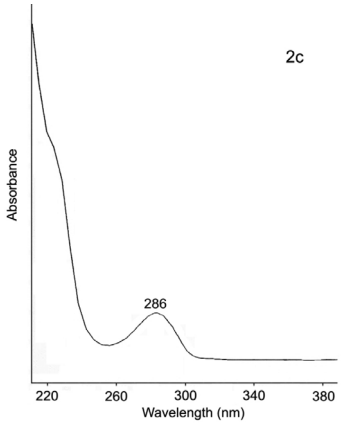
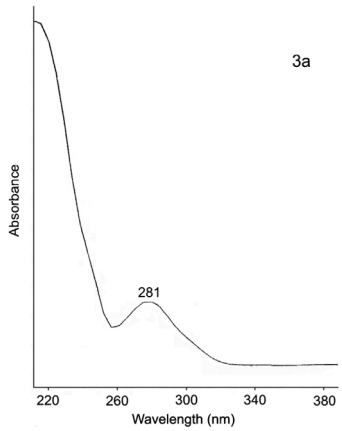
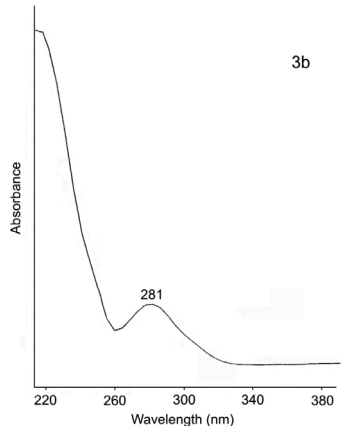
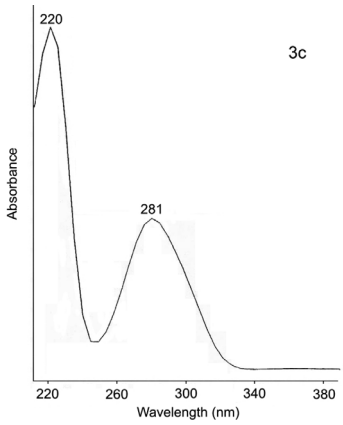




FIGURE 2. UV-DAD spectra of compound/compounds present on SE-HPLC chromatogram from fractions 1 and 2 a, b, c – marks related to the peaks from Figure 1.



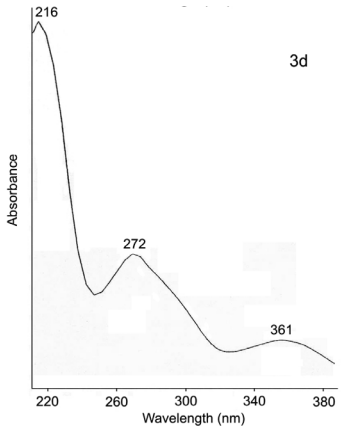




FIGURE 3. UV-DAD spectra of compound/compounds present on SE-HPLC chromatogram from fractions 3 a, b, c, d – marks related to the peaks from Figure 1.

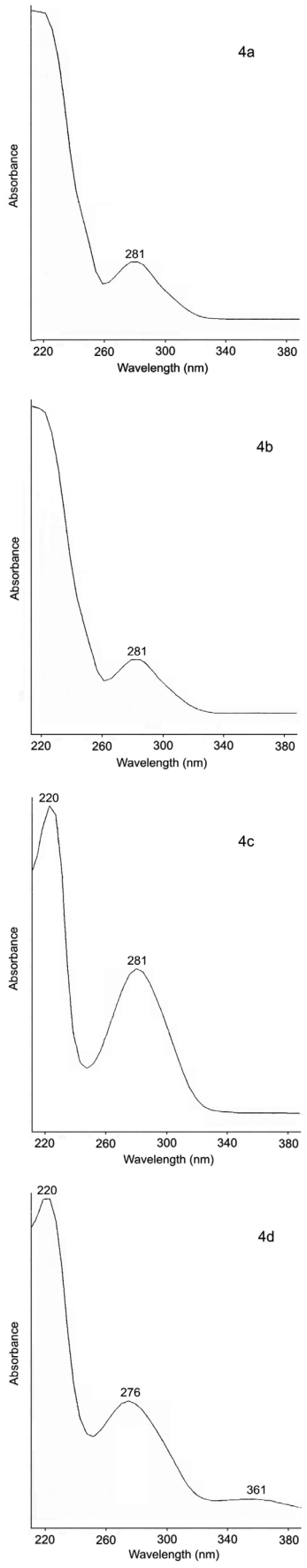



FIGURE 4. UV-DAD spectra of compound/compounds present on SE-HPLC chromatogram from fractions 4 a, b, c, d – marks related to the peaks from Figure 1.

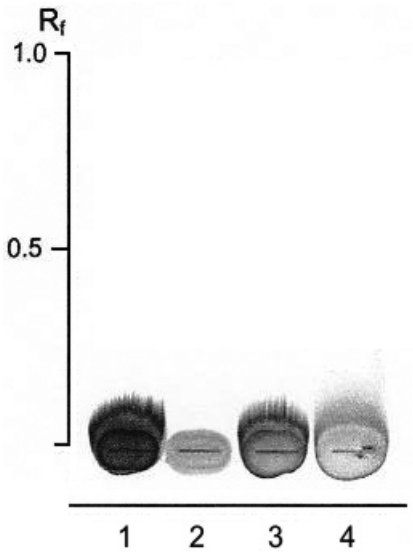



FIGURE 5. TLC chromatogram of tannin fractions 1-4 separated from the dechlorophyllized crude extract of bearberry leaf using Sephadex LH-20 column chromatography.

lecular-weight phenolic compounds. The presence of these compounds in the chromatogram is difficult to explain: according to Strumeyer & Malin [1975], all low-molecular--weight phenolic compounds should have been eluted with ethanol from the column packed with Sephadex LH-20 gel, but this was not the case.

A majority of UV-DAD spectra of compounds recorded as peaks on the SE-HPLC chromatograms were characterised by a maximum at 281 nm (Figures 2-4). Compound/ compounds recorded at the retention time of 12.01 min in fraction 2 exhibited only a shoulder at 260 nm. UV-DAD spectra with maxima at 276 and 361 nm, as well as 272 and 360 nm were noted for peaks separated from fractions 3 and 4. The wavelength of 286 nm was observed in the UV spectrum for the compound separated as peak 'c' with a retention time of 11.80 min from fraction 2.

UV spectra with a maximum at 281 nm are typical of proanthocyanidins (*i.e.*, condensed tannins) and have previously been reported for tannin fractions separated from plant material using Sephadex LH-20 column chromatography [Karamać *et al*., 2007]. UV-DAD spectra with a clear maximum have been reported for SE-HPLC fractions separated from buckwheat and buckwheat groats [Karamać, 2007]. Spectra with maxima at 272/276 and 361 nm might originate from complexes of hydrolysable tannins with flavonols or other flavanoids. Such tannin complexes in oaks of North America have been reported by Yarnes *et al.* [2006].

The high polarity of condensed tannins present in the bearberry-leaf extract was confirmed using thin-layer chromatography. Compounds separated from the dechlorophyllized extract using Sephadex LH-20 column chromatography and applied to TLC gave red spots on the line of application after treatment with the vanillin-HCl reagent. This confirms that they are condensed tannins and that they are more polar than catechins and catechin oligomers [Amarowicz & Shahidi, 1996].

## CONCLUSIONS

SE-HPLC with a TSK G2000SW$_{XL}$ column resulted in a good separation of condensed tannins present in the bearberry-leaf extract according to their molecular masses. The chromatography revealed the presence of additional phenolic compounds in the tannin fractions which are not proanthocyanidins (*i.e.* condensed tannins) This observation is in accordance with the results of a previous study [Pegg *et al*., 2005].

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