PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **12**, 336–339 (2001)
DOI: 10.1002/pca.602

A Single Extraction Step in the Quantitative Analysis of Arbutin in Bearberry (*Arctostaphylos uva-ursi*) Leaves by High-Performance Liquid Chromatography

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A fast and simple extraction procedure coupled with a simple HPLC method has been developed in order to determine the arbutin content of leaves of *Arctostaphylos uva-ursi* plants grown at four different geographical sites and collected during two different seasons. Using the optimised analytical system, the arbutin content of bearberry leaves was found to vary from 6.30 to 9.16% expressed on a dry weight basis. Autumn is shown to be a better period than spring for the collection of plant material in order to obtain the highest yield of arbutin. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: extraction methods; high performance liquid chromatography; arbutin; *Arctostaphylos uva-ursi*; Ericaceae.

INTRODUCTION

The crude drug of *Arctostaphylos uva-ursi* (L.) Sprengel (Ericaceae), also known as bearberry, contains three main groups of pharmaceutically relevant compounds, phenols, tannins and flavonoids, with arbutin (hydroquinone-β-D-monoglucopyranoside) being the main phenolic constituent. Besides being prized as a garden plant, the leaves of *A. uva-ursi* are used as a urinary antiseptic and an astringent (Karikas *et al.*, 1987). The pharmacological active compound is hydroquinone which originates from arbutin by *in vivo* glucoside cleavage. However, the mechanism of action is not clear at present because arbutin alone is reported to show little anti-microbial effectiveness (Jahodár *et al.*, 1985).

Several methods for the quantitative analysis of arbutin, including spectrophotometry (Jahodár *et al.*, 1986), capillary zone electrophoresis (Kenndler *et al.*, 1990), and Nguyen–Hiep's chromatospectrophotometry (Assaf *et al.*, 1987) have been previously described. Nonetheless, a reversed-phase HPLC method seems to be more suitable as it can be applied directly to a crude extract, even from plants other than bearberry (Assaf *et al.*, 1987; Lutterbach and Stockigt, 1992, 1993; Keller *et al.*, 1996), whereas the former methods require a purification step which may lead to the loss of some arbutin. Furthermore, existing HPLC methods have been developed to quantify arbutin in cell suspension cultures (Lutterbach and Stockigt, 1992, 1993) and in urine samples (Jahodár *et al.*, 1985).

In the present work, different extraction parameters and chromatographic conditions have been combined in order to establish a less complex, faster and cheaper method for the extraction and determination of arbutin in bearberry leaves than those previously described.

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Contract/grant sponsor: LIFE programme of the European Commission; Contract/grant number: ENV/E/000260.

EXPERIMENTAL

Plant material. Leaves of *Arctostaphylos uva-ursi* were collected from four natural populations growing at different altitudes in the Catalan Pyrenees (Spain), namely, Adraén/A (1750 m), Adraén/B (1600 m), Cloterons (2000 m), and Guils de Cerdanya (1750 m). Voucher specimens are deposited in the Herbarium Ilerdense (HBIL; Institut d'Estudis Ilerdencs, Lleida, Spain). The ontogenic cycle of the bearberry is characterised by two growth periods interrupted by a summer dormancy. The plant begins to sprout from the end of April until the beginning of May, and the second growth period takes place from August to September (Jahodár *et al.*, 1986). The plant material used in this study was collected in autumn 1997 and spring 1998. The leaves of each plant were dried, powdered and passed through a sieve in order to produce an homogeneous powder for the analyses.

Extraction parameters. Although a number of HPLC methods for the determination of arbutin in bearberry leaves have been previously reported, additional experiments were carried out in order to establish the most efficient extractive protocol, and to determine the effect of extraction solvent on the peak resolution of arbutin by HPLC. All the trials were carried out with homogeneous plant material collected from the same site and during the same period.

In order to establish the complete extraction of arbutin, two simple methods were used. *Method A*: dried

Received 22 June 2000
Accepted 18 December 2000

powdered plant material (50 mg) was sonicated with 5 mL of solvent at 25°C for 10 min, and the mixture centrifuged at 7600 ***g*** for 10 min. The supernatant was analysed by HPLC (see below) in order to determine the arbutin level, the marc extracted four more times under the same conditions using new solvent, and the arbutin content determined after every extraction process. *Method B*: dried powdered plant material (50 mg) was sonicated with 25 mL of solvent at 25 °C for 30 min, and the mixture centrifuged at 7600 ***g*** for 10 min. The supernatant was analysed by HPLC (see below) and extraction of the marc was repeated with 25 mL of new solvent in order to determine if all of the arbutin had been extracted.

In order to study the effect of the eluent on the resolution of the arbutin peak, four solutions of standard arbutin were prepared in water, methanol, and water: methanol (50:50 and 95:5). On the basis of initial results, the effects of altering some further extraction parameters were studied in order to optimise the complete extraction procedure. Two different temperatures (25 and 45°C), solvents [water, and water:methanol (95:5)] and weights of sample (50 and 100 mg) were considered. Triplicate assays were performed for all experiments, and a two-way analysis of variance (ANOVA; Statistics for Windows 4.0 Microsoft, Redmon, WA, USA) was used to assess the observed differences in the arbutin concentrations. Differences were considered to be statistically significant when $p < 0.05$.

Optimised extraction procedure. According to the results obtained in the initial experiments, method B, employing water:methanol [95:5; both solvents of HPLC grade (Scharlab, Barcelona, Spain)], was selected for the extraction of arbutin as it was shorter and less complex, thus avoiding possible errors in sample manipulation, and the same solvent could be used for both extraction and as the HPLC mobile phase. In the final optimised method, dried powdered plant material (50 mg) was extracted with 25 mL of water:methanol (95:5) at 25 °C in an ultrasonic bath for 30 min. After centrifugation at 7600 ***g*** for 10 min, the supernatant was adjusted to 25 mL in a measuring flask. Each sample was extracted in triplicate, and for each replicate two vials were filled and aliquots from each injected twice. Thus the value obtained for each sample is the mean of 12 HPLC analyses. Samples were quantified immediately after extraction in order to avoid possible chemical alterations. Blanks and standards containing known concentrations of arbutin were placed between the samples to monitor the quantification.

Analytical HPLC method. Reversed phase-HPLC analyses were carried out on a Hewlett-Packard (Palo Alto, CA, USA) series HP 1050 chromatograph equipped with a quaternary pump, diode array detector, autoinjector and a model HP 3396 Chemstation for control and data processing. Quantitative analysis was carried out using a Nucleosil (Teknokroma, Spain) C_8 column (20 × 3.9 mm i.d.; 5 μm) and a pre-column (250 × 4 mm i.d.) similarly packed. The mobile phase was water:methanol (95:5), previously filtered through 0.45 μm filter (Millipore, Massachusetts, USA), at a flow-rate of 1 mL/min; the injection volume was 10 μL and UV detection was at 280 nm.

The concentration of arbutin was determined using a calibration curve established with six concentrations of standard arbutin (Sigma-Aldrich, Saint Louis, MO, USA) from 25 to 400 μg/mL. The calibration curve parameters were: area = 2.15702 × μg injected − 1.05998 [$n = 5$; $r = 0.99997$; residual standard deviation = 3.00558 (0.03%)]

RESULTS AND DISCUSSION

Extraction of arbutin from plant material

The results of the preliminary assays showed that the efficiency of extraction of method B was better than that of method A. In method A, the majority of arbutin was obtained in the first extraction, whilst that extracted in the first three steps was 99.01% of the total (as obtained in all five steps). The arbutin peak was almost imperceptible in the fourth extraction and was not detectable in the last. In method B, all of the arbutin was extracted in the first step since no arbutin peak could be detected in the chromatogram of the second extraction.

Significant differences in extraction efficiencies were obtained by using different temperatures (Table 1). Higher levels of arbutin could be determined following extraction at 25°C compared with 45°C, and the reproducibility of extraction was also better when the plant material was extracted at 25°C. Although no statistically significant differences in the determined concentration of arbutin could be detected using a 50 mg sample compared with a sample of 100 mg (Table 1), trials made with 50 mg sample showed a better reproducibility of the analyses. Finally, the two extraction solvents employed showed no statistically significant differences one from the other.

HPLC analysis of arbutin

In the HPLC analysis, arbutin eluted as a single symmetrical peak (retention time 3.7 min) when the mobile phase was also the injection solvent. The use of extraction

Table 1. Levels of arbutin determined using different parameters in the establishment of the extraction process.

		Arbutin (μg/mL)[a] Weight of sample extracted	
Extraction temperature (°C)	Extraction solvent	50 mg	100 mg
25	Water	253.81 ± 2.03	502.39 ± 16.19
25	Water:methanol (95:5)	258.27 ± 1.33	506.06 ± 5.19
45	Water	230.17 ± 18.90	450.44 ± 58.42
45	Water:methanol (95:5)	203.66 ± 27.10	474.11 ± 41.62

[a] Mean values ± SD ($n = 6$).

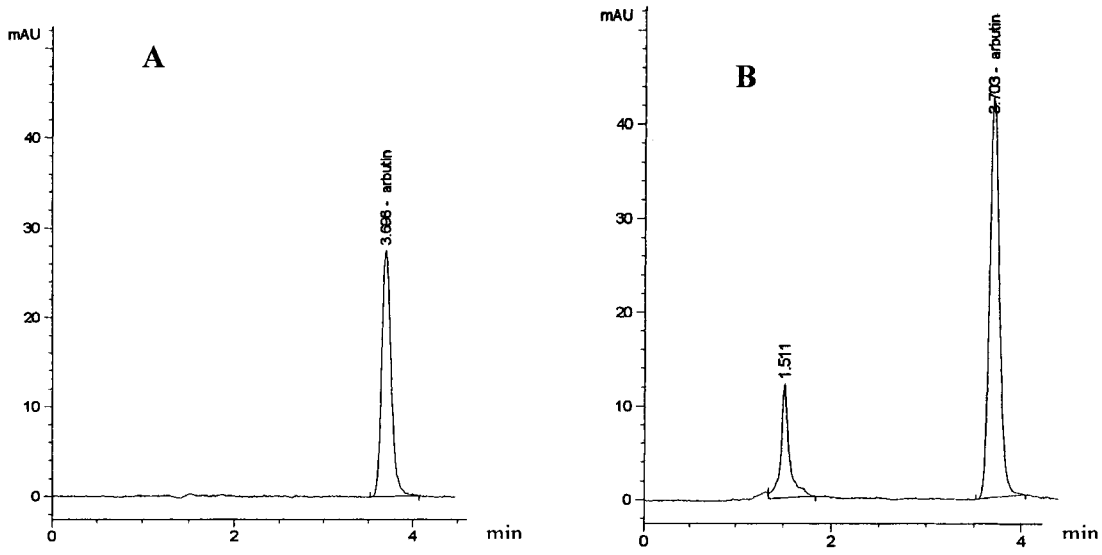

Figure 1. HPLC chromatograms of (A) a standard solution of arbutin (100 μg/mL), and (B) a sample of bearberry leaves extracted according to the proposed method. (For extraction and chromatographic protocols see Experimental section.)

solvents different from those used in the mobile phase resulted in severe peak distortions because of the different viscosities (Castells *et al.*, 1997). In order to check if other compounds eluted with arbutin, preliminary analyses performed at different wavelengths ranging from 250 to 340 nm and for 1 h run time showed that traces of other compounds appeared in the chromatogram only at 250 nm. HPLC chromatograms of standard arbutin and of a bearberry sample are shown in Fig. 1.

Accumulation of arbutin in leaves of bearberry

Figure 2 shows the levels of arbutin in leaves of *A. uva-ursi* collected from different natural populations of bearberry during spring and autumn. The levels were different in the four wild populations, and were also different according to the period of collection. Thus, bearberry leaves collected from Adraén/A showed the highest autumn content of arbutin (91.57 μg/mg dry weight), whilst leaves from Cloterons had the highest spring level (80.48 μg/mg dry weight). For the same collection periods, the lowest arbutin levels were found in leaves from Guils de Cerdanya (69.95 μg/mg dry weight) and Adraén/B (62.98 μg/mg dry weight), respectively. However, in all samples the levels of arbutin in leaves collected in autumn were higher than those in leaves collected in spring, being on average 82.18 and 70.18 μg arbutin/mg dry weight, respectively. Significant differences were found between arbutin levels during the two periods of collection at all of the sites exception for Guils de Cerdanya, the differences being much greater at the sites Adraén/A and Adraén/B than at Cloterons. Overall, the content of arbutin varied between 6.30 and 9.16% dry weight, values which are quite similar to those found by other workers which ranged from 5.95% (Matsuda *et al.*, 1996) to 7.60% (Kenndler *et al.*, 1990). On the basis of the obtained results, the collection of bearberry leaves should be carried out preferably during the autumn period because of their higher arbutin content, but the yield of arbutin can be different according to the characteristics of the sites where the plants grow.

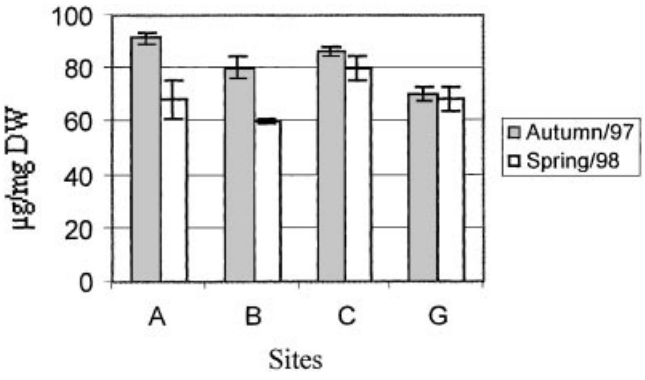

Figure 2. The arbutin content of leaves of bearberry collected from four different sites (A = Adraén/A; B = Adraén/B; C = Cloterons; G = Guils de Cerdanya) and during two different seasons (hatched bars = autumn; open bars = spring). The results shown are mean values ($n = 12$) and the bars represent the standard deviations.

In summary a simple, fast and accurate method has been established for the determination of arbutin in leaves of *Arctostaphylos uva-ursi*. The HPLC protocol described involves isocratic elution which is very favourable since results are easily reproduced and the necessity of re-equilibrating the column between injections is eliminated. The method offers a good reproducibility (±2.8% based on the peak area of arbutin during the period of analysis) and precision (relative standard deviation deviation = 0.34%), and the solvent system is environmentally acceptable. In comparison with reported methods, the described protocol is shorter than that of Matsuda *et al.* (1996), previously developed for *A. uva-ursi*, where a 4 h reflux is required, and that of Assaf *et al.* (1987), developed for *Origanum majorana*, where the retention time of arbutin is 25 min. The described method is also easier and cheaper than that used for *Solanum tuberosum* (Keller *et al.*, 1996) where a two-step linear gradient is required.

Acknowledgements

This work was financed by the LIFE programme of the European Commission (project ENV/E/000260).

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