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Short communication

# Quantitative determination of naphthaquinones of *Arnebia densiflora* (Nordm.) Ledeb. by an improved high-performance liquid chromatographic method

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**Abstract**

An improved HPLC procedure for quantitative determination of the alkannin derivative naphthaquinones, β,β-dimethylacrylalkannin (I), teracrylalkannin (II) and isovalerylalkannin (III)+α-methyl-*n*-butylalkannin (IV), contained in *Arnebia densiflora* roots and extracts was introduced. This procedure, as the mainstay of the study, enabled separation of the compounds within only 12 min on a reversed-phase column. © 1997 Elsevier Science B.V.

*Keywords:* *Arnebia densiflora*; Naphthaquinones; Alkannin derivatives

## 1. Introduction

*Arnebia densiflora*, widespread over Anatolia, belongs to the Boraginaceae family. The roots of the Boraginaceae family are known to be used not only for treatment of burns and skin diseases, but also as red-dye in the drug, cosmetic and textile industries, in the Far East and Europe [1].

Two natural isomeric compounds contained in the roots of some species of this family of plants, alkannin (L-form) and shikonin (*R*-form), possessing an isohexenylnaphthazarin structure [2], are known as natural colorants and are represented in the Colour Index as Natural Red 20 and Shikonin or Tokyo Violet (CI. 75520 and 75530), respectively.

Today, shikonin and its derivatives are commercially produced by cell culture method and are specifically used as natural colorants in the pharmaceutical industry [3,4].

This family of plants does not solely contain alkannin and/or shikonin in their roots, but includes their natural derivatives which are also known to have coloring properties and some biological activity [1–4]. *Arnebia densiflora*, for instance, contains alkannin derivatives (Fig. 1), namely β,β-dimethylacrylalkannin (I), teracrylalkannin (II) and isovalerylalkannin (III)+α-methyl-*n*-butylalkannin (IV).

Although there seems to be many studies on the isolation of isohexenylnaphthaquinones, their structure identification and biological activities, works describing their relevant analytical aspects in a quantitative manner are extremely limited [5–10].

Ikeda et al. [6] studied the separation, using a HPLC system equipped with a chiral column, of alkannin and its optical isomer, shikonin, of a

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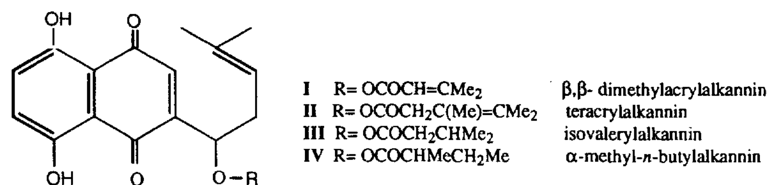


Fig. 1. Alkannin derivatives isolated from *Arnebia densiflora* roots.

Boraginaceae species different to the one we have used.

Nickel and Carrol [5], following their own work on the separability of seven different isohexenylnaphthaquinone derivatives (alkannin, teracrylalkannin, β,β-dimethylacrylalkannin, α-methy-*n*-butylalkannin+isovalerylalkannin, acetyl-alkannin, β-hydroxyisovalerylalkannin, deoxyalkannin) by normal-phase, reversed-phase and ion-pair HPLC, concluded that these naphthaquinones could only be separated by the ion-pair method, and they could not even be eluted from the column by the normal-phase and the reversed-phase (various compositions of methanol–water and acetonitrile–water) applications. Separation of their seven isohexenylnaphthaquinone derivatives, using either tetramethylammonium chloride or nonylamine as the ion-pair reagent, could be achieved over 60 min, at 70°C in a MicroPak MCH-10 (Varian, 300×4 mm I.D.) column, with a 2 ml/min flow-rate.

Although Fujita et al. [7] claimed that they have separated seven of the shikonin derivatives (shikonin, β-hydroxyisovalerylshikonin, acetylshikonin, deoxyshikonin, β,β-dimethylacrylshikonin, α-methy-*n*-butylshikonin+isovaleryl-shikonin) at 40°C using HPLC with an HLC 802-UR (Toyo-Soda) column and with a mobile phase consisting of acetonitrile–water–triethylamine–acetic acid (70:30:0.3:0.3), with a 1.1 ml/min flow-rate, Nickel and Carrol [5] experimentally proved that elution of these naphthaquinones has never been accomplished by commercial columns.

Therefore, it is still important to develop a simple, rapid and sensitive quantitative method for determination of alkannin and shikonin derivatives in roots, extracts and preparations.

Using the HPLC method which was modified in this study, the alkannin derivative naphthaquinones (Fig. 1), namely, β,β-dimethylacrylalkannin (I), teracrylalkannin (II) and isovaleryl alkannin (III)+α-methyl-*n*-butylalkannin (IV), isolated from *Arnebia densiflora* roots [11], could be separated within 12 min with a high resolution and precision. It is known from the literature, not only cited in this paper but also in many others, that, the components III and IV, can usually be isolated as a mixture and are interpreted as a single peak, as emerged from HPLC results. In fact, for the general purposes of our study, we had no reason to try to separate III and IV.

## 2. Experimental

β,β-Dimethylacrylalkannin (I), teracrylalkannin (II) and isovaleryl alkannin (III)+α-methyl-*n*-butylalkannin (IV) were isolated from *Arnebia densiflora* roots using chromatographic methods and their structures were identified by spectroscopic methods [11]. Isolated compounds were used as reference standard substances. All the analytical grade reagents, used for HPLC measurements, were of Merck quality.

### *2.1. Extraction*

The alkannin derivatives were extracted from air-dried roots using *n*-hexane as solvent in a Soxhlet apparatus operated for 2 h. Yield of extraction was found to be 5.7±0.1%, on a dry-weight basis.

### *2.2. Standard stock solutions*

Standard stock solutions were prepared by dissolving 50 mg samples of teracrylalkannin and isovaleryl alkannin+α-methyl-*n*-butylalkannin, and 25 mg β,β-dimethylacrylalkannin, each, in 50 ml ethyl acetate.

### *2.3. Sample solutions*

The sample solutions containing alkannin deriva-

tives were prepared by dissolving 20 to 100 mg of the extracted samples in ethyl acetate, in a 100-ml volumetric flask.

### 2.4. HPLC assay

The LC system used was a Varian Model 2010, equipped with a Rheodyne Model 7125 syringe-loading valve fitted with a 10-μl sample loop. HPLC experiments were conducted using a Ultracarb ODS C-20 (5 μm particle size, 25 cm×4.6 mm I.D., Phenomenex, USA) column with a flow-rate of 1 ml/min at ambient temperature. The mobile phase was methanol–water–formic acid (95:5:0.1). A Shimadzu SPD6 AV UV-Vis detector, set at 520 nm, was used. A detector range setting of 1.25 mA was used together with a Shimadzu C-R4A Model Chromatopac Integrator.

### 2.5. Standard curve

Stock solutions, containing 0.5, 0.75, 1, 1.5 and 2 ml of alkannin derivatives, were diluted with ethylacetate in a 10-ml volumetric flask. A 10-μl volume of each solution was injected into the column. Peak areas of the chromatograms were plotted against concentrations (mg/ml) of alkannins injected. Results were expressed as the average of three injections.

## 3. Results and discussion

Naphthaquinones in *Arnebia densiflora* roots were quantitatively determined by HPLC. These alkannin derivatives were well separated with a Ultracarb ODS-20 column and eluted within 12 min.

A liquid chromatogram of an *n*-hexane extract is shown in Fig. 2. The peaks with retention times of about 5.7 min, 6.5 min and 9.0 min correspond to β,β-dimethylacrylalkannin (I), teracrylalkannin (II) and isovaleryl alkannin (III)+α-methyl-*n*-butylalkannin (IV), respectively. The peak areas of each compound were found to range linearly against measured concentrations. Range of the calibration curves, was set according to the concentration levels

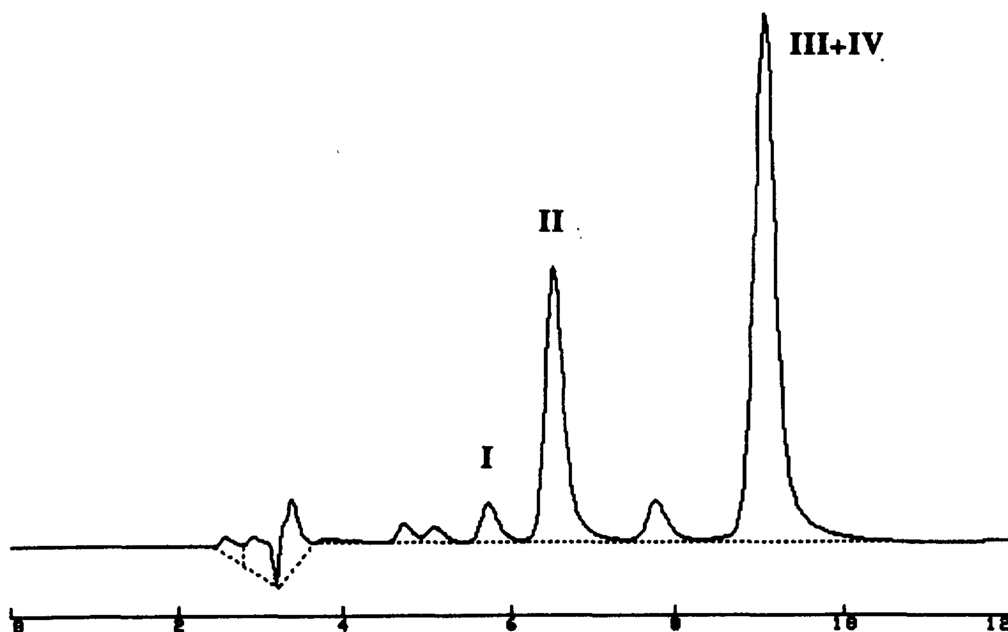


Fig. 2. HPLC chromatogram of naphthaquinones in *n*-hexane extract of *Arnebia densiflora* roots. Conditions: column: 5 μm Ultracarb ODS C-20 (25 cm×4.6 mm I.D.); mobile phase: methanol–water–formic acid (95:5:0.1); flow-rate: 1 ml/min; detector: Shimadzu SPD6AV, 520 nm. Peaks: I=β,β-dimethylacrylalkannin; II=teracrylalkannin; III+IV=isovaleryl alkannin+α-methyl-*n*-butylalkannin.

of the naphthaquinones contained in extracts, under the conditions of this work.

Regression equations, corresponding to the maximum absorption wavelength, 520 nm, of the three naphthaquinones, were formulated as I $y=3\,433\,761x-21.95$ ($r=0.999$); II $y=2\,726\,635x+5575$ ($r=0.999$); III+IV $y=4\,274\,891x+1026$ ($r=0.999$), where $y$ is the integration unit and $x$ is the weight, in mg per ml, of naphthaquinones.

To check the precision of the recovery experiments, conducted for determining the teracrylalkannin concentrations in roots, the experiments, in certain cases, were accompanied by addition of 50 ng and 80 ng of standard teracrylalkannin to the root extraction media at the beginning of the extraction period. The average recovery ratio was found to be 95.9% (C.V. 6%, $n=10$), while limit of detection (LOD) of the method was 3.5 ng/ml.

Relative contents (in percent), along with the corresponding standard error (S.E.) values of the naphthaquinones I, II, III+IV in the extracts were found to be 2.18 (±0.07), 21.5 (±0.3) and 30.5 (±0.3), respectively, for $n=10$.

## 4. Conclusions

The four alkannin derivative naphthaquinones, found in relatively high amounts in the roots of Anatolian *Arnebia densiflora*, were determined quantitatively, using HPLC.

Although the naphthaquinones of Boraginaceous plants have previously been studied by HPLC [5–7], the modified method described here has not only provided good separation and precision, but has also been found to be less time-consuming due to the suitability of the polarities of the eluents, column selectivity and the other conditions under which the experiments were conducted, and may therefore be used in quantitative evaluation of alkannin and shikonin derivatives contained in either plants, extracts or preparations.

The naphthaquinones (two individual and two in mixture), mentioned in this paper, can be successfully eluted and separated, within just 12 min, by reversed-phase chromatography with an ODS C-20 column and a methanol–water–formic acid (95:5:0.1) mobile phase, in spite of the Nickel and Carrols aforementioned statement.

Accuracy of the data of this work has been confirmed by comparing the HPLC results with those obtained by (non-published) thin-layer chromatography (TLC) experiments.

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