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HPLC determination of peroxisomicine A_1 in *Karwinskia* fruits: comparison of off-line solid phase extraction and columns switching heart-cut sample preparation

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Peroxisomicine A_1 (T-514) (3,3′-dimethyl-3,3′,8,8′,9,9′-hexahydroxy-3,3′,4,4′-tetrahydro-(7,10′)-bianthracen-1,1′-(2*H*,2*H*′)-dione) is a toxic compound occurring in plants of genus *Karwinskia* (family Rhamnaceae) [1]. It exhibits selectively *in vitro* toxicity on tumour cells, suggesting that this compound could have a potential antineoplastic effect. It is currently under preclinical screening [2].

We have already reported an HPLC assay for the determination of T-514 in different parts of genus *Karwinskia* plants (leaves, stems, roots) [3]. An extraction with ethyl acetate and a clean-up by SPE have been used for the sample pretreatment.

The aim of the presented work was to develop an effective preseparation technique for the determination of peroxisomicine A_1 in *Karwinskia* fruits. Samples were extracted with ethyl acetate [3]. The pooled and evaporated extracts were dissolved in acetonitrile/water 40 : 60. A C_{18} analytical column with the mobile phase consisting of methanol and McIlvaine buffer (pH 3) 78 : 22 was used. The retention time of T-514 was 7.7 ± 0.2 min. A chromatogram of a fruit extract is illustrated in Fig. 1a. The sample was also treated by off-line SPE [3] and the chromatogram obtained was very similar. It is obvious from Fig. 1a that peaks eluting before and after T-514 did not disappear. These components could be other dimeric anthracenones having similar characteristics as T-514 (according to their UV spectra).

Column-switching in the heart-cut mode is one of the possible methods for removing matrix constituents. The mobile phase II was methanol/McIlvaine buffer (pH 3) 78 : 22. Because it was important to wash all compounds having similar properties as T-514 from the precolumn, a mobile phase I containing the same components as the main mobile phase (methanol and McIlvaine buffer) was chosen. A methanol content of 50–55% was tested using a precolumn directly connected to the detector. A content of 53% methanol was chosen to achieve the satisfactory resolution of T-514 zone from additional matrix components. A time of 10 min was sufficient for removing weakly retained compounds from the precolumn. After that, the valve was switched to the injection position to transfer the analyte from the precolumn onto the analytical column. A time of 0.8 min was adequate for a complete transfer of the T-514 zone. Subsequently, the valve was switched back and the precolumn was washed for 3 min with the mobile phase I to remove strongly retained compounds and to prepare it for the next analysis. No memory effect was observed using the same precolumn several times. The chromatogram of the fruit extract after the column-switching is demonstrated in Fig. 1b. Peroxisomicine A_1 was eluted in 8.9 ± 0.1 min. Column-switching in the heart-cut mode allowed to remove all undesirable sample components and increased the analytical column lifetime. Recovery was determined comparing T-514 peak areas using only the analytical column and the analytical column combined with the precolumn. A recovery of $101.1 \pm 1.4\%$ was obtained. The analysis time including column-switching treatment was 20 min. The sample throughout was approximately 4 samples/h.

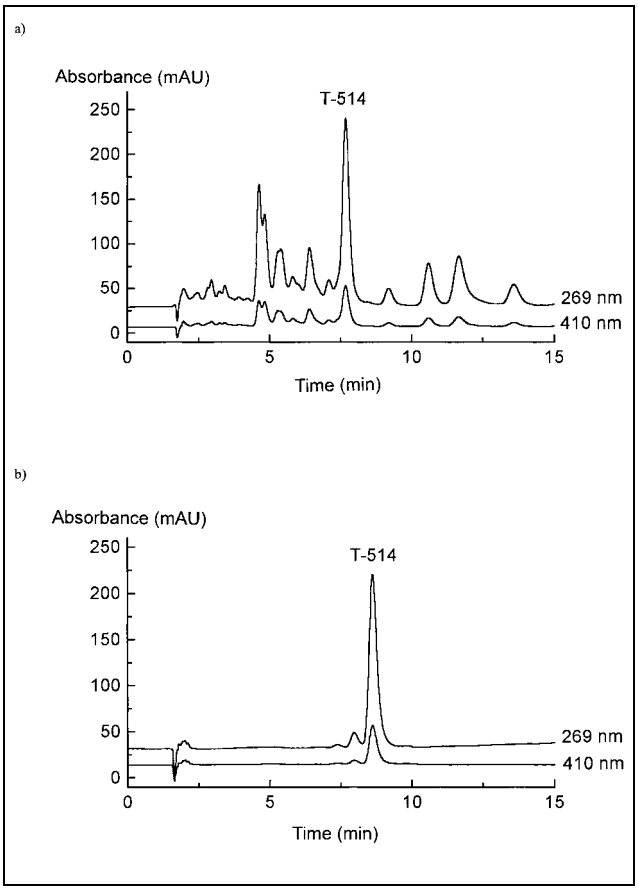


Fig. 1: Chromatogram of a) *Karwinskia* fruit extract, b) *Karwinskia* fruit extract after column-switching treatment. HPLC conditions: chromatographic column NovaPak C18 (150 × 3.9 mm; 4 μm), precolumn NovaPak C18 (20 × 3.9 mm), mobile phase I: methanol-McIlvaine buffer (pH 3) 53 : 47 (v/v), flow rate 1.4 ml/min, mobile phase II: methanol-McIlvaine buffer (pH 3) 78 : 22 (v/v), flow rate 0.6 ml/min, DAD detection at 269 and 410 nm, injection volume of 20 μl, column temperature 23 °C

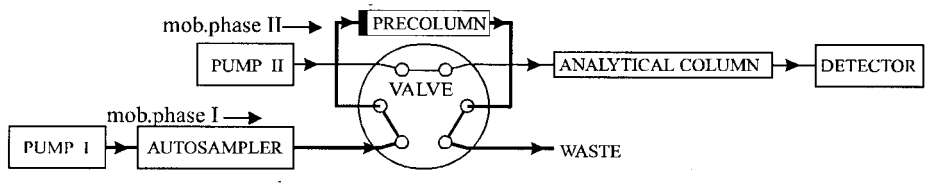
Repeatabilities of whole sample treatments were measured by 2-fold injection of three parallel liquid extractions of fruit samples and determined as relative standard deviations-RSD (8.5%).

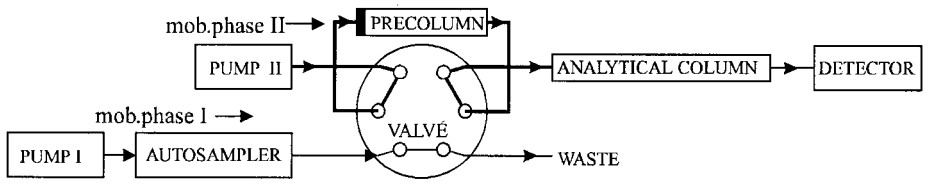
A linear dependence of the T-514 peak area on the T-514 concentration was obtained in the range of 0.126–12.6 μg/ml ($r = 0.9991$) with the linear regression equation: $y = bx$, $b = 61.8 \pm 0.6$, y-T-514 peak area [mAU.s]; x-concentration of T-514 [μg/ml]. Limits of T-514 quantification (ratio signal/noise 10) in plant samples were 120 ng/ml at 410 nm and 28 ng/ml at 269 nm, respectively.

Experimental

1. Chemicals

Standard of T-514 and fruit samples of *Karwinskia* were obtained from Departamento de Farmacología y Toxicología Facultad de Medicina U.A.N.L., Montery, N.L., México, methanol and acetonitril (gradient grade) from





Time	Autosampler	Valve	Action
0	inject	load	injection of the 1st sample, cutting of the impurities
10		inject	transfer of the analyte
10.8		load	recondition of the precolumn
14	inject	load	injection of the 2nd sample

Fig. 2: Scheme and timetable of column-switching heart-cut system

Merck (Germany), ethyl acetate, citric acid and $Na_2HPO_4 \cdot 12\,H_2O$ (all p.a. grade) from Lachema (Czech Republic).

2. Apparatus

The autosampler Basic-Plus Marathon Spark (Holland), the pump Waters 501 (USA) and the HP 1100 system Hewlett Packard (USA), the chromatographic column NovaPak C18 (150 × 3.9 mm; 4 µm) Waters (USA), the precolumn NovaPak C18 (20 × 3.9 mm) Waters (USA), the switching valve Rheodyne 7125 (USA), the rotary vacuum evaporator RVC-64 (Czech Republic), the shaker T-22 Lověna (Czech Republic), the Dorcus SPE vacuum manifold Tessek (Czech Republic), the filter paper Filtrak No. 368 (Germany) and the Bakerbond C18 (100 mg) SPE cartridges Mallinckrodt Baker (Germany).

3. Preparation of solutions

A stock solution of T-514 (ca. 100 µg/ml) was prepared in methanol and stored in a refrigerator at −20 °C. Working solutions were prepared diluting the stock solution with CH_3OH. McIlvaine buffer (pH 3) was prepared mixing 795 ml of 0.1 M citric acid and 205 ml of 0.2 M Na_2HPO_4.

4. Chromatographic conditions

HPLC conditions were: DAD detection at 269 and 410 nm, the injection volume of 20 µl, column temperature 23 °C. The mobile phase I was CH_3OH/McIlvaine buffer (pH 3) 53 : 47 (v/v), flow rate 1.4 ml/min, the mobile phase II: CH_3OH/McIlvaine buffer (pH 3) 78 : 22 (v/v), flow rate 0.6 ml/min.

5. Sample preparation

5.1. Liquid-liquid extraction

Peroxisomicine A1 was isolated from homogenized fruits of *Karwinskia* (0.1 g) by four-fold extraction at ambient temperature, each time with 5 ml of ethyl acetate for 30 min. The extracts were filtered through a filter paper, pooled and evaporated. Dry residues were dissolved in 20 ml of CH_3CN/H_2O 40 : 60.

5.2. Solid-phase extraction

An SPE cartridge (C18) was conditioned with 3 ml of CH_3OH and 3 ml of deionized H_2O. The extract (1 ml) was loaded. After washing with 2 ml of CH_3CN and H_2O (20 : 80), peroxisomicine A_1 was eluted with 4 ml of CH_3OH and McIlvaine buffer pH 3 (90 : 10). The eluat was evaporated to dryness and dissolved in 1 ml of the mobile phase.

5.3. Column-switching

The column-switching HPLC procedure is illustrated in Fig. 2.

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