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Short communication

High-performance liquid chromatographic method for the determination of juglone in fresh walnut leaves

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Abstract

An efficient high-performance liquid chromatographic gradient method for the determination of juglone in fresh walnut leaves was developed and validated. The leaves were collected and extracted by maceration in chloroform for 1 h at room temperature. The chloroform solution was evaporated under reduced pressure to give a dry residue recovered by methanol. The methanol solution was chromatographed on an LiChrospher-RP 18 end-capped 125×4 mm column, with a gradient of mobile phase: water–phosphoric acid–acetonitrile; juglone was detected by visible absorbance at 420 nm. © 1998 Elsevier Science B.V.

Keywords: Plant material; Juglone; Naphthoquinones

1. Introduction

Juglone is a naphthoquinone compound present in fresh leaves and hulls of the walnut tree (*Juglans regia* L.) [1,2]. Little work has been done on the determination of juglone content in walnut leaves. Wojcik et al. indicated a spectrophotometric method [3], and Lemberkovics et al. described a spectrophotometric method combined with preparative layer chromatography [4]. Van Damme and De Nève have also described a fluorescence assay of hydroxynaphthoquinones [5]. The main disadvantage of these methods is a lack of specificity, as other constituents of the plant material can react with the reagents employed. A GLC method was suggested by Hedin et al. to determine juglone in pecan nuts, but a TLC has to be run before GLC analysis because of interfering substances [6]. Hence this method is critical and the results therefore unreliable.

No HPLC method has been described in the literature, prompting us to develop an HPLC method to determine juglone content in an extract of fresh leaves. The advantage of HPLC is principally its greater selectivity compared with that of the methods previously mentioned. Using HPLC, it is possible to inject the whole plant extract and so determine juglone content alone in the extract. This method has been developed and validated as prescribed in good analytical practice [7].

2. Experimental

2.1. Chemicals

Standard juglone was obtained from Extrasynthèse (Genay, France). All organic solvents were of HPLC

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grade from C.I.L. (Ste. Foy-la-Grande, France). Phosphoric acid (84%) was purchased from Prolabo (Paris, France).

2.2. Instrumentation and chromatographic conditions

The HPLC system comprised a Waters Model 590 and 510 dual pump system controlled by a Waters automated gradient controller, a Waters U6K manual injector, and a Waters 991 photodiode array detector (Waters Associates, Milford, MA, USA). Detector output was monitored and analysed with a Waters 991 chromatography acquisition program operated by an NEC APC IV Power Mate 2 digital microcomputer (NEC System, Boxborough, MA, USA).

Chromatographic separations were performed on a LiChrospher 100 RP-18 end-capped column (125×4 mm, particle size 5 μm) (Merck, Darmstadt, Germany), protected by a precolumn of the same type (4×4 mm). The mobile phase was a mixture of A: water–phosphoric acid (95.5:0.5, v/v) and B: acetonitrile–water (90:10, v/v), with a linear gradient programmed as follows: t_0: 75% A–25% B; $t_{30\ min}$: 60% A–40% B; $t_{40\ min}$: 25% A–75% B; $t_{45\ min}$: 25% A–75% B (isocratic). This solvent and this gradient were adjusted from a work previously carried out in our laboratory in order to separate some flavonoid aglycones. These analytical conditions were developed to obtain a short retention time and a good resolution of the peak corresponding to juglone. A 45-min long elution was necessary to elute nonpolar components present in small quantities in the chloroform extract of the leaves. Flow-rate was 2 ml/min. The injected volume was 5 μl in each assay.

The spectra of compounds were recorded from 250 to 500 nm, and the detection wavelength was 420 nm, which is the λ_{max} of juglone in methanol solution.

2.3. Preparation of solutions

A calibration standard of juglone was prepared with juglone from Extrasynthèse: about 3.50 mg exactly weighed in 10.0 ml of methanol.

Walnut leaves were collected by us in Lempdes (France), in May 1996, and treated in the hour following harvesting. Leaves were cut into small fragments (about 1 cm). A 1.2-g amount of plant material was extracted by maceration in 150 ml of chloroform for 1 h, at room temperature. We have shown that 1 h was sufficient to quantitatively extract juglone from the leaves, by carrying out extractions at five durations; 0.5 h, 1 h, 2 h, 4 h and 24 h. Juglone contents were respectively: 0.442, 0.498, 0.501, 0.500, 0.450 g/100 g. There is no significant difference between results obtained after 1 h, 2 h or 4 h, so 1 h was chosen in our procedure. The chloroform solution was filtered and evaporated under reduced pressure at 50°C to give a dry residue. This residue was recovered in 10.0 ml of methanol and the methanol solution was injected for analysis.

As usual in pharmacognosy, the juglone content is expressed in relation to the dry plant material (weight of fresh leaves after deduction of water content). The determination of water in fresh leaves was carried out on each sample, following the procedure indicated in [8] .

3. Results and discussion

3.1. Juglone detection

In our experimental conditions, the retention time of juglone was about 9.1 min (Fig. 1), and its identification in the sample solution was carried out by comparison of the spectrum of the peak corresponding to the retention time of juglone with that of standard juglone, by means of a photodiode array detector (Fig. 2). Peak purity was determined by recording three spectra (250–500 nm) at three peak positions (9.06, 9.11 and 9.2 min, respectively), and superimposing these spectra with that of standard juglone. Examination of data showed that purity was 99.93%.

3.2. Stability

The stability of juglone in methanol solution was examined over a 1-week period. Once daily, the concentration of juglone in this original solution was calculated from a standard curve derived from a freshly prepared solution. Results showed that juglone is not stable in methanol solution, and so it is necessary to prepare the calibration standard just before analysis (after 24 h, the loss is about 20%). We also noted that the chloroform extract of the

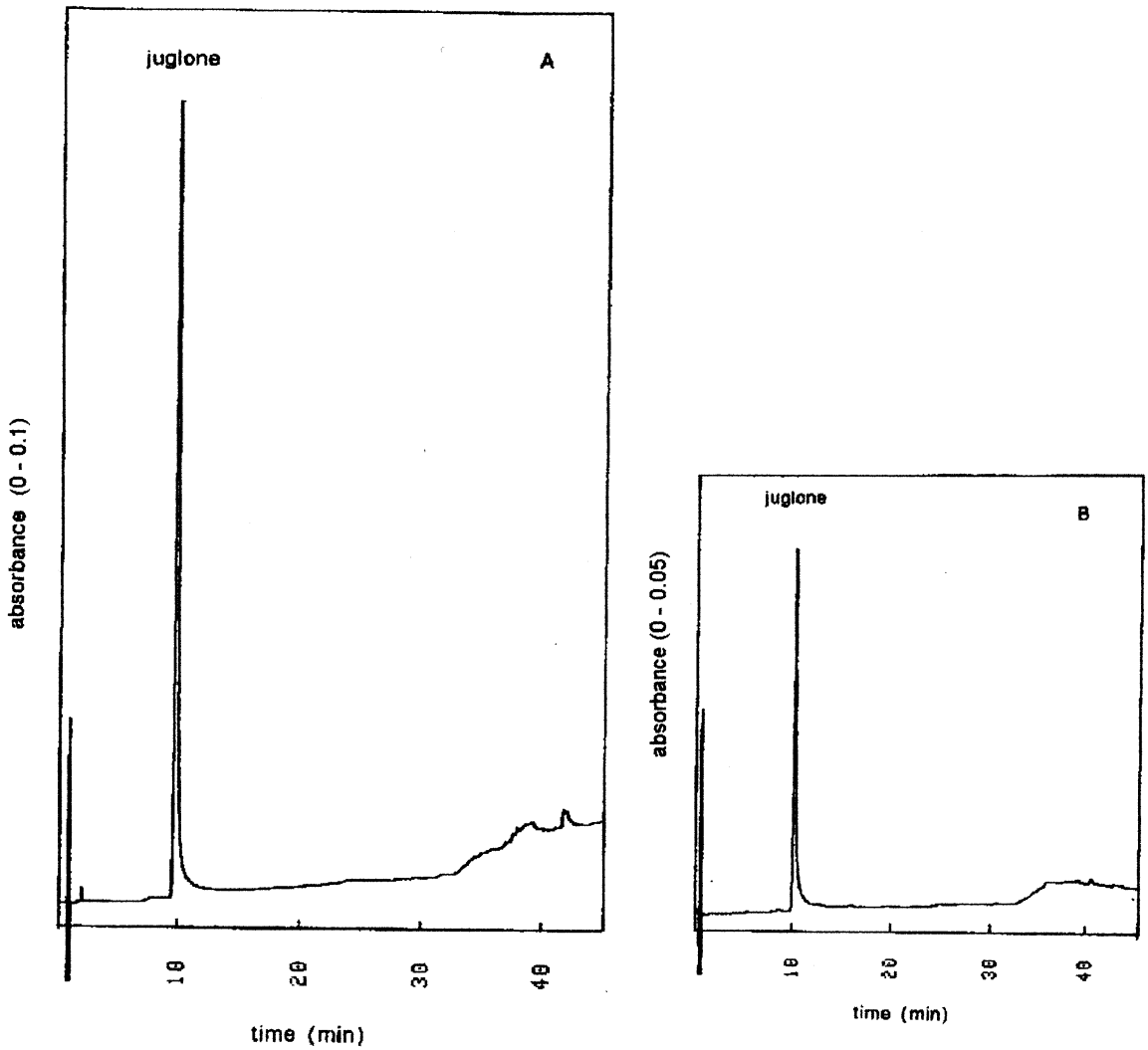

Fig. 1. Chromatograms of a chloroform extract from walnut leaves (A) and of a standard juglone (B) ($\lambda_{detection}$=420 nm).

leaves could not be conserved, but the dry residue of this extract could be kept up to 24 h at 4°C in the dark. To avoid these problems of preservation, the leaves were extracted in the hour following harvesting, and the extract was analysed on the same day.

3.3. Validation

3.3.1. Standard juglone

Linearity was established by analysing methanol solutions (n=5) with a concentration level of juglone between 0.20 and 0.50 mg/ml. The R.S.D. was 1.3% and coefficient of correlation was 0.9978.

Repeatability was evaluated by analysis of methanol solutions (n=5), with a concentration level 0.3–0.4 mg/ml. The R.S.D. was 1.1%.

Limit of detection: the smallest injected quantity of juglone yielding a significantly higher signal than background noise was 0.05 μg.

3.3.2. Assay sample

Linearity of the total procedure: the juglone content was determined on five assay samples between 0.7428 and 1.7482 g of plant material extracted as indicated in Section 2.3. The results were: R.S.D.=2.8%; coefficient of correlation=0.9957.

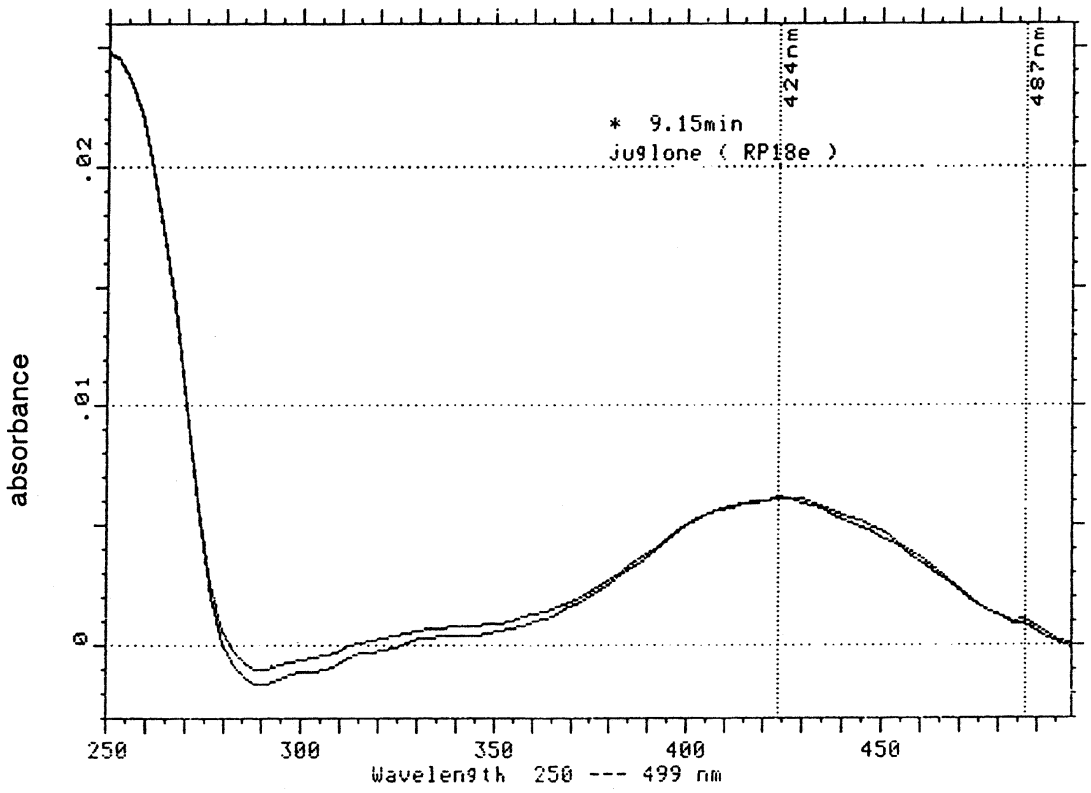

Fig. 2. Superimposition of the spectrum of the peak corresponding to juglone in assay sample with the spectrum of standard juglone.

Repeatability was evaluated by analysing replicate assay samples ($n=5$) obtained after extraction from plant material (about 1.2 g). The mean juglone content was 0.498 ± 0.0164 g/100 g (R.S.D.=3.3%).

Limit of detection: the smallest injected quantity of juglone yielding a significantly higher signal than background noise was 0.065 μg.

All the R.S.D. and the coefficients of correlation values were consistent with those required in analytical procedures [7].

4. Conclusion

We developed an HPLC method for the determination of the juglone content in fresh walnut leaves. This method was developed and validated as prescribed for analytical procedures. Compared with previously published methods, the HPLC method is simple, rapid, and more specific. Hence it could be a valuable tool for the determination of juglone content in a large numbers of fresh walnut leaves, harvested at different stages of vegetation.

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