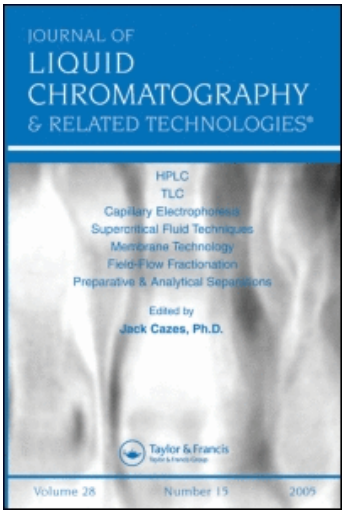

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Sun, Chongde , Li, Xian , Xu, Changjie , Zhang, Shanglong , Chen, Kunsong , Chen, Qingjun and Liu, Chunrong(2007) 'Determination of 9(10*H*)-Acridone by HPLC with Fluorescence Detection', Journal of Liquid Chromatography & Related Technologies, 30: 2, 245 — 254

To link to this Article: DOI: 10.1080/10826070601064409

URL: http://dx.doi.org/10.1080/10826070601064409

Journal of Liquid Chromatography & Related Technologies®, 30: 245–254, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070601064409

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Abstract: Acridone alkaloids are important bioactive compounds found mainly in the Rutaceae family. An easy to use, rapid, and accurate high performance liquid chromatography (HPLC) method with fluorescence detection was developed for determination of 9(10*H*)-acridone. The HPLC assay was performed on a reversed phase C_{18} column with a gradient of methanol in the water as the mobile phase, and eluent flow rate of $1.0\,mL\,min^{-1}$. The wavelengths of excitation and emission were 395 and 435 nm, respectively. The linear relationship between the peak area and the 9(10*H*)-acridone concentration with the correlative coefficient of 0.999 was observed. This method is highly sensitive with a detection limit of $0.67\,ng\,mL^{-1}$ ($S/N = 3$), and comparison of the 9(10*H*)-acridone content in the

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citrus roots among four varieties proved that it is suitable for the detection of acridone in plant tissues.

Keywords: Column liquid chromatography, Reversed phase, Fluorescence HPLC, Qualification, Quantification, Acridone

INTRODUCTION

Acridone alkaloids are secondary plant metabolites that were first isolated from plant growing in the Australian rain forest in 1948.[1] Thereafter, more acridone alkaloids were isolated from more than 20 plant genera, among which the plant of the Rutaceae family has been reported to be rich in these compounds.[2,3]

Recently, acridone alkaloids have attracted considerable attention due to their pronounced bioactivities. Activities such as antivirus,[4] anticancer, and antiproliferaty,[5–7] cytotoxity,[8,9] antimalaria,[10,11] antibiotic and antifungal,[10,12] have been reported by scientists worldwide. For example, acridones have been reported to inhibit the growth of different types of virus such as HSV, HCMV, EBV, HIV-1, and Adv 6, and such inhibition effect varied dramatically due to different structures of various acridones;[13] *In vivo* antitumor experiments done with 17 acridones extracted from *C. grandis*,[4,14] *C. sinensis*, *G. citrifolia*,[15] and *G. pentaphylla*[16]all showed inhibition effect against the growth of tumors on the back of the mice that were initiated by dimethylbenz [a] anthracene (DMBA) and promoted by 12-O-tetradecanonylphorbol-13-antigen (TPA). Simple acridones, such as 1,3-Dihydroxy-*N*- methylacridone, inhibited the growth of fungi effectively, and the inhibition effect was much higher than that of coumarins and flavone of an equivalent amount.[17,18] Kawaii et al.[7] reported acridones as inducers of human promyelocytic leukemia cell (HL-60) differentiation, which resulted in suppressed HL-60 cell growth. In addition, acridones play important roles in plant defense[19,20] and plant taxonomy.[21]

Several methods have been developed to extract and identify acridone alkaloids from plant tissues. Wu et al.[14,15,21–25] used ^{1}H NMR, IR, and MS methods to identify several acridone alkaloids from the root and bark of *C. grandis*, *C. sinensis* and *C. depressa*. Kuzovkina et al.[26] used HPLC-^{1}H NMR to identify and determine acridone alkaloid glucosides in the tips of transformed roots of *Ruta graveolens*. Unfortunately, these methods are not applicable for routine usage due to the high cost of the required equipment or the complicated procedures of operation. In addition, such detection methods have encountered serious limitations because of the complicated physicochemical natures of these chemicals from various plant resources.

Due to the specific skeleton structure, acridone alkaloids were reported to have a characteristic of fluorescence absorption.[26,27] However, there is no

fluorescence method used directly for the determination of acridones. The objective of this study is to develop a simple and rapid method for the detection of one of acridones, i.e., 9(10*H*)-acridone, in citrus by HPLC with fluorescence detection.

EXPERIMENTAL

Chemicals

9(10*H*)-acridone standard was purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile and methanol of HPLC grade were purchased from Caledon laboratories LTD (Georgetown, Canada). Ethanol and other chemicals were of analytical grade.

Instrumentation

A fluorescence spectrophotometer (PELS-50B, Perkin-Elmer Ltd, USA) was used to determine the wavelengths of fluorescence excitation and emission of the 9(10*H*)-acridone standard. The Beckman HPLC system was used for the detection of acridone. It consists of a reverse-phase C_{18} column (4.3 mm × 25 cm), 125 pump, 166 UV monitor, and fluorescence monitor (FP2020, Jasico, Japan). A Heidolp evaporator (Laborata 4000, Germany) was used to evaporate the extracted samples from citrus roots.

Standard Solution

A stock solution of 1 mg mL^{-1} was prepared from 9(10*H*)-acridone standard in ethanol and the concentrations of 250, 500, 1000, 2000, and 4000 ng mL^{-1} were prepared by serial dilution of the stock solution with the proper amount of ethanol.

Chromatographic Conditions

A fluorescence detector was used for the HPLC detection of 9(10*H*)-acridone. The reversed-phase HPLC method was carried out using a mobile phase composed of a gradient methanol in double distilled water (% methanol): 0–4 min (0–62%), 4–19 min (62–83%), 19–23 min (83–100%), 23–32 min (100%), 32–32 min (100–0%), and 32–35 min (0%) to finish a cycle. The flow rate was 1.0 mL min^{-1} and analytes were separated in a C_{18} column (4.3 mm × 25 cm) operated at room temperature (25 ± 1°C). A sample of 20 μL each time was injected to the HPLC system and the

concentrations of acridone in each sample were calculated by comparing the peak area with that of the standard.

Validation of the Assay

The linearity of the method was established by injections in the range of 250–4000 ng mL^{-1} with five replicates. A calibration plot was constructed by plotting the peak area ratio against a concentration of 9(10*H*)-acridone and calculating the regression equation. Limit of detection (LOD) was determined for the 9(10*H*)-acridone standard.

To determine the intra-day precision, five injections of each concentration were done on the same day. These studies were repeated on three consecutive days to determine the inter-day precision.

To determine the extraction recovery, root samples (n = 3) containing 1.25, 2.50, and 5.00 μg 9(10*H*)-acridone standard were prepared and analyzed. Ratios of the assay value to the spiked amount for 9(10*H*)-acridone were calculated.

Application to Citrus Samples

Roots of *C. changshanensis*, *C. grandis*, *C. unshiu*, and *C. reticulata* were obtained from Quzhou Citrus Institute in Zhejiang province, China from April to November, 2005.

Citrus roots divided into three parts (fibre, phloem, and xylem) were dried at 50°C and then ground, respectively. Two grams of the sample was placed in a Soxhlet extractor containing 40 mL ethanol and, was then, extracted by an ultrasonic device at 30°C for 1 hour. The extraction was filtered and the residue was extracted one more time with the same procedure. Both extracts were combined and evaporated close to dryness at 35°C. The residue was dissolved in 2 mL ethanol, from which 1 mL was then transferred to a 1.5 mL Eppendorf tube. All analytes were filtered through 0.30 μm filters (ϕ13, organism) prior to HPLC analyses. Standard deviations (S.D.) were calculated by Origin (Microcal Software Inc., Northampton, MA, USA). Duncan's new multiple range method test (DPS version 3.11) was calculated for mean separations.

RESULTS AND DISCUSSION

Identification

The 9(10*H*)-acridone standard showed fluorescence excitation and emission at wavelengths of 395 and 435 nm, respectively (Figure 1). A gradient

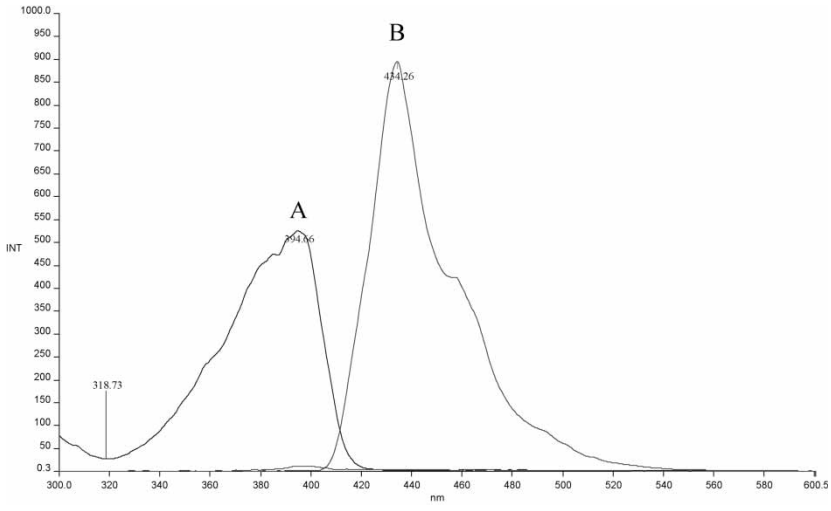

Figure 1. Excitation curve (A) and emission curve (B) of 9(10*H*)-acridone standard by fluorescence spectrophotometer.

reversed-phase HPLC method with fluorescence detection at these wavelengths was, thus, carried out for determination of 9(10*H*)-acridone standard and those in the root of *C. changshanensis* (Figure 2, A and B). The retention time of the 9(10*H*)-acridone was 11.2 min and no interfering peaks were observed in all the samples examined.

Validation of the Developed Method

Linearity and Range

A linearity study was carried out to determine whether this method could measure different concentrations of 9(10*H*)-acridone accurately. Five stock solutions containing 250, 500, 1000, 2000, and 4000 ng mL^{-1} of 9(10*H*)-acridone were tested individually, with five repetitions for each concentration. The calibration curve was linear and the regression equation between the peak area ratio (y) against concentration (x) was: $y = 0.0087 + 34.007x$ ($r = 0.999$, $n = 5$).

Limit of detection (LOD) was 0.67 ng mL^{-1}, based on the determination of the lowest acridone amount with a signal-to-noise ratio (S/N) of 3.

Reproducibility and Accuracy

Reproducibility and accuracy were determined for five standard samples as described above (Table 1). The intra-day coefficients of variation ranged

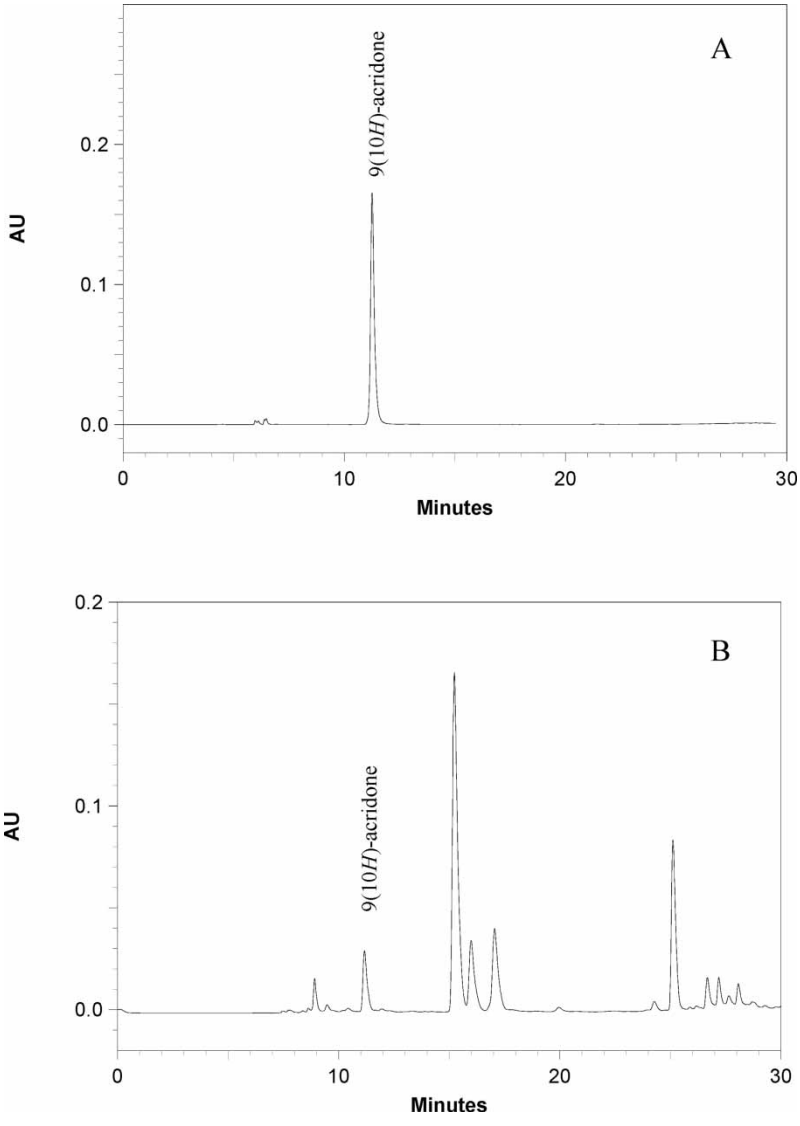

Figure 2. Fluorescence HPLC chromatography of 9(10*H*)-acridone standard (A) and 9(10*H*)-acridone in the root of *C. changshanensis* (B). Wavelengths for fluorescence excitation and emission were 395 and 435 nm, respectively. C_{18} column: flow rate 1.0 mL min^{-1}, 20 μL for each injection, methanol gradient elution.

from 1.29 to 2.59% (n = 5). The inter-day coefficients of variation for analysis of the same acridone samples on three consecutive days ranged from 1.42 to 2.85%. The accuracy (%) of the method, expressed as the mean deviation of all concentrations from theoretical value, ranged from 0.36 to 2.01%.

Table 1. Reproducibility and accuracy of analysis of 9(10*H*)-acridone (n = 5)

Nominal concentration (ng mL^{-1})	Assay value (mean ± SD) (ng mL^{-1})	Coefficient of variation (%)	Accuracy (%)
Intra-day[a]			
250	244.97 ± 6.35	2.59	−2.01
500	495.89 ± 12.50	2.52	−0.82
1000	995.11 ± 16.60	1.67	−0.50
2000	2015.17 ± 25.99	1.29	+0.76
4000	4014.20 ± 70.92	1.77	+0.36
Inter-day[b]			
250	252.30 ± 6.69	2.65	+0.92
500	491.20 ± 14.01	2.85	−1.76
1000	1008.83 ± 23.84	2.36	+0.88
2000	2008.87 ± 28.49	1.42	+0.44
4000	3967.60 ± 65.50	1.65	−0.81

[a]The sample was analyzed five times during one day.
[b]The sample was analyzed over three consecutive days.

Extraction Recovery

To determine extraction recoveries, a measured amount of 1.25, 2.50, and 5.00 μg 9(10*H*)-acridone standards were added to the root of *C. changshanensis* and analyzed as described above (n = 3). The ratios of the assay value to the spiked amount for 9(10*H*)-acridone standard were calculated. The extraction recoveries ranged from 97.12 to 101.56% for the three levels (Table 2).

Application to Biological Samples

The developed method was applied to the determination of acridone in the root of citrus. Figure 2 (B) showed the presence of 9(10*H*)-acridone in the root of

Table 2. Recovery tests of the analytic method for 9(10*H*)-acridone (n = 3)

Spiked amount (μg)	Assay value (mean ± SD) (μg)	Recovery rate (%)
1.25	1.27 ± 0.05	101.56 ± 4.35
2.50	2.43 ± 0.08	97.31 ± 3.04
5.00	4.86 ± 0.14	97.12 ± 2.86

Table 3. Content of 9(10*H*)-acridone in different root tissues of four citrus cultivars. Different letters refer to significant differences among data both across the columns and rows ($\alpha = 0.05$)

	Content of 9(10*H*)-acridone ($\mu g\ g^{-1}$ DW)	
Citrus varieties	Root phloem	Fibre
C. changshanensis	2.49 ± 0.12 ab	2.69 ± 0.24 a
C. reticulata	1.49 ± 0.07 d	1.57 ± 0.04 d
C. grandis	2.01 ± 0.08 c	2.21 ± 0.08 bc
C. unshiu	0.02 ± 0.00 e	0.03 ± 0.00 e

C. changshanensis. The content of 9(10*H*)-acridone in root phloem and fibre of four citrus cultivars ranged from 0.02 to 2.69 $\mu g\ g^{-1}$ DW (Table 3). Significant differences in 9(10*H*)-acridone content was found in the citrus root of different cultivars, however, it showed no significant difference in different root parts within each cultivar. The root phloem and fibre of *C. changshanensis* contained the highest 9(10*H*)-acridone content when compared with their counterparts of other cultivars. 9(10*H*)-Acridone was undetectable in the root xylem of all the four cultivars tested. Such results showed that, without the presence of expensive and complicated facilities such as NMR, IR, and MS, the fluorescence HPLC can be efficiently used for qualification and quantification of acridones. This method can be extended to the study of different acridones from various plant sources in cases where more acridone references are available. In addition, the contribution of acridones in the bioactivity study of plant based food or Chinese herbal medicine can be clarified with such a convenient detection method.

CONCLUSION

The developed method using a gradient reversed-phase HPLC method with fluorescence detection effectively separated 10*H*)-acridone from the samples. This is the first time using a fluorescence-HPLC method to detect acridone directly, and it proved to be an easy, reliable, cost effective, and efficient method for the determination of acridones in plant tissues. It can be used for further study and integrative utilization of acridones.

ACKNOWLEDGMENTS

This work was supported by the 111 project (B06014) and the Span project of State Agriculture Ministry (No.2004-17).

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Received September 13, 2006
Accepted October 23, 2006
Manuscript 6939