Simultaneous Determination of Two Isomers of Asarone in *Piper sarmentosum* Roxburgh (Piperaceae) Extracts using Different Chromatographic Columns

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Abstract

**Purpose:** To develop a rapid and reliable reverse phase high performance liquid chromatography (RP-HPLC) method to quantify the two isomers of asarones in *P. sarmentosum* extracts using two different columns under similar analytical conditions.

**Methods:** Two isomers, α- and β-asarone, were analyzed using two types of C-18 columns with 0.1 % orthophosphoric acid: acetonitrile: methanol (50: 40: 10) as mobile phase. The developed method was applied to determine the contents of α- and β-asarone in extracts of different parts of *P. sarmentosum*.

**Results:** Column A retention times for the elution of α- and β-asarone were 11.890 ± 0.008 and 10.80 ± 0.004 min, respectively, and were significantly shorter than those of column B (15.110 ± 0.024 and 13.290 ± 0.018, respectively, p < 0.001). Column B showed better resolution (1.82 ± 0.025 of the isomers than column A (1.10 ± 0.01, p < 0.001). Both columns showed comparable sensitivity, precision and selectivity of the compounds investigated. α-Asarone level was in the range 0.36 - 5.14 % in ethanol and 50 % ethanol extracts, but absent in all water extracts. β-Asarone occurred in the range of 0.01 - 0.15 % in ethanol and 50 % ethanol extracts but was absent in all water extracts of *P. sarmentosum*.

**Conclusion:** The results indicate that the developed method is a suitable quality assurance method for determining α- and β-asarone isomers in herbal extracts and food preparations.

**Keywords:** α- Asarone, Isomers, Piper sarmentosum, Herbal extracts, Retention time

INTRODUCTION

*Piper sarmentosum* Roxburgh (family: Piperaceae) is a herbaceous shrub, and is most widely distributed in Southeast Asia including Malaysia, Cambodia, Laos, Philippines, Myanmar, Thailand, Vietnam and China [1]. The plant has been investigated for a number of pharmacological activities such as hypoglycemic [2], anti-amoebic [3], antibacterial [4], neuromuscular blocking [5], anti-malarial [6], antioxidant [7], anti-tuberculosis [8], anticancer [9] and anti-angiogenic activity [10].

The chemical constituents identified from the plant include (2E, 4E)-N-isobutyldodecanamide, N-(2-phenylpropanoyl) pyrrole, sarmentine, sarmentosine, 1-(3,4-methylenedioxyphenyl)-(1E)-tetradecane, 1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene, asaricin, α-asarone, γ-
asarone, hydrocinnamic, oxalic acids, sitosterol and naringenin [11,12]. Asarones are mostly found in isomers i.e. cis- and trans-forms. The trans-asarone or α-asarone chemically known as (E)-1,2,4-trimethoxy-5-(prop-1-en-1-yl) benzene while cis-asarone or β-asarone known as (Z)-1,2,4-trimethoxy-5-(prop-1-en-1-yl)benzene.

Apart from showing biological activities, i.e. anthelmintic, pesticidal and neuroprotective [13,14], both α- and β-asarones are also known as carcinogenic and genotoxic compounds [15,16]. β-asarone possesses toxic and sterilizing effects [17] whereas α-asarone showed greater cytotoxic effect [18].

In food and herbal industries it is required that the quantity of asarones be limited to ascertain the products safety [19-21]. Therefore, analysis of these asarone isomers in P. sarmentosum is important for its quality control in herbal remedy preparation.

This study was conducted in order to develop and validate a simple and robust RP-HPLC method for the quantification of α- and β-asarones in different extracts of P. sarmentosum without using large quantity of organic solvents and special stationary phase or modifiers for the separation of asarone isomers.

**EXPERIMENTAL**

**Preparation of raw material**

P. sarmentosum plant was collected from Batu Gajah, Malaysia. The plant samples were identified by Dr. Rahmad Zakaria from School of Biological Sciences, Universiti Sains Malaysia and the specimen vouchers were deposited at School of Biological Sciences, Universiti Sains Malaysia with voucher number USM/Herbarium/11481. The plant samples which were divided into four different parts (leaf, stem, root and fruit) were ground into powder form using an electric grinder SM-100 (Retsch, Germany).

**Chemicals and reagents**

All solvents used were of HPLC grade and were acquired from Merck Sdn. Bhd. (Selangor, Malaysia). Authentic α- and β-asarones were obtained from Sigma-Aldrich (M) Sdn. Bhd (Kuala Lumpur, Malaysia). Two C-18 columns (250 x 4.6 mm, 5 µm): (A) ZORBAX Eclipse Plus Phenyl-Hexyl and (B) ZORBAX Eclipse Plus were purchased from Agilent Technologies (USA).

**Preparation of extracts**

All extracts were prepared by maceration of powdered material with 99 % ethanol, 50 % ethanol and water solvents at 60 °C for 48 h. All extracts were filtered; the solvent was evaporated to dryness using rotary evaporator R-100 (Buchi, Switzerland) at 40 °C. Extracts were labeled as PSL-E, PSL-EW, PSL-W, PSS-E, PSS-EW, PS-S-W, PS-E, PSR-E, PSR-EW, PS-W, PSF-E, PSF-EW and PSF-W, respectively, where PS = P. sarmentosum, L = leaves, S = Stem, R = roots, F = fruits, EW = ethanol: water (50: 50), W = water, E = ethanol.

**Instrumentation and HPLC conditions**

All experiments were performed using an Agilent Technologies 1260 infinity (USA) HPLC system with UV detector, quaternary pump, online degasser, and column incubator. Separation was done on two different C-18 columns (250 x 4.6 mm, 5 µm) (A) ZORBAX Eclipse Plus Phenyl-Hexyl, C-18 column and (B) ZORBAX Eclipse Plus C-18 made by Agilent Technologies (USA), using an isocratic mobile phase consisting 0.1 % phosphoric acid : acetonitrile : methanol 50 : 40 : 10, the flow rate was set at 1 ml/min, column temperature was maintained at 30 °C, and detection was performed at 210 nm. Injection volume was 10 µL and data acquisition was carried out by Agilent ChemStation software for LC systems.

**Preparation of the standard mixture**

A stock solution of a mixture of α- and β-asarone was prepared at 1 mg/mL in HPLC grade methanol, and serial dilutions were prepared in the range 0.44 – 250 µg/mL. P. sarmentosum extracts were also prepared at 10 mg/mL in the same solvent and were further diluted to 1 mg/mL with methanol. Both, samples and standard stock solution were filtered through 0.45 µm syringe filters.

**Method validation**

The proposed method was validated on both columns according to the ICH guidelines [22]. The following validation characteristics were evaluated: selectivity, linearity, precision, accuracy and the limits of detection and quantification (LOD and LOQ).

**Linearity**

Linearity was determined by injecting 10 µL of the standard mixture in the concentration range
0.448 - 250 µg/mL. The calibration curves were obtained by plotting peak area versus concentration, and linearity (R2) was determined by regression analysis of the calibration graphs.

**Selectivity**

The selectivity of method was determined by comparing the retention time of target compounds obtained in the sample extracts with their reference counterparts, and by spiking the extracts with known concentration of the reference compounds.

**Precision**

Precision was determined as the coefficient of variation (% CV) of peak area and retention time. The standard mixture was analyzed at 5 concentrations in the range 7.81 - 125 µg/mL, and the intraday, interday and intermediate precisions were determined (n = 5).

**Accuracy**

Accuracy was determined as a percentage recovery of α- and β-asarone at 3.16, 6.25 and 12.5 µg/mL added to the ethanol extract at 1000 µg/mL. The peak area corresponding to the compounds in the ethanol extract (B), the individual reference compounds (C) and their combinations (A) were recorded. The percentage recovery was then calculated using the following formula: % Recovery = ((A – B) / C) × 100. The results are presented average ± SD (n = 3).

**Limits of detection (LOD) and of quantification (LOQ)**

The LOD and LOQ were calculated from the slope and standard deviation method as in Eqs 1 and 2.

\[
\text{LOD} = (3.3 \times \delta)/S \quad \text{........................................... (1)}
\]

\[
\text{LOQ} = (10 \times \delta)/S \quad \text{........................................... (2)}
\]

Where \( \delta \) is the standard deviation of the Y intercept of the linear regression equation. S is the slope of the linear regression equation [22].

**Determination of α- and β-asarone concentration in P. sarmentosum extracts**

Three different extracts from 4 parts of P. sarmentosum were analyzed for asarone contents (Figures 2 and 3). Concentrations of α- and β-asarones were calculated by applying the linear regression equations of the reference compounds. The identifications of asarones were done by comparing the retention times to those of standards, as well as adding the individual standard to the samples. P. sarmentosum extracts (10 µL) were injected at 1000 µg/mL, and the peak areas corresponding to α- and β-asarone were recorded. The linear regression equations of the standard calibration curves were applied to calculate the concentrations of the marker compounds, and the results are presented as mean % wt/wt using the formula: % wt/wt = (the found concentration/1000 µg/mL) × 100

**Statistical analysis**

Statistical calculations were carried out using SPSS 20.0 software package. Independent sample t-test was applied and the differences were considered significant at \( p < 0.05 \).

**RESULTS**

It is reported that trans/α- isomers stays for a longer time in column as compared cis/β-isomer [23]. So in the present study, it was observed that with each column the cis/β-asarone had a lower retention time than the trans/α-asarone. In terms of column comparison, we found that column A has significantly shorter retention time for the elution of α- and β-asarone which were 11.89 ± 0.008 min and 10.80 ± 0.004 min compared to column B 15.11 ± 0.024 min and 13.29 ± 0.018 min, (Figure 1). The dimethylphenylhexylsilane stationary phase in column A was able to elute the target compounds faster than column B which contained dimethyl-n-octadecylsilane stationary phase. Column B showed better resolution (1.82 ± 0.025 min difference between) of the isomers as compared to column A (1.10 ± 0.01 min difference) which was statistically significant, \( p < 0.001 \). We also found that both columns showed comparable sensitivity, precision and selectivity of the compounds investigated. Column A had higher sensitivity with lower LOD and LOQ compared to column B.

**Selectivity**

The selectivity of the method was determined by comparing the retention times of α- and β-asarone obtained in the sample extracts with those of the reference compounds on both columns. The retention time of α- and β-asarone was 11.8 ± 0.002 and 10.79 ± 0.021 min on column A while it was 15.09 ± 0.01 and 13.26 ± 0.02 min for column B respectively.

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Figure 1: HPLC chromatograms of α- and β-asarone at 210 nm. A = ZORBAX Eclipse Plus Phenyl-Hexyl C18 column, B = ZORBAX Eclipse Plus C18 column

Their counterparts in P. sarmentosum extracts were eluted at 11.81 ± 0.002 and 10.8 ± 0.03 min on column A while they eluted 15.28 ± 0.67 and 13.33 ± 0.03 min, respectively, on column B. Spiking the extracts with α- and β-asarone increased the peak areas of the compounds without any shift in the retention times and appearance of extra peaks. This further confirmed the identity of the compounds and the method’s selectivity on both columns.

Linearity

Linearity was presented in terms of the regression coefficient ($R^2$) of the regression equations of reference compounds ($R^2$) was 0.999 ± 0.001 for both α- and β-asarone which indicate good linearity of the proposed method using two different columns.

Precision

The precision was calculated in terms of the % CV of the retention time and peak area (Table 1 and 2). The α- and β-asarone were eluted at 11.800 ± 0.002 and 10.790 ± 0.021 min on column A with CV of < 0.05 %, and 15.09 ± 0.01 min and 13.26 ± 0.02 min with a % CV of < 0.42 % on column B, respectively. The % CV of the peak areas were calculated in the concentration range 7.81 - 125 µg/mL with an average of < 0.05 and < 0.20 % on columns A and B respectively. These results indicated good reproducibility of retention times and peak areas on both columns.

Accuracy and recovery

The results are presented as recovery of the reference compounds, viz, 31.25, 62.5, and 125 µg/mL ranging from 97 ± 0.50 and 99 ± 0.5 % for columns A and B, respectively.

LOD and LOQ

The LOD and LOQ of α- and β-asarone with column A and B were calculated and presented in Table 2.

α- and β-asarone in extracts of P. sarmentosum

Asarone contents in the extracts of different parts of P. sarmentosum varied between the samples and are shown in Table 3. The α-asarone was found from 0.36 - 5.14 % in ethanol and 50 % ethanol extracts of P. sarmentosum parts and absent in all water extracts. β-asarone was found in lesser amounts in the ranges from 0.01 - 0.15 % in ethanol and 50 % ethanol extract and was absent in all water extracts of different parts of P. sarmentosum.
Table 1: Precision analysis of α- and β-asarone using both columns

|          | Precision | Column A | Column B |
|----------|-----------|----------|----------|
|          | Conc. (µg/mL) | PA (mAU*S) AV±SD | % CV | RT (min) AV±SD | % CV | PA (mAU*S) AV±SD | % CV | RT (min) AV±SD | % CV |
| **Intraday** | 125 | 8021.0 ± 1.42 | 0.02 | 11.89 ± 0.005 | 0.04 | 8005.16 ± 3.10 | 0.04 | 15.08 ± 0.01 | 0.08 |
|          | 62.5 | 4236.8 ± 0.45 | 0.02 | 11.89 ± 0.005 | 0.04 | 4263.42 ± 2.31 | 0.05 | 15.09 ± 0.00 | 0.03 |
|          | 31.25 | 2080.8 ± 0.84 | 0.04 | 11.90 ± 0.007 | 0.05 | 2160.56 ± 1.89 | 0.09 | 15.15 ± 0.01 | 0.04 |
|          | 15.625 | 1075.8 ± 1.09 | 0.10 | 11.89 ± 0.004 | 0.03 | 1057.52 ± 2.78 | 0.25 | 15.14 ± 0.01 | 0.04 |
|          | 7.8125 | 542.0 ± 0.00 | 0.00 | 11.87 ± 0.043 | 0.36 | 568.20 ± 1.07 | 0.19 | 15.15 ± 0.01 | 0.06 |
| **Interday** | 125 | 8022.4 ± 1.14 | 0.01 | 11.90 ± 0.01 | 0.01 | 7967.56 ± 1.67 | 0.02 | 15.09 ± 0.00 | 0.02 |
|          | 62.5 | 4235.8 ± 1.09 | 0.02 | 11.89 ± 0.02 | 0.02 | 4263.87 ± 1.99 | 0.05 | 15.09 ± 0.01 | 0.04 |
|          | 31.25 | 2082 ± 0.70 | 0.03 | 11.90 ± 0.03 | 0.01 | 2138.23 ± 2.49 | 0.12 | 15.09 ± 0.01 | 0.07 |
|          | 15.625 | 1075.2 ± 0.83 | 0.07 | 11.90 ± 0.01 | 0.01 | 1097.75 ± 1.97 | 0.18 | 15.09 ± 0.01 | 0.06 |
|          | 7.8125 | 543.8 ± 0.83 | 0.15 | 11.89 ± 0.03 | 0.00 | 563.98 ± 3.42 | 0.61 | 15.09 ± 0.01 | 0.05 |
| **Inter-medium** | 125 | 8022.4 ± 1.14 | 0.01 | 11.90 ± 0.09 | 0.02 | 7986.36 ± 1.00 | 0.01 | 15.09 ± 0.01 | 0.04 |
|          | 62.5 | 4235.8 ± 0.99 | 0.01 | 11.89 ± 0.02 | 0.02 | 4263.65 ± 0.22 | 0.01 | 15.09 ± 0.00 | 0.01 |
|          | 31.25 | 2082 ± 0.70 | 0.03 | 11.90 ± 0.03 | 0.01 | 2138.23 ± 2.49 | 0.12 | 15.09 ± 0.01 | 0.07 |
|          | 15.625 | 1075.2 ± 0.83 | 0.07 | 11.90 ± 0.01 | 0.01 | 1097.75 ± 1.97 | 0.18 | 15.09 ± 0.01 | 0.06 |
|          | 7.8125 | 543.8 ± 0.83 | 0.15 | 11.89 ± 0.03 | 0.00 | 563.98 ± 3.42 | 0.61 | 15.09 ± 0.01 | 0.05 |
| **β-asarone** | 125 | 8389.6 ± 1.1 | 0.01 | 10.79 ± 0.005 | 0.05 | 7977.84 ± 1.71 | 0.02 | 13.31 ± 0.00 | 0.00 |
|          | 62.5 | 4485.8 ± 1.7 | 0.03 | 10.79 ± 0.005 | 0.05 | 4190.78 ± 2.60 | 0.06 | 13.31 ± 0.00 | 0.00 |
|          | 31.25 | 2243.6 ± 1.1 | 0.05 | 10.79 ± 0.008 | 0.08 | 2193.30 ± 3.99 | 0.18 | 13.30 ± 0.01 | 0.04 |
|          | 15.625 | 1237.8 ± 1.4 | 0.13 | 10.79 ± 0.005 | 0.05 | 1116.04 ± 2.76 | 0.25 | 13.30 ± 0.00 | 0.00 |
|          | 7.8125 | 646.2 ± 0.83 | 0.12 | 10.80 ± 0.004 | 0.04 | 7977.84 ± 1.71 | 0.02 | 13.31 ± 0.00 | 0.00 |
|          | 125 | 8389.8 ± 1.2 | 0.02 | 10.8 ± 0.00 | 0.06 | 8018.86 ± 3.25 | 0.04 | 13.27 ± 0.02 | 0.18 |
| **Interday** | 62.5 | 4487.4 ± 1.5 | 0.03 | 10.79 ± 0.00 | 0.05 | 4198.37 ± 3.82 | 0.09 | 13.27 ± 0.02 | 0.18 |
|          | 31.25 | 2243.6 ± 1.1 | 0.05 | 10.79 ± 0.00 | 0.05 | 2196.84 ± 3.31 | 0.15 | 13.27 ± 0.02 | 0.18 |
|          | 15.625 | 1235.4 ± 1.3 | 0.10 | 10.79 ± 0.00 | 0.05 | 1115.91 ± 2.46 | 0.22 | 13.26 ± 0.02 | 0.18 |
|          | 7.8125 | 647.0 ± 0.00 | 0.00 | 10.8025 ± 0.00 | 0.04 | 581.46 ± 2.36 | 0.41 | 13.26 ± 0.02 | 0.18 |
| **Inter-medium** | 125 | 8389.7 ± 0.14 | 0.00 | 10.79 ± 0.00 | 0.03 | 7998.35 ± 1.09 | 0.01 | 13.29 ± 0.02 | 0.13 |
|          | 62.5 | 4486.6 ± 1.13 | 0.02 | 10.79 ± 0.00 | 0.00 | 4194.58 ± 0.86 | 0.02 | 13.29 ± 0.02 | 0.13 |
|          | 31.25 | 2243.6 ± 0.00 | 0.00 | 10.79 ± 0.00 | 0.01 | 2195.07 ± 0.48 | 0.02 | 13.29 ± 0.01 | 0.10 |
|          | 15.625 | 1236.6 ± 1.69 | 0.13 | 10.79 ± 0.00 | 0.01 | 1115.98 ± 0.21 | 0.02 | 13.28 ± 0.02 | 0.12 |
|          | 7.8125 | 646.6 ± 0.56 | 0.08 | 10.8022 ± 0.00 | 0.00 | 581.89 ± 0.06 | 0.01 | 13.28 ± 0.01 | 0.09 |
Table 2: Summary of α- and β-asarone calibration data on column A and B. The regression equation is \( y = ax + b \), where \( a \) is the slope and \( b \) is the y-intercept

| Precision | Column A | α-Asarone | Column B | β-asarone |
|-----------|----------|------------|----------|-----------|
|           | \( a \) (AV±SD) | \( b \) (AV±SD) | LOD (µg/mL) (AV±SD) | LOQ (µg/mL) (AV±SD) | \( a \) (AV±SD) | \( b \) (AV±SD) | LOD (µg/mL) (AV±SD) | LOQ (µg/mL) (AV±SD) |
| Intraday  | 63.928 ± 0.01 | 94.712 ± 0.63 | 0.032 ± 0.00 | 0.099 ± 0.00 | 63.558 ± 0.13 | 142.06 ± 4.25 | 0.22 ± 0.00 | 0.67 ± 0.001 |
| Interday  | 63.928 ± 0.01 | 95.170 ± 0.66 | 0.034 ± 0.00 | 0.10 ± 0.00 | 62.926 ± 0.87 | 143.54 ± 0.00 | 0.30 ± 0.001 | 0.90 ± 0.010 |

| Intraday  | 66.058 ± 0.01 | 200.64 ± 0.54 | 0.027 ± 0.00 | 0.081 ± 0.00 | 62.950 ± 0.03 | 162.60 ± 2.86 | 0.09 ± 0.001 | 0.27 ± 0.004 |
| Interday  | 66.066 ± 0.01 | 200.24 ± 0.68 | 0.034 ± 0.01 | 0.103 ± 0.00 | 63.252 ± 0.12 | 155.64 ± 0.01 | 0.19 ± 0.001 | 0.59 ± 0.001 |

Table 3: Summary of α- and β-asarone concentration in three extracts of different parts of *P. sarmentosum*

| Samples | Column A | α-Asarone (% ± SD) | Column B | β-asarone (% ± SD) |
|---------|----------|---------------------|----------|---------------------|
|         | RT (min) | α-Asarone (% ± SD) | RT (min) | β-Asarone (% ± SD) |
| PSL-E   | 11.95    | 1.56 ± 0.001        | 10.78    | 0.08 ± 0.001        |
| PSL-EW  | 11.95    | 0.85 ± 0.001        | 10.78    | 0.09 ± 0.003        |
| PSS-E   | 11.92    | 0.42 ± 0.000        | N/D      | N/D                 |
| PSS-EW  | 11.93    | 0.38 ± 0.002        | N/D      | N/D                 |
| PSR-E   | 11.92    | 5.14 ± 0.001        | 10.78    | 0.15 ± 0.001        |
| PSR-EW  | 11.96    | 1.14 ± 0.002        | 10.8     | 0.01 ± 0.001        |
| PSR-W   | N/D      | N/D                 | N/D      | N/D                 |
| PSF-E   | 11.96    | 3.84 ± 0.001        | 10.8     | 0.11 ± 0.001        |
| PSF-EW  | 11.96    | 3.75 ± 0.001        | 10.8     | 0.11 ± 0.001        |
| PSF-W   | N/D      | N/D                 | N/D      | N/D                 |

N/D: Not detected
Figure 2: HPLC chromatograms of α- and β-asarone and *P. sarmentosum* extracts at 210 nm on ZORBAX Eclipse Plus Phenyl-Hexyl C-18 column. A = PSL-E, B = PSL-EW, C = PSL-W, D = PSR-E, E = PSR-EW, F = PSR-W, G = PSS-E, H = PSS-EW, I = PSS-W, J = PSF-E, K = PSF-EW and L = PSF-W

Figure 3: HPLC chromatograms of α- and β-asarone and *P. sarmentosum* extracts at 210 nm on ZORBAX Eclipse Plus C-18 column. A = PSL-E, B = PSL-EW, C = PSL-W, D = PSR-E, E = PSR-EW, F = PSR-W, G = PSS-E, H = PSS-EW, I = PSS-W, J = PSF-E, K = PSF-EW and L = PSF-W
DISCUSSION

For a long time, *P. sarmentosum* has been used as herbal remedy and food supplement, thus reliable procedures are needed for the quantitative analysis of its phytochemical constituents. In this study, *P. sarmentosum* extracts are analysed for their content of α- and β-asarone. Both α- and β-asarone are phenylpropanoids which have been reported to have cytotoxic, genotoxic, carcinogenic, psychoactive effects in both in vitro and in vivo models [18,21].

Based on the HPLC data, α-asarone is present as the major isomer in *P. sarmentosum* extracts compared to β-asarone. This study showed that in different parts of *P. sarmentosum* the concentration of α- and β-asarone were different and also varied between the extraction methods.

The importance of measuring asarone in *P. sarmentosum* is to determine the best extraction method in order to produce a safer extract which is free from asarone or produce extract with asarone concentration within acceptable limit set by the food regulation bodies, i.e., 115 µg/day [20].

One case study on *Acorus calamus* herbal preparation which contained asarone has produced side effects such as tachycardia, dizziness, tremor, irregular breathing, pallor, anxiety, nausea and vomiting [24]. From this analysis, we found that water extracts of *P. sarmentosum* are free from asarones and practically safe for consumption.

CONCLUSION

The developed HPLC method is simple, rapid and compatible with two different C-18 columns for the quantification of α- and β-asarone in *P. sarmentosum* extracts.

The analysis of different extracts of various parts of the plant showed variations in asarone content. The method is promising for determining asarone isomers in medicinal herbs.

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