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Comparison of GC-MS and TLC techniques for asarone isomers determination[1]

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Abstract

Two chromatographic methods (GC-MS and TLC) have been developed for separation and determination of α and β asarone from essential oils and alcoholic extracts. The study has been performed on the *Acorus calamus* (**I**) and *Asarum europaeum* (**II**) essential oils of Romanian origin and the alcoholic extract of *Acorus calamus* L (**III**) and it is a consequence of the International Boards exigency regarding the presence of β asarone in food, beverages and pharmaceuticals. The isomers were determined using both internal and external standard methods. Both SIM and SCAN techniques were used and the results were compared regarding the chromatographic resolution and interference compounds. The method exhibits good repeatability and low detection limit but is expensive and time consuming. The two isomers concentrations are 5.2–6.7 μg ml^{-1} (**I**), 460–510 μg ml^{-1} (**II**) and 2.7–5.7 μg ml (**III**) for α asarone and 91–98 μg ml^{-1} (**I**), 24–29 μg ml^{-1} (**II**) and 88–97 μg ml^{-1} (**III**) for β asarone. The TLC method was developed as an alternative for the GC method. The separation was performed on silica gel plates using toluene: ethyl acetate 8:2 as mobile phase. The evaluation of the chromatograms was made by densitometry using multiple wavelength. The sum of the two isomers are between 80–120 μg ml^{-1} (**I**) and 127–145 μg ml^{-1} (**III**) using spectrophotometric detection and between 73–93 μg ml^{-1} (**I**) and 99–105 μg ml^{-1} (**III**) using fluorimetric detection. The results of the two chromatographic methods were compared. Even the GC is more sensitive, mathematical computations for spots optimization and interference elimination could improves the TLC quality results. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: α-Asarone; β-Asarone; Gas chromatography-mass spectrometry; TIC; SIM; Thin layer chromatography; Densitometry

1. Introduction

Asarone is a typical component of the *Acorus calamus* L. and *Asarum europaeum* L. Owing to their therapeutically and odoriferous properties,

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the two plants are utilized both in phytotherapy and in the alimentary industry for food and beverages spicing and preparing.

In the nomenclature of the Romanian pharmaceutical products there are two drugs which contain calamus and asarum rhizome. The study of the chemical composition of the two plants rhizomes has put into evidence the presence of two geometrical isomers of asarone: α-asarone (trans-1,3,4-trimethoxi-5(1-propenyl) benzene and β-asarone (*cis*-1,3,4-trimethoxi-5(1-propenyl) benzene (Fig. 1)

The pharmacological studies on the essential oils extracted from the two plants have put into evidence, besides their beneficial effects, their toxicity too. In 1974, Federal Drug Administration (FDA) interdicted the utilization of the *Acorus calamus* L. owing to the hepatic carcinomas appeared at the rats on the long time administration of volatile oil [1]. At the same time, FDA proved that is only β-asarone which is susceptible of carcinogen action. The European Council limited the utilization of β-asarone: maximum 0.1 mg kg^{-1} in non alcoholic drinks and 1 mg/kg in alcoholic drinks and food stuffs containing *Acorus calamus* L and *Asarum europaeum* L [2].

The methods published up to the present utilize the most various techniques for α- and β-asarone determination [3,4] but, generally, chromatographic methods are preferred: thin layer chromatography (TLC) [5], gas chromatography (GC) [6], and high performance liquid chromatography (HPLC) [7–9]. The most reliable technique for the asarone isomer determination from essential oils remains the gas chromatographic-mass-spectrometric (GC-MS) method [10]

The study has been performed on the *Acorus calamus* (**I**) and *Asarum europaeum* (**II**) essential oils of Romanian origin and the alcoholic extract of *Acorus calamus* L (**III**). The researches of a previous study [11] concerning the β-asarone determination by UV spectrophotometry method have been taken again and one has tried to eliminate the interference by the apparent content curves method [12]. For the rapid dosing of the asarone isomers from alcoholic extracts and drinks, a TLC method has been imagined and the results being compared with those obtained by GC-MS. The TLC procedure can be used as a fast screening method in order to quantitate the total asarone because FDA interdicted the use of the products that contains this compound, either in α or β form. The evaluation of the GC-MS method has been performed by full scan (total ion current-TIC) using internal and external standards and by selected ion monitoring (SIM). The TLC results have been performed by densitometry (reflectance and fluorescence).

2. Materials and methods

2.1. Samples

The essential oils are obtained by steam distillation of fresh rhizome.

The alcoholic extract of *Acorus calamus* is obtained as follows: dried rhizomes were left in contact with ethanol for 48 h. Of this extract 10 g were diluted with 20 ml of water and than extracted twice with 20 ml of petroleum ether. The organic phase were concentrate under low pressure and the residuum were diluted in benzene for obtaining a 1% solution.

2.2. Reagents

All solvents (hexane, benzene, toluene, ethyl acetate, and ethanol) were of chromatographic grade and were purchased from Merck (Darmstadt, Germany). α-asarone and β-asarone references and eugenol as internal standard were obtained by Roth (Karlsruhe, Germany).

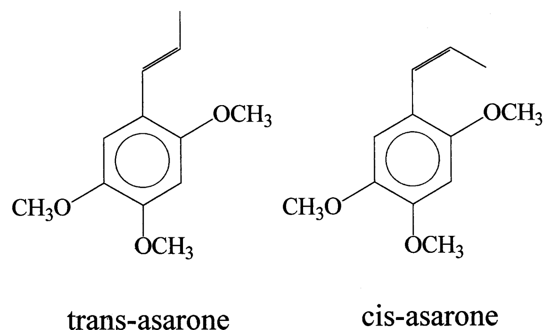

Fig. 1. Asarone isomers structures.

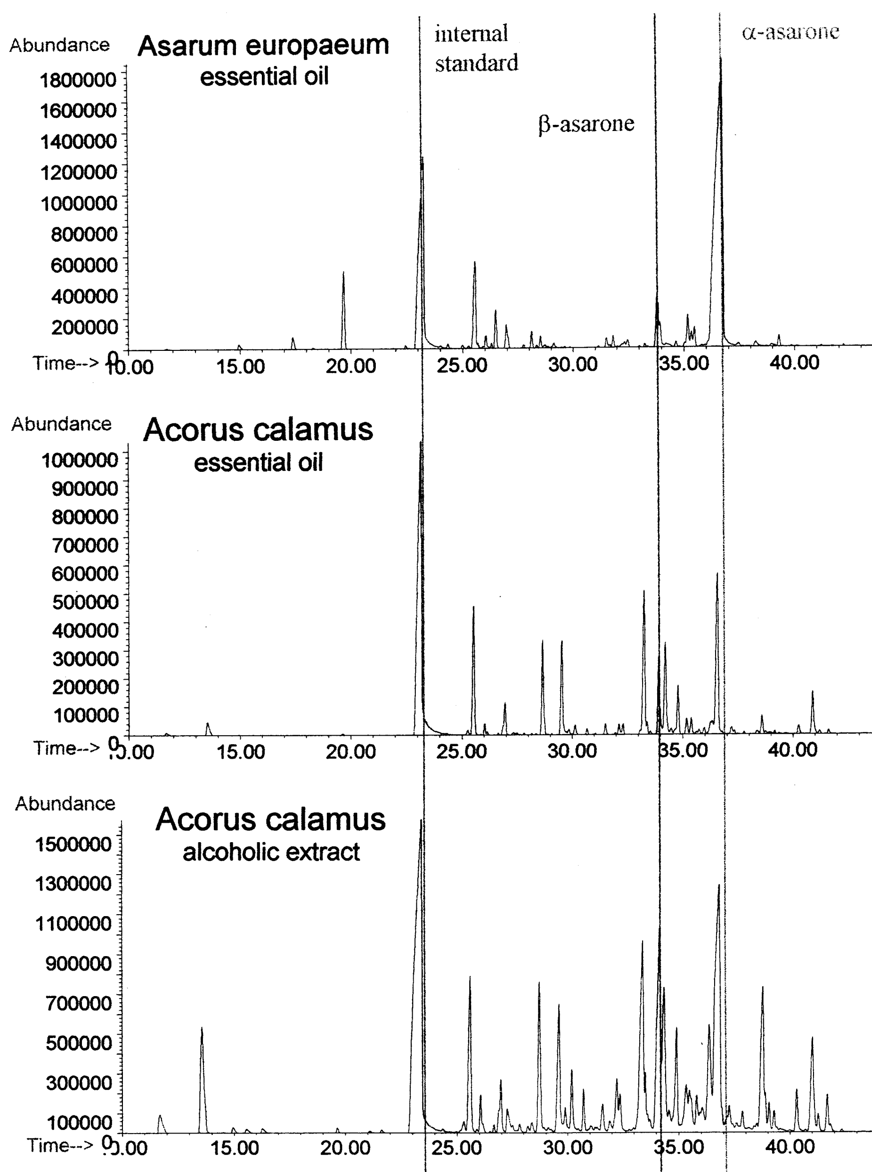

Fig. 2. Chromatograms of essential oils extracted from *Acorus calamus* L. and *Asarum europaeum* L.

2.3. Solutions

The reference solutions for GC-MS were obtained dissolving 1, 3, 5, 10 and 20 mg α- and, respectively, β-asarone in 20 ml hexane. Eugenol (1 mg) as internal standard was added and the solutions were making up to 100 ml with hexane. The reference solutions for TLC were obtained dissolving 1, 3, 5, 10 and 20 mg β-asarone in 1000 ml benzene.

The sample solutions of essential oils for GC-MS were prepared dissolving 5 mg essential oil in 20 ml hexane. Eugenol (1 mg) as internal standard was added and the solutions were making up to

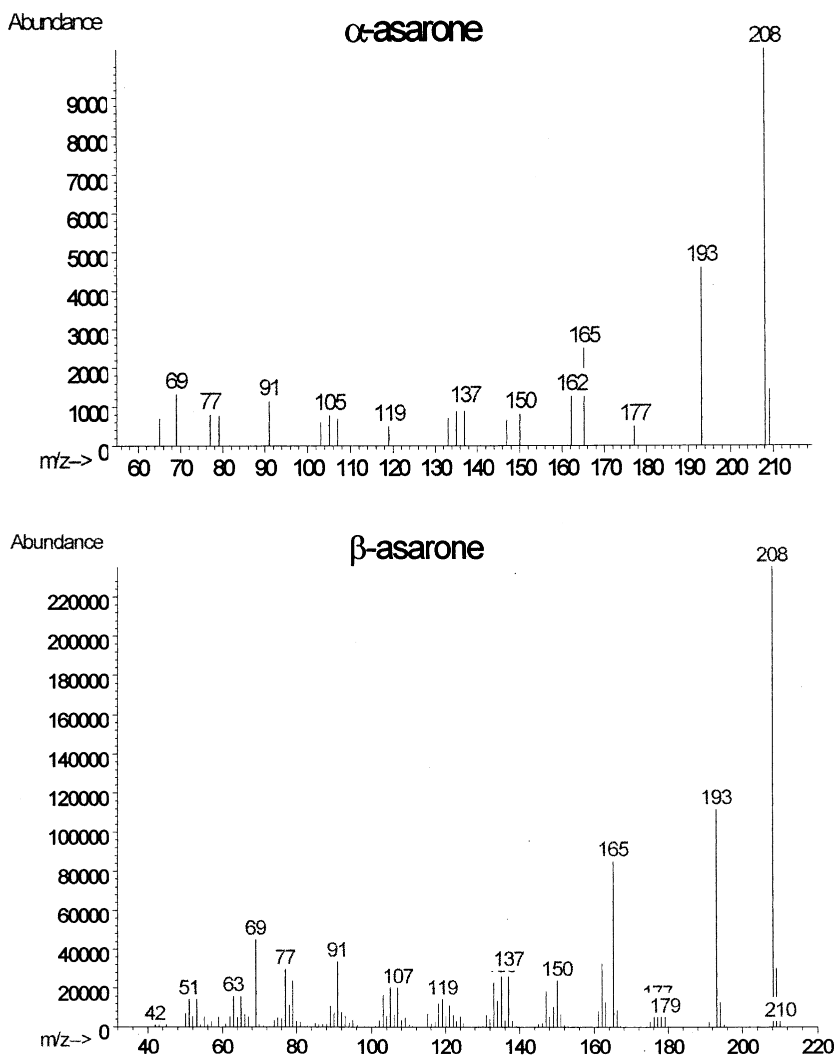

Fig. 3. Mass spectra of α- and β-asarone.

50 ml with hexane. The sample solutions for TLC were obtained dissolving 1 mg essential oil in 100 ml benzene.

2.4. Instrumentation

GC-MSD analyses were performed on a Hewlett–Packard 5890 series II-5972 MSD using a HP-MS 5 column, 0.26 mm i.d. × 30 m, 0.25 μm coating thickness. The GC was operated under the following conditions: manual injection; split 1:20; injector temperature, 250°C; carrier gas, He; flow, 1 ml min^{-1}; linear velocity, 36.4 cm s^{-1}; oven temperature programmed from 60 to 240°C at 3°C min^{-1}; detector temperature, 280°C; time run, 60 min. The MSD was operated under 70 eV; scan range 41–300 amu; scan, TIC and SIM. MSD were tuned before each injection using PFTBA (perfluorotributylamine) as tuning standard.

TLC analyses were performed on silica gel plates GF_{254} 10 × 10 cm (Merck, Darmstadt, Germany) using as mobile phase toluene–ethyl acetate (8:2). The spots were applied to the plates

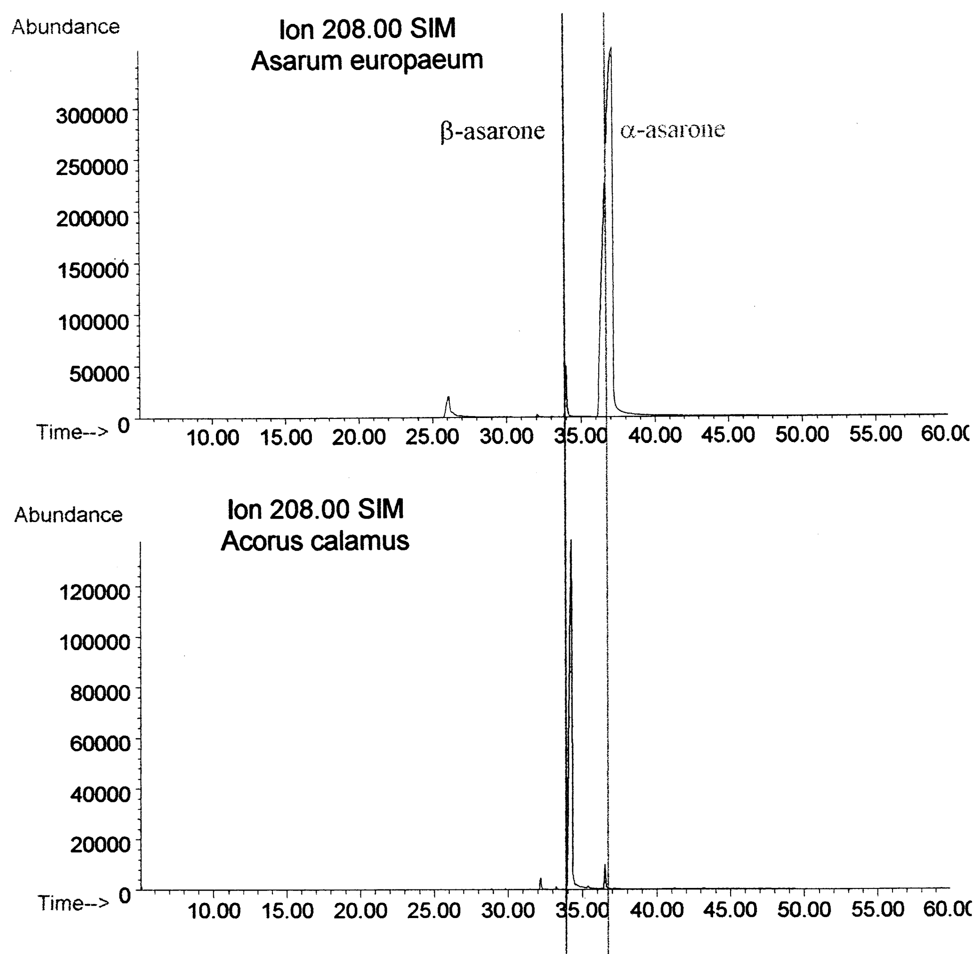

Fig. 4. GC-MS SIM of calamus and asarum essential oils.

using a Hamilton syringe 1 cm from the bottom edge of the plate. The plates were developed in standard separating chamber Desaga with chamber saturation for 1 h and the distance run was 7.5 cm. Evaluation was performed 'in situ' using a Desaga Densitometer CD 60. The measurement were performed in reflectance at 275 nm, slit 2×0.2 mm, step width 0.1 mm and in fluorescence, excitation wavelength 365 nm and measurement wavelength > 370 nm (edge filter), slit 2×0.2 mm, step width 0.1 mm. The essential oils and alcoholic extract were applied direct to the plate.

3. Results and discussion

3.1. GC-MS

The asarone isomers are well separated on the HP-5 column (Fig. 2). Their identification by means of the mass spectra is practically impossible, these being very similar (Fig. 3). For identification we used the Kováts retention index (I) calculated by means of a series of n-alkanes. The obtained results are within 1637–1651 for β-asarone and 1690–1695 for α-asarone.

The previous analyses concerning the composi-

Table 1
Determination of asarone isomers by GC-MS

Plant	Sample	α-Asarone (μg ml^{-1})		β-Asarone (μg ml^{-1})	
		TIC	SIM	TIC	SIM
Acorus calamus L.	Essential oil	6.5 ± 0.2	5.4 ± 0.2	93.1 ± 1.5	97.1 ± 0.9
Asarum europaeum L.	Essential oil	485 ± 25.6	489 ± 12.6	26.2 ± 1.6	28.0 ± 0.4
Acorus calamus L.	Alcoholic extract	2.8 ± 0.1	5.5 ± 0.2	94.5 ± 2.9	89.3 ± 1.4

tion of the essential oils obtained from *Acorus calamus* and *Asarum europaeum* of Romanian origin have proved that there do not contain eugenol, what suggested us to utilize them as internal standard, having in view the structural similarities with the asarone isomers. By injecting five samples of mixture eugenol:α-asarone:β-asarone 1:1:1 (w/w/w), the response factor has been found to be 1.01 ± 0.02. For the effected determinations, the response factor has been considered 1.

The chromatograms of standards have been measured in full scan. The calibration curves have been obtained for five standard solutions of different concentrations (0.01, 0.03, 0.05, 0.1, 0.2 mg ml^{-1}) of α-asarone and β-asarone respectively which contain eugenol as internal standard. The calibration graph of the standards integrated peaks related to those of the internal standard versus concentration of the two isomers has led us to linear calibration curves: $y = (101.72 \pm 2.5)x - 0.29 \pm 0.25$ ($R^2 = 0.92$) for α-asarone and $y = (93.803 \pm 0.9)x - 0.081 \pm 0.09$ ($R^2 = 0.97$) for β-asarone, respectively.

By SIM method one have monithorized the molecular ion m/z 208 characteristic of the asarone isomers by electronic impact at 70 eV (Fig. 4). The linear regression of the normalized inten-

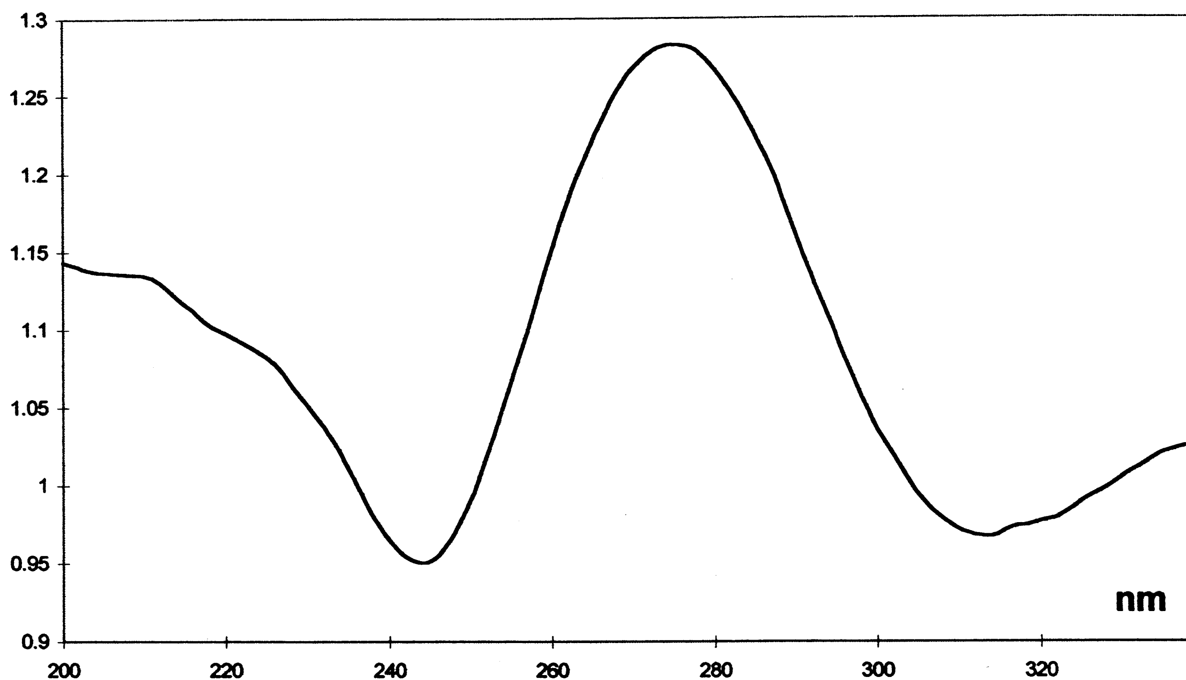

Fig. 5. Remission spectra of β-asarone.

Table 2
Determination of asarone by TLC

Plant	Sample	Asarone (μg ml^{-1})			
		UV spectrophotometry		Fluorescence	
		Height	Area	Height	Area
Acorus calamus L.	Essential oil	134 ± 6.6	136 ± 9.2	102 ± 2.5	102 ± 3.2
	Alcoholic extract	91 ± 11.2	111 ± 7.3	75 ± 2.4	92 ± 1.8

sity of the ion m/z 208 versus concentration of the five standard solutions (0.01, 0.03, 0.05, 0.1, 0.2 mg ml^{-1}) are represented by the equations $y = (16.709 \pm 0.21)x - 0.18 \pm 0.05$ ($R^2 = 0.95$) for α-asarone and $y = (16.063 \pm 0.29)x + 0.029 \pm 0.06$ ($R^2 = 0.91$) for β-asarone, respectively. The as-

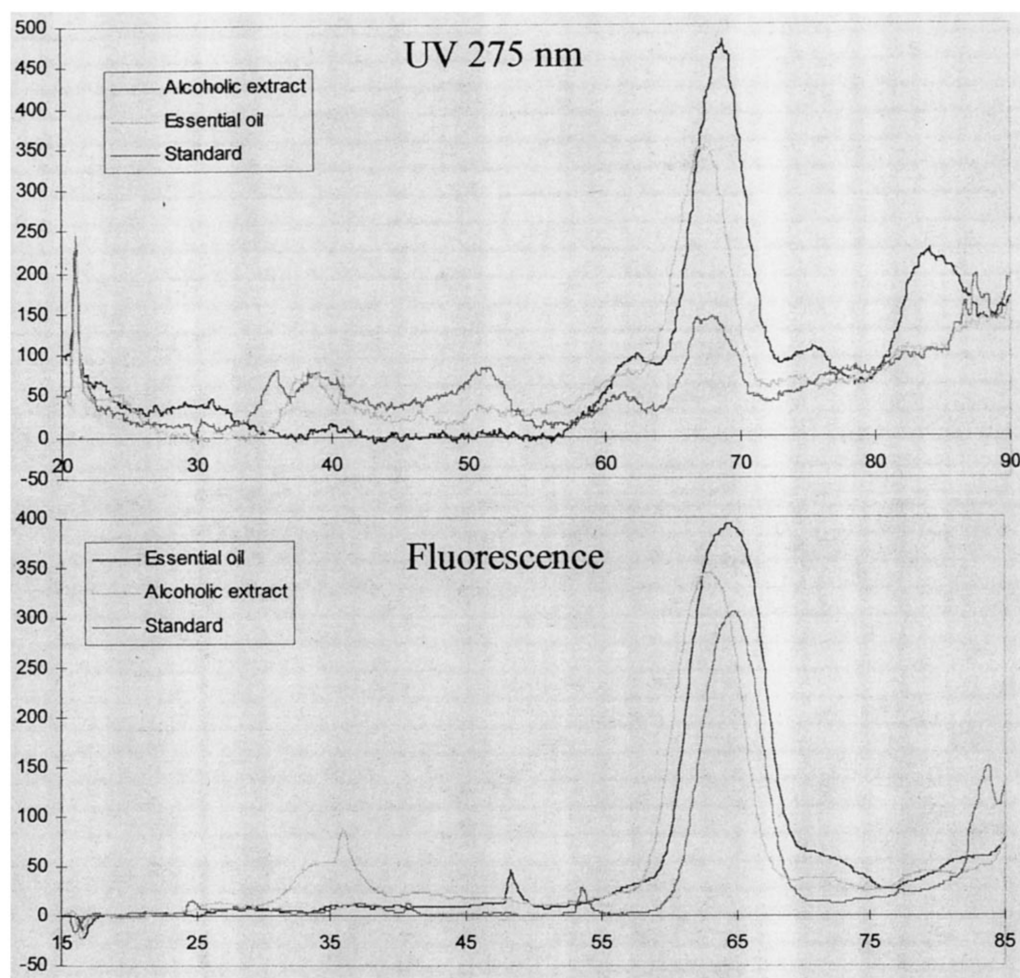

Fig. 6. TLC separation of components from *Acorus calamus* L. essential oil and alcoholic extract

arone isomers content of the samples (**I**), (**II**) and (**III**) is presented in Table 1.

The obtained results are congruent with the literature date concerning the composition of the essential oils of *Acorus calamus* and *Asarum europaeum* [6,7,10].

3.2. TLC

The asarone isomers could not be separated by chosen TLC method, that is why there were determined together. The calibration has been performed with five β-asarone solutions in benzene of the concentration 1, 3, 5, 10 and 20 μg ml^{-1}. The reflection spectra of β-asarone presents a peak at 275 nm (Fig. 5). Also, this exhibits a blue fluorescence by excitation of 365 nm. The chromatograms performed in reflectance and in fluorescence are presented in Fig. 6. The remission determination with external standard has been performed at 275 nm and for the calibration one has used both the heights and the areas of the separated peaks. The calibration equations calculated by least-squares regression according to Kubelka-Munk liniarisation are:

- remission
  $y = (31.948 \pm 1.12)x - 3.65 \pm 11.6$
  ($R^2 = 0.9963$) (height)
  $y = (85.568 \pm 1.35)x + 1.26 \pm 14.04$
  ($R^2 = 0.9992$) (area)
- fluorescence
  $y = (21.619 \pm 0.60)x - 1.64 \pm 6.22$
  ($R^2 = 0.9977$) (height)
  $y = (95.483 \pm 2.89)x - 16.48 \pm 29.9$
  ($R^2 = 0.9973$) (area)

The results obtained by interpolation of the heights and areas of the separated compounds with the same R_f with β-asarone are presented in Table 2.

Between results there are significant differences. This can be explained by the poorer resolution of the TLC separation than that of the GC-MS and the obtained peaks are not pure. Also, the results obtained by remission are about 20% higher than those obtained by fluorescence.

4. Conclusions and perspectives

The results of the asarone isomer determination from the *Acorus calamus* and *Asarum europaeum* essential oils confirm the literature data concerning their composition. The GC-MS method is precise owing to the good resolution of the capillary column. The TLC method is less exact and has the disadvantage of not being able to separate the isomers but it can be utilized for the rapid screening of the total quantity of asarone from vegetable extracts, food stuffs, drinks and drugs.

The following stages of the study will have in view solving the problems concerning the interferences contribution to the final results for the TLC method as well as the statistical evaluation of the results for the validation of the methods.

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