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Note

Reversed-phase high-performance liquid chromatography for the determination of β-asarone

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In drink and liquor formulations, aromatic substances play an important role because they introduce individual flavours that many affect consumer preferences. These aromatic substances originate mostly from natural sources (essential oils, extracts of roots, leaves, flowers, etc.), although these natural products are tending to be replaced with synthetic substances.

A natural aroma used in vermouths, bitters and liquors is calamus essential oil from *Acorus calamus*. This product is at present under close studied because recent investigations have revealed carcinogenic properties of one of its constituents, β-asarone (*cis*-2,4,5-trimethoxy-1-propenylbenzene). On account of this, the U.S. Food and Drug Administration has prohibited the use of calamus in any form (root, extract or oil) in any food or drug[1,2], and the Committee of experts for aromatic substances, including delegates from Great Britain, Belgium, Denmark, France, G.F.R., Italy, The Netherlands, Sweden, Switzerland, have recently fixed 0.1 mg/kg as the maximum content of β-asarone in foods and soft drinks and 1 mg/kg in alcoholic beverages.

Methods for the determination of β-asarone include gas–liquid chromatography[3–5] and thin-layer chromatography with densitometric valuation of the spots[6] and spectrofluorimetry[7]. These methods are unsatisfactory owing to the possibility of interferences, which are difficult to eliminate even with complex sample purification procedures; further, the sensitivity is not adequate for the requirements imposed by the above limits, which require the availability of very sensitive methods. The method described in this paper combines the high separation efficiency of high-performance liquid chromatography (HPLC) with the sensitivity and selectivity of the fluorimetric detector and allows the simple and rapid determination of β-asarone at concentrations down to 0.001 mg/l.

EXPERIMENTAL

A Perkin-Elmer Model 601 liquid chromatograph equipped with two 3000-p.s.i. pumps was used. For sample introduction a Rheodyne 7120 injection valve with a loop of 10 μl was used. The liquid chromatograph was connected in series with a spectrophotometric and a spectrofluorimetric detector. The spectrophotometric detector (Perkin-Elmer Model LC 55 UV-Vis, single beam with variable wavelength) was

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connected with a Perkin-Elmer Model 123 recorder and with the LC 55-S scanner for recording the spectra. The spectrofluorimetric detector was a Perkin-Elmer Model MPF 3 with a microcell of 20 μl (063-0547) equipped with a Perkin-Elmer Model 56 recorder. For spectrophotometric detection a wavelength of 254 nm was used, and for spectrofluorimetric detection an excitation wavelength of 310 nm and an emission wavelength of 355 nm were used.

The chromatographic separations were carried out with a stainless-steel column (25 cm × 2.6 mm I.D.) packed with octadecylsilane (HC-ODS/Sil-X, Perkin-Elmer, n 089-0716).

Reagents

All solvents were of analytical-reagent grade. Hexane (Carlo Erba, Milan, Italy) and absolute methanol (J. T. Baker, Phillipsburgh, NJ, U.S.A.) were used without further purification.

The mobile phase was methanol–water (6.2:3.8). The solvents were preliminarly filtered with Millipore filters. Pure α-asarone and β-asarone were supplied by C. Roth (Karlsruhe, G.F.R.).

A calibration graph was prepared by dilution of a standard solution containing 1 mg/ml of β-asarone in methanol. The solution remained stable for many months.

Preparations of samples

A 100-ml volume of an alcoholic beverage was placed in a 500-ml flask together with 200 ml of deionized water, the mixture was distilled and 250 ml of distillate were collected. The distillate was transferred into a separating funnel and 250 ml of saturated sodium chloride solution were mixed with it. Extraction was effected with two 25-ml volumes of n-hexane and the combined n-hexane phases were extracted with 20 ml of 1 *M* sodium hydroxide solution, 20 ml of 1 *M* hydrochloric acid and twice with 20 ml of water, in that order.

The n-hexane extract was dried with sodium sulphate and evaporated to dryness at room temperature under reduced pressure on a rotary evaporator. The residue was dissolved in 3 ml of methanol. The resulting solution was filtered on a FHLP 01300 Millipore filter directly into a glass vessel which was hermetically sealed via a perforable rubber septum. The sample was then ready for chromatographic analysis.

Chromatographic analysis

The analysis was carried out by reversed-phase HPLC, injecting 10-μl volumes of the sample by means of the injection valve, and by eluting with methanol–water (6.2:3.8). The column was thermostated at 65 °C. The flow rate was 0.75 ml/min at a pressure with of 1000 p.s.i.

β-Asarone was identified by comparing the chromatographic behaviour of the samples with that of a standard solution of β-asarone. Confirmation was obtained determining the excitation and emission spectra directly on the eluted fraction kept in the microcell, using the stopped-flow method.

The amount of β-asarone in the samples was obtained by interpolation on a calibration graph obtained by plotting the height of the β-asarone peak against the amount injected. A linear response of the detector was observed up to 5 mg/l.

TABLE I

RECOVERY OF β-ASARONE

Sample No.	β-Asarone (mg/l)				Recovery (%)
	Present	Added	Total	Found	
15	0.910	0.153	1.063	1.064	100.6
9	0	0.153	0.153	0.155	101.3
10	0	0.256	0.256	0.277	108.0
16	0	0.051	0.051	0.052	102.0
17	0	0.102	0.102	0.102	100.0
13	0.140	0.051	0.191	0.195	107.0
*	0	0.101	0.101	0.111	108.0
*	0	0.153	0.153	0.158	103.0

* Aqueous alcoholic solution.

The calibration graph was checked every time immediately before the analysis of a sample.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained for a standard mixture of α- and β-asarone and for two samples of alcoholic drinks. For comparison, Fig. 1 also shows the chromatograms obtained simultaneously with the spectrophotometric detector connected in series with the spectrofluorimetric detector.

Comparison of the graphs clearly shows the superiority of the fluorimetric detector and indicates that the spectrophotometric detector cannot be used for the determination of β-asarone owing to its insufficient sensitivity and the presence of numerous interfering substances at 254 nm.

In order to evaluate the reproducibility accuracy of the proposed method, to the samples of commercial drinks that had previously been analysed for their

TABLE II

β-ASARONE CONCENTRATIONS DETERMINED IN COMMERCIAL SAMPLES

Sample		β-Asarone (mg/l)	Sample		β-Asarone (mg/l)
Type	No.		Type	No.	
Vermouth	1	0.018	Bitters	11	0.006
	2	0.005		12	0.003
	3	Absent		13	0.140
	4	Not measurable		14	0.004
	5	0.002		15	0.910
	6	Not measurable		16	Absent
	7	0.003			
	8	0.021	Alcoholic aperitif	17	Absent
	9	Absent			
	10	Absent			

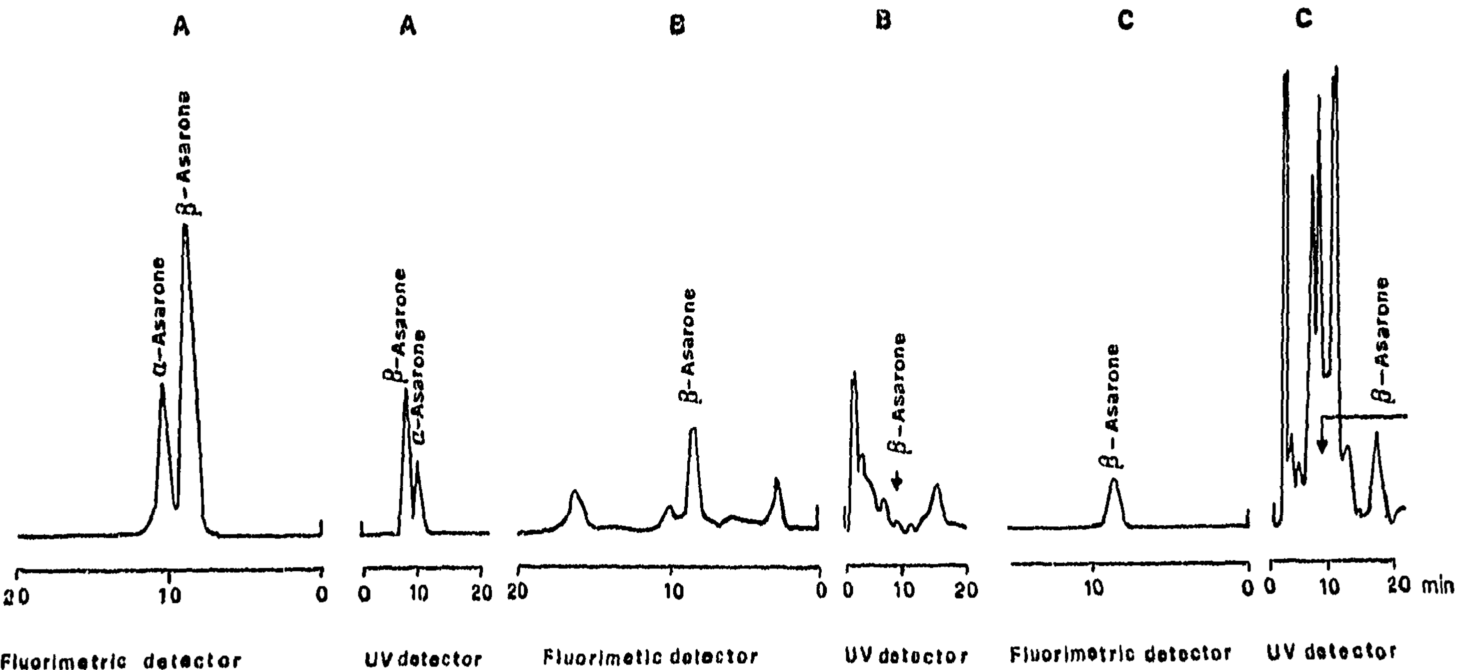

Fig. 1. Chromatograms obtained by spectrophotometric and spectrofluorimetric detectors connected in series: (A) standard α- and β-asarone mixture; (B) and (C) different samples of alcoholic beverages. The arrow shows the retention time of β-asarone. Column, HC-ODS/Sil-X (25 × 0.26 cm I.D.); mobile phase, methanol-water (6.2:3.8); flow rate, 0.75 ml/min; pressure, 1000 p.s.i.; temperature, 65°C; detection, UV (254 nm), fluorimetric (λ_{exc} 310 nm, λ_{em} 355 nm).

β-asarone contents were added known and variable amounts of pure β-asarone. The fortified samples were analysed using the described method. The results are summarized in Table I.

In Table II the β-asarone concentrations obtained by analysing some samples of vermouth and bitters purchased from local commercial sources are reported. The reported values are the averages of two determinations.

As is readily apparent from Table II, β-asarone was absent from or not measurable in 7 of the 17 examined samples; in two samples the β-asarone content was higher than 0.1 p.p.m., but in the remaining samples it was below this value.

The method was also applied to the analysis of a sample of European calamus oil, a solution in methanol ($1:2\cdot10^4$) being injected directly into the column. The β-asarone content found was 7.56 g per 100 ml.

The chromatogram obtained by both the fluorimetric and the spectrophotometric detectors is shown in Fig. 2. Also in this instance the complete separation of the two isomers is evident and the advantage of the spectrofluorimetric detector is also apparent. Confirmation of the peaks was obtained by the stopped-flow technique, measuring both excitation and emission fluorescence spectra and using "UV scanning" with the LC 55 spectrophotometer. Fig. 3 shows the UV spectra obtained for the two peaks of α- and β-asarone.

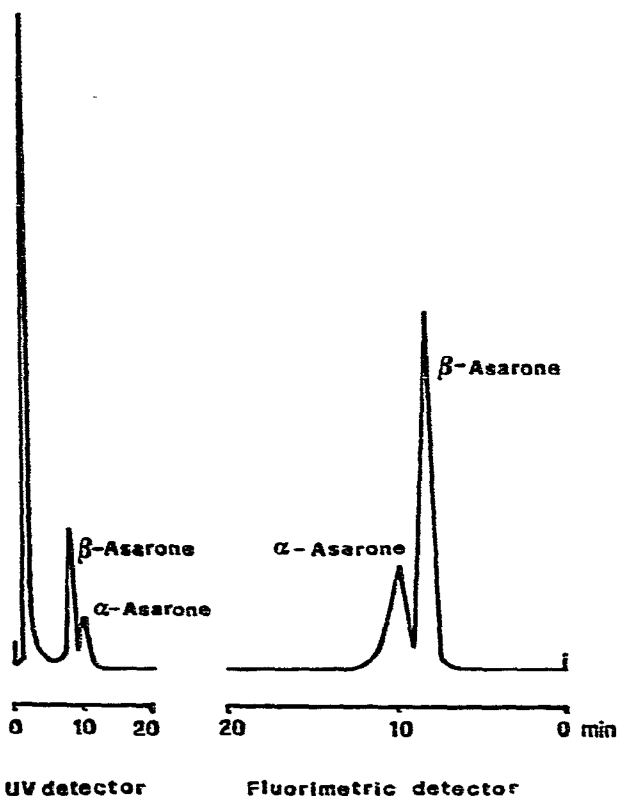

Fig. 2. Chromatograms of calamus oil in methanol ($1:2\cdot10^4$). Conditions as in Fig. 1.

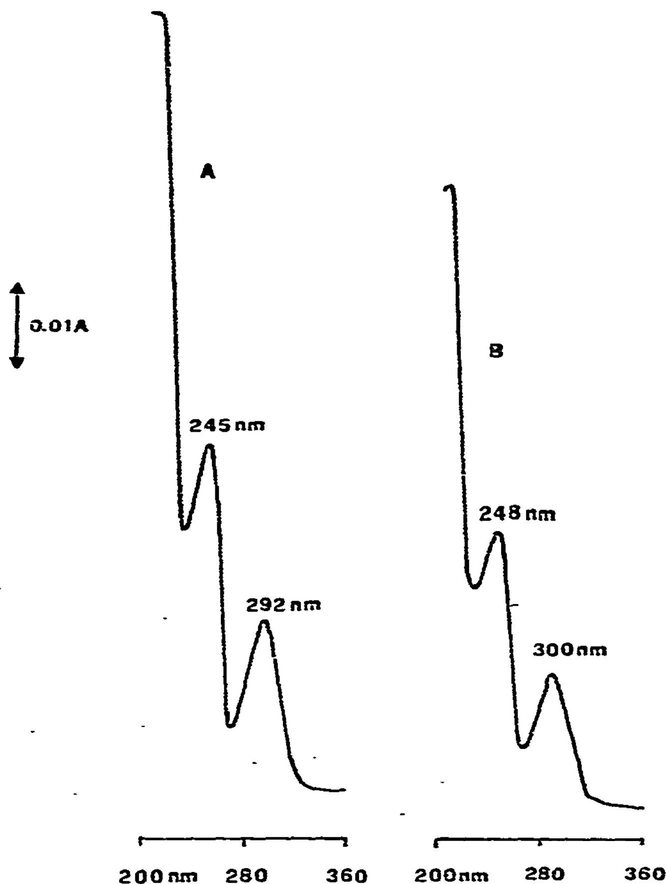

Fig. 3. UV spectra of β-asarone (A) and α-asarone (B) obtained by the stopped-flow technique on a calamus oil sample.

CONCLUSION

The obtained results show that the described method is rapid, accurate, reproducible and selective. The use of a fluorimetric detector enhances the sensitivity and lowers interferences, making possible the determination of β-asarone in complex mixtures.

ACKNOWLEDGEMENT

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