Journal of Chromatography, 219 (1981) 332–337
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,186

Note

High-performance liquid chromatographic analysis of carvacrol and thymol in the essential oil of *Thymus capitatus*

V. SOLINAS* and C. GESSA
Istituto di Chimica Agraria, Università di Sassari, Sassari (Italy)
and
L. FALCHI DELITALA
Cattedra di Botanica, Facoltà di Medicina Veterinaria, Università di Sassari, Sassari (Italy)
(First received May 11th, 1981; revised manuscript received June 22nd, 1981)

Phenolic compounds are widely distributed in plants, but their functions are not yet known[1–3]. Most of them are formed via the shikimic acid pathway, which leads also to amino acids (phenylalanine, tryptophan), growth substances (indole acetic acid) and secondary products such as lignins, coumarins, flavones and anthocyanins. The metabolism of phenolic substances is related to phenylalanine ammonialyase (PAL), the activity of which is affected by external and internal factors. In particular, the different amounts of thymol in the essential oil of *Thymus serrulatus* grown in different environments has been related to water supply[4].

In *Thymus capitatus* the phenolic fraction, which is the main component of the essential oil (60–90%), comprises only two isomeric phenolics, carvacrol (2-*p*-cymenol) and thymol (3-*p*-cymenol), whose synthesis can be related to external factors[5]. Several researchers have attempted to separate and quantitate these two isomers by using gas-chromatographic (GC) or chemical methods in which phenolics were first separated from non-phenolics by alkaline extraction[6–10]. The results obtained were not satisfactory because the chromatographic peaks were not completely resolved and/or the alkaline extraction was not quantitative. Recently, the use of glass capillary columns in GC has allowed a very efficient separation of these two phenols[11].

For the same purpose, we have developed a fast and accurate method involving reversed-phase high-performance liquid chromatography (HPLC), which has already proved successful in the separation of different phenolics[12–16].

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph consisted of: two Waters Assoc. Model 6000 pumps, a Model U6K universal injector, and a Model 660 solvent programmer; two columns, (1) 300 × 3.9 mm I.D., packed with μBondapak C_{18} (Waters Assoc.), (2) 250 × 4.6 mm I.D., RP-18 (Brownlee Labs.) packed with 10-μm LiChrosorb (E. Merck); a variable-wavelength detector, Perkin-Elmer LC-55 UV–visible spectrophotometer with a 8-μl sample cell; a Leeds & Northrup Speedomax XL 68 Mark II recorder.

0021-9673/81/0000–0000/$02.50

Reagents

A carvacrol and thymol standard was obtained from Fluka. The solvents were acetonitrile RS and methanol RS for HPLC (Carlo Erba, Milan, Italy). Twice distilled water was filtered through a 0.2-μm Millipore system. The phenolic standard solutions were 200 ppm carvacrol and thymol in methanol. Acetonitrile–water (40:60) was employed as mobile phase.

Extraction of essential oil

The essential oil of *T. capitatus* was extracted by steam distillation at atmospheric pressure and then separated from the aqueous phase with petroleum ether. The ethereal phase was washed with water, dried over anhydrous Na_2SO_4 and the solvent removed in a rotary evaporator at low pressure and 30°C.

Separation of "phenolic fraction"

The phenolic fraction was separated by the alkaline method[9,10] shown schematically in Fig. 1. The yields of phenolic and non-phenolic fractions from 2 g of essential oil were 1.55 g and 0.45 g, respectively.

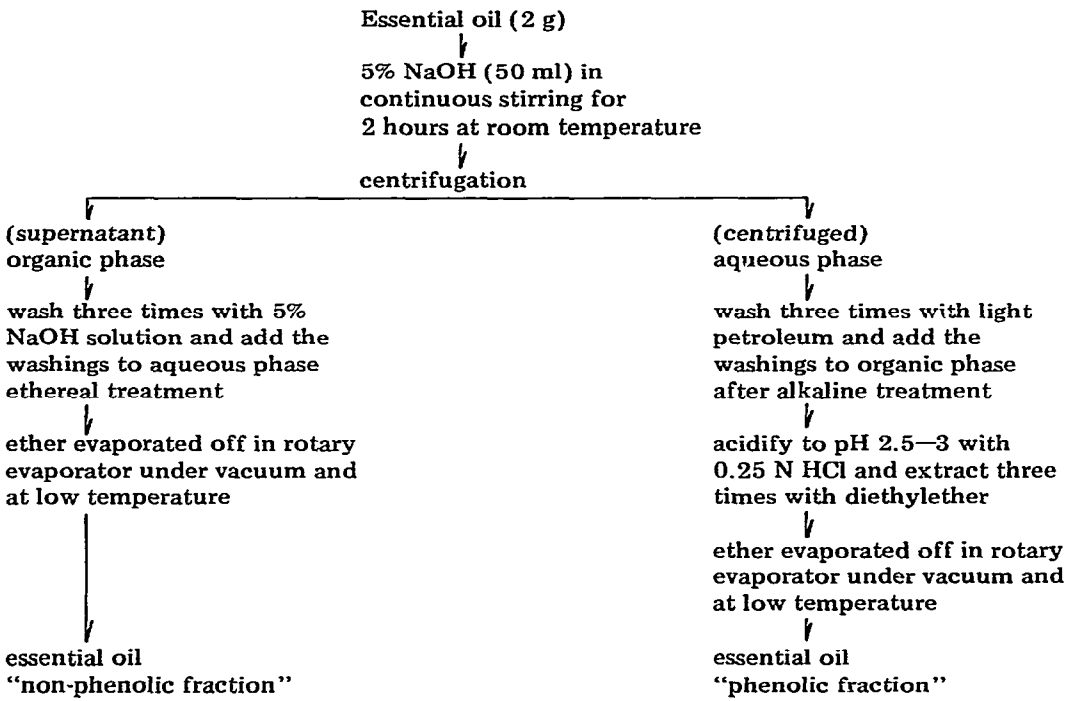

Fig. 1. Flow diagram illustrating the alkaline separation of the "phenolic fraction" from the "non-phenolic fraction" of the essential oil.

Determination

In order to find the best chromatographic conditions for separation of the two isomers, the retention time, t_R, capacity factor, k', relative retention, α, and resolution, R_s, were calculated for the two columns tested (Table I).

TABLE I

RETENTION TIMES, t_R, CAPACITY FACTORS, k', RELATIVE RETENTIONS, α, AND RESOLUTIONS, R_s, OF CARVACROL AND THYMOL ON μBONDAPAK C_{18} AND RP-18 USING WATER–ACETONITRILE AS ELUENT

Compound	*μBondapak* C_{18}				*RP-18*			
	t_R *(min:sec)*	k'	α	R_s	t_R *(min:sec)*	k'	α	R_s
Carvacrol	17:10	7.55	1.14	1.45	17:50	7.75	1.12	1.39
Thymol	19:30	8.65			19:40	8.70		

Calibration curves were constructed by using standard solutions containing different ratios of the two phenols and plotting the amount of pure component against the peak area (Fig. 2). Each point of the curve was calculated as the average value from three injections. Good results were also obtained by plotting the amount of each compound against the heights or weights of the peaks, as demonstrated by the correlation coefficients calculated by using a simple regression equation.

For the quantitation of the two phenolics, solutions in methanol at different concentrations (200 and 2000 ppm) of the essential oil and of the two fractions obtained by alkaline extraction were subjected to HPLC.

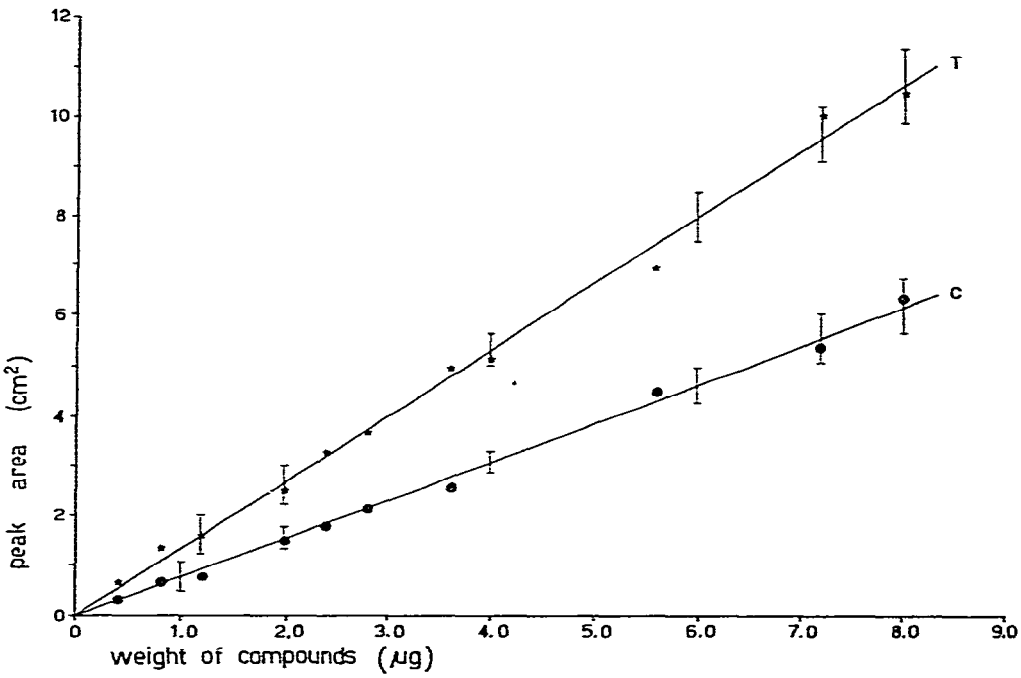

Fig. 2. Calibration graphs for carvacrol (C) and thymol (T). Each point shown is the average from three determinations. The lines indicate the 95% confidence limits for some points on the calibration curves.

RESULTS AND DISCUSSION

The HPLC separation of the two isomers carvacrol and thymol was attempted in reversed-phase mode because of the low polarity and solubility of both chemicals in water. Excellent results were obtained when a μBondapak C_{18} column, an acetonitrile–water (40:60) mobile phase and a flow-rate of 1.5 ml/min were used. No improvements were achieved by varying either the flow-rate or the mobile phase composition. The analysis time and the resolution of two bands decreased as the acetonitrile content increased. The analysis time was unnecessarily long and resolution did not improve by increasing the water content.

No appreciable variations were obtained under the same chromatographic conditions but with the RP-18 column, the packing of which is somewhat more retentive than that of μBondapak C_{18}. The two columns had approximately equal resolution powers for the two isomeric phenolics as demonstrated by the α parameter, 1.12 on RP-18 and 1.14 on μBondapak C_{18} (Table I).

Thus, all results reported are those obtained with μBondapak C_{18}.

The correlation coefficients between the amount of compound and the area, height or weight of the peaks (Fig. 2) indicate linearity over the investigated concentration ranges (0–8 μg).

Some chromatograms of mixtures of carvacrol and thymol at different ratios are reported in Fig. 3. The two isomers are well separated as demonstrated by the R_s values (about 1.4), which indicate less than 1 % overlap of bands. From the same tests it is possible to calculate the detection limits under these chromatographic conditions as the amount of sample which gives a response equal to the noise of the detector[17]: these are 0.02 μg for carvacrol and 0.15 μg for thymol.

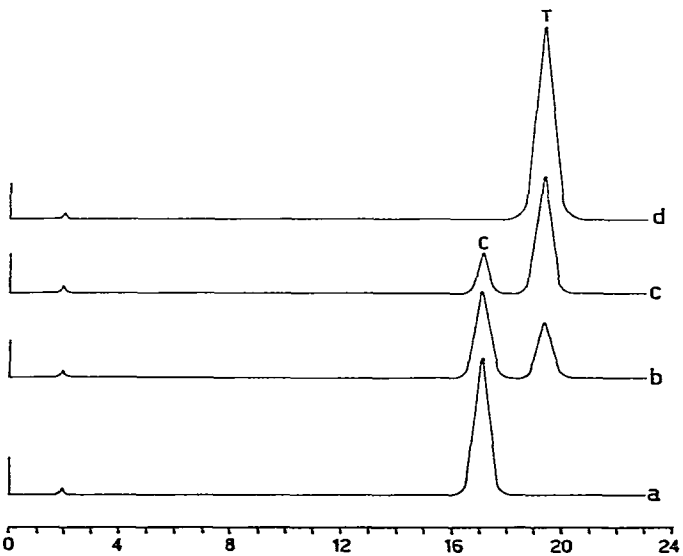

Fig. 3. HPLC chromatograms of 8-μg mixtures of carvacrol (C) and thymol (T) in different ratios: a, 100% C; b, 70% C + 30% T; c, 30% C + 70% T; d, 100% T. Conditions: mobile phase, water–acetonitrile (60:40); column, μBondapak C_{18}, 300 × 3.9 mm I.D.; flow-rate, 1.5 ml/min; pressure, 700–1000 p.s.i.; detector, UV at 283 nm.

In order to check the effectiveness of the alkaline extraction, equal amounts of the "non-phenolic fraction" and of the "phenolic fraction", obtained according to Fig. 1, were injected. The respective chromatograms (Fig. 4) show that the two fractions have about the same percentage of phenolics. This demonstrates that the alkaline extraction is not quantitative, as already pointed out[10], and that the phenols present in the essential oil of *T. capitatus* are partitioned between the aqueous and organic phases, with a preference for the former. Consequently, in this case, the alkaline extraction should be avoided if the exact content of phenolics is to be determined.

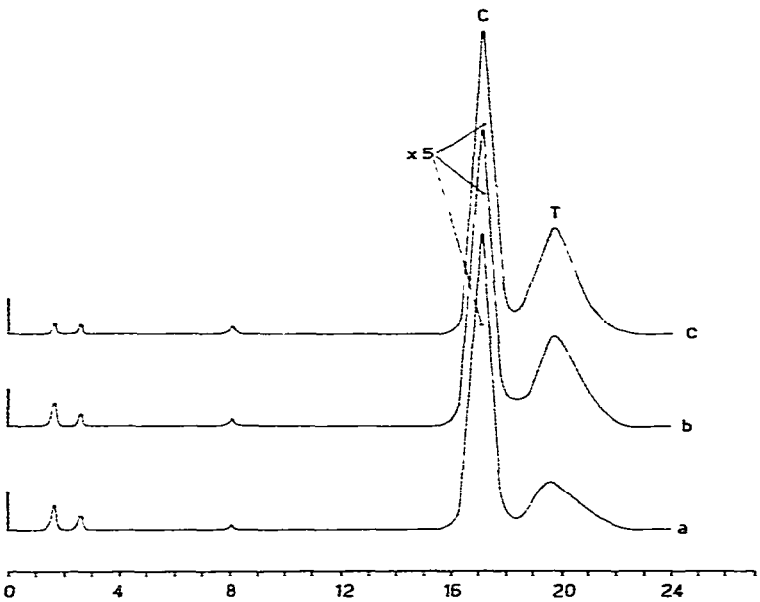

Fig. 4. HPLC chromatograms of 80 μg of the "non-phenolic fraction" (a), "phenolic fraction" (b) and "essential oil" (c) of *Thymus capitatus*. Peaks: C = carvacrol; T = thymol. Conditions as in Fig. 3.

A chromatogram of the essential oil which was not subjected to alkaline extraction is also shown in Fig. 4. A large amount of oil (80 μg) was injected, in order to reveal the thymol, which is present in very small amount in comparison with carvacrol. The separations were not as good as those obtained in calibration tests, as demonstrated by the lower R_s value (about 1.0). Nevertheless, this value indicates that the separation is still efficient and that reasonable estimations of the contents can be made.

On the other hand, the retention times did not change in the concentration range examined.

Only the thymol was quantitated from the chromatogram in Fig. 4c, whereas carvacrol was determined from a chromatogram (not shown) obtained by injecting 8 μg of the essential oil.

By means of the calibration graphs, it has been calculated that 94% of the essential oil of *T. capitatus* is constituted by the "phenolic fraction", of which 91% is carvacrol and 9% is thymol.

In conclusion, the present study has demonstrated that:

(1) the sample of the essential oil must be injected without alkaline separation,

(2) the elimination of preliminary purification and separation steps notably shortens the analysis time and reduces the risk of sample loss,

(3) HPLC allows the separation and quantitation of phenolic compounds, even of isomers, in some essential oils.

REFERENCES

1 R. G. S. Bidwell, *Plant Physiology*, Macmillan, New York, 1979.
2 M. B. Wilkins (Editor), *The Physiology of Plant Growth and Development*, McGraw-Hill, London, 1969.
3 P. M. Smith, *The Chemotaxonomy of Plants*, Edward Arnold, London, 1976.
4 P. Rovesti, *Riv. Ital. Essenze, Profumi Piante Off.*, 5 (1958) 215.
5 J. Tucakov, *Riv. Ital. Essenze, Profumi Piante Off.*, 8 (1964) 376.
6 C. Runti and G. Bruni, *Boll. Chim. Farm.*, 99 (1960) 435.
7 L. Falchi Delitala, *Boll. Chim. Farm.*, 105 (1966) 873.
8 R. Granger, J. Passet and R. Verdier, *C.R. Acad. Sci. (Paris)*, 258 (1964) 5539.
9 Y. R. Naves, *La France et ses Parfums*, 2 (1959) 23.
10 A. P. Burchfield and E. E. Storrs, *Biochemical Applications of Gas Chromatography*, Academic Press, New York, 1962.
11 J. D. Miquel, H. M. J. Richard and F. G. Sandret, *J. Agr. Food Chem.*, 24 (1976) 833.
12 W. A. Court, *J. Chromatogr.*, 130 (1977) 287.
13 J. F. K. Huber, in E. L. Johnson and R. Stevenson (Editors), *Basic Liquid Chromatography*, Varian, Palo Alto, CA, 1972, pp. 9–40.
14 R. L. Rouseff, *Trends in Fluorescence*, 1 (1978) 10.
15 W. M. Walter, A. E. Purcell and G. K. McCollum, *J. Agr. Food. Chem.*, 27 (1979) 938.
16 L. W. Wulf and C. W. Nagel, *J. Chromatogr.*, 116 (1976) 271.
17 C. F. Simpson, *Practical High Performance Liquid Chromatography*, Heyden & Son, New York, 1976.