Pergamon 0031-9422(94)00657-1 Phytochemistry, Vol. 38, No. 2, pp. 391–396, 1995
Elsevier Science Ltd
Printed in Great Britain
0031 9422/95 $9.50 + 0.00

CHEMICAL POLYMORPHISM OF THE ESSENTIAL OIL OF *THYMUS CARNOSUS* FROM PORTUGAL*

LIGIA SALGUEIRO, ROSER VILA,†‡ XAVIER TOMAS,§ FELIX TOMI,¶ SALVADOR CAÑIGUERAL,‡ JOSEPH CASANOVA,¶ ANTONIO PROENÇA DA CUNHA and TOMAS ADZET‡

Laboratorio de Farmacognosia, Faculdade de Farmacia, Universidade de Coimbra, Rua do Norte, 3000-Coimbra, Portugal; ‡Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Universitat de Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain; §Departament de Quimiometria, Institut Químic de Sarrià, E-08017 Barcelona, Spain; ¶Laboratoire d'Hélioénergétique, URA CNRS 877, Université de Corse, Route des Sanguinaires, 20000-Ajaccio, France

(*Received in revised form* 14 *June* 1994)

Key Word Index—*Thymus carnosus*; Lamiaceae; essential oil; GC-MS; ^{13}C NMR; multivariate analysis; infraspecific variability.

Abstract—The composition of the essential oils of 11 populations of *Thymus carnosus* from Portugal and their infraspecific variability were investigated by GC, GC-MS and ^{13}C NMR. The results obtained were submitted to Principal Component and Chemometric Cluster Analyses. Borneol was the main constituent in all the populations except in one, which had a high content of linalool. This compound showed high percentages in samples originating from the region of Estremadura. Multivariate analysis allowed the distinction between three different groups of essential oils, (i) borneol/*cis*-sabinene hydrate/terpinen-4-ol, (ii) linalool/borneol/*trans*-sabinene hydrate and (iii) borneol/camphene.

INTRODUCTION

The infraspecific variability of the essential oils of the genus *Thymus* has been the object of previous works of our research groups [1–5] and has been recently reviewed by Stahl-Biskup [6].

As part of an exhaustive investigation of the chemical polymorphism of the essential oils of *Thymus* species from Portugal, carried out by the Laboratory of Pharmacognosy of the University of Coimbra, we report herein the results of the investigation of several populations of *T. carnosus*. This species is endemic to south western Iberia, growing in coastal sands, mainly localized in Huelva (Spain), Algarve, Baixo Alentejo and Estremadura (Portugal). While the composition of the volatile oil of populations of Spanish origin has been previously reported [7, 8], only carvacrol has been mentioned in the taxon from Portugal [9].

Qualitative and quantitative analysis of the essential oils of representative samples of each population were performed by GC, GC-MS and ^{13}C NMR. The latter has proved to be a useful technique in the investigation of the composition of essential oils [10], particularly in the identification of stereoisomers [11] and compounds that are poorly separated by GC, as well as in the field of chemical polymorphism [5]. On the basis of previous work [12], we have developed a technique which allows the identification of the main compounds of an essential oil (detection limit: 0.5–1%) by ^{13}C NMR, without previous separation. This identification is performed by computer-aided analysis of the ^{13}C NMR spectrum by comparing the signals obtained in the spectrum with those of pure compounds included in a library created in our laboratory [10].

In a second step, to study the infraspecific variability, the oil of individual plants of each population was analysed by GC and the results obtained were submitted to a Chemometric Cluster and Principal Component Analyses. A Principal Component Analysis (PCA) was performed to establish the discriminating power for selected oil constituents. The first objective of PCA [13] is to identify what constituents are involved with the main dispersion of values of the whole sample data set. So, if one or more patterns are formed, differences between individuals or groups of individuals can be possibly explained in terms of those few constituents. Cluster Analysis [14] evaluates the similarity of samples referred to the whole constituents data set. If some cluster is detected, it is possible to study which characteristica are responsible of such a group of similar samples, e.g. genetics, location, climate, etc., as well as to define its main chemical pattern composition. In addition, coherence in the achieved results obtained by both independent

*Part of results presented as a poster in the 12èmes Journées Internationales des Huiles Essentielles, Digne les Bains. France, September 1993.

†Author to whom correspondence should be addressed.

techniques can validate the conclusions of that chemometric study.

RESULTS AND DISCUSSION

Analysis of essential oil

The average yield of essential oil of the air-dried aerial parts of the 11 populations of *T. carnosus* investigated was 1.9% (v/w). The qualitative and quantitative analytical results are shown in Table 1. More than 94.5% of the volatile oil was identified in each sample, a total of 74 components being identified. Oxygenated monoterpenes were the main group of constituents in all populations. Borneol was the major compound in all samples, ranging from 18.6 to 32.0%, except for sample E, in which linalool had a higher percentage than borneol (25.5 and 15.0%, respectively).

Samples E, G and H were characterized by their high linalool content, 25.5, 15.8 and 9.5%, respectively, compared with the other populations in which the highest percentage detected was 0.4%. This result is of special interest, because these three samples come from the region of Estremadura, while the others have other origins.

Other important constituents in the essential oils were *trans*-sabinene hydrate (2.0–17.0%), camphene (2.5%–13.0%), *cis*-sabinene hydrate (2.5%–11.2%) and terpinen-4-ol (4.0–11.1%).

Infraspecific variability

Results of PCA of the whole data set show a suitable projection of samples in the space formed by the three first principal components, because 83.8% of the total variance is retained in this projection. Correlation between constituents and these three principal components indicates the specific discriminant power of linalool, borneol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, terpinen-4-ol and camphene.

From all the individual samples submitted to multivariate analysis, three well-defined groups of essential oils were differentiated by both the Cluster Analysis and PCA (Figs 1 and 2, respectively). Cluster I: those characterized by a high percentage of borneol, *cis*-sabinene hydrate and terpinen-4-ol; 54.2% of the samples analysed. Cluster II: those having linalool, borneol and *trans*-sabinene hydrate as major constituents; 9.6% of samples analysed. Cluster III: those containing a high content of borneol and camphene; 36.2% of samples analysed.

The mean chemical composition of the essential oil of each cluster is presented in Figs 3–5. As indicated by PCA, the main differences are in the contents of linalool, borneol, *cis*-sabinene hydrate, *trans*-sabinene hydrate, terpinen-4-ol and comphene.

Within clusters I and III, samples of different geographic origin are included. In cluster II, all the individuals which are characterized by their content in linalool were collected in the region of Estremadura, which is in accordance with the results found in the analysis of the

Table 1. Constituents of essential oils of Portuguese populations of *Thymus carnosus*

Components	Per cent in samples										
	A	B	C	D	E	F	G	H	I	J	K
Monoterpene hydrocarbons	23.7	23.8	28.7	30.7	12.1	32.6	20.8	25.7	28.8	35.0	46.6
Tricyclene	0.2	0.3	0.3	0.4	t	0.6	0.2	0.2	0.5	0.5	0.9
α-Thujene*	1.4	1.1	2.7	2.5	0.5	2.6	1.0	2.1	1.1	1.9	2.8
α-Pinene*	2.0	2.5	3.0	4.0	1.5	5.1	2.9	3.1	3.9	5.3	6.5
Camphene*	7.0	6.0	8.1	7.0	2.5	9.9	5.5	6.8	7.4	11.5	13.0
β-Pinene*	1.1	1.6	1.9	2.1	0.3	2.7	1.0	1.6	1.8	2.3	3.6
Sabinene*	0.9	0.9	1.4	1.4	0.3	1.8	0.8	1.4	2.0	1.4	2.1
Myrcene	0.4	0.5	0.5	0.9	0.3	0.8	0.5	0.6	0.5	0.6	1.0
α-Phellandrene	0.1	t	0.1	0.1	t	t	t	t	t	t	0.1
α-Terpinene*	1.8	1.3	2.0	2.3	0.1	0.7	1.1	1.5	1.8	1.9	2.2
Limonene*	1.5	1.6	1.4	2.0	1.0	2.3	0.8	1.8	1.3	1.1	3.1
β-Phellandrene	0.2	0.5	0.1	0.3	0.1	0.2	0.1	0.2	0.3	0.2	0.5
cis-Ocimene	0.3	0.5	0.2	0.2	0.2	0.4	0.2	0.3	0.2	0.3	0.5
γ-Terpinene*	3.5	3.5	3.9	4.3	1.0	2.1	2.0	2.7	3.9	4.0	4.9
trans-Ocimene*	1.2	1.5	0.9	1.2	1.5	2.5	2.2	2.1	1.2	2.1	3.0
p-Cymene*	1.3	1.1	1.3	0.9	2.5	0.4	1.9	0.6	2.2	1.0	1.4
Terpinolene	0.7	0.8	0.9	1.1	0.2	0.4	0.5	0.7	0.7	0.8	1.0
α-*p*-Dimethylstyrene	0.1	t	t	t	t	t	t	—	—	t	—
Oxygenated monoterpenes	68.2	66.7	63.5	60.4	76.7	56.8	65.7	66.3	62.2	58.6	44.7
1.8-Cineole	0.2	t	t	t	t	t	t	t	t	t	0.1
cis-Linalool oxide	—	—	—	—	t	—	t	0.1	t	—	—
trans-Sabinene hydrate*	6.0	4.8	5.3	6.5	17.0	3.4	8.5	10.1	6.0	5.2	2.0
trans-Linalool oxide	—	—	—	—	0.1	—	t	t	—	—	—
Campholenal	0.1	0.1	t	0.1	t	t	t	t	0.1	0.1	t
Camphor*	2.0	2.9	1.7	0.9	0.6	1.7	1.7	2.9	1.8	3.4	2.5

Continued overleaf

Table 1. *Continued*

Components	Per cent in samples										
	A	B	C	D	E	F	G	H	I	J	K
Linalool*	t	0.2	0.1	0.1	25.5	0.1	15.8	9.5	t	0.4	0.1
cis-Sabinene hydrate*	10.6	8.5	10.5	10.7	4.0	5.5	3.8	11.2	10.2	10.0	2.5
Linalyl acetate	—	—	—	—	0.1	—	t	t	—	—	—
Pinocarvone	t	t	t	t	t	t	0.1	t	0.1	t	t
Bornyl formate	0.1	0.1	0.1	0.1	t	t	0.1	0.1	0.2	0.1	0.3
Bornyl acetate*	3.5	6.8	4.2	2.0	5.0	4.6	3.8	1.5	5.3	4.3	4.8
Terpinen-4-ol*	12.2	10.0	11.1	11.0	5.9	4.0	6.5	7.2	8.0	8.0	5.9
cis-Dihydrocarvone	0.7	0.7	0.4	0.6	t	0.2	0.1	t	0.3	0.1	0.1
Bornyl propionate	t	t	t	t	t	t	t	t	t	t	t
cis-Verbenol	0.2	0.5	0.3	0.7	0.2	0.3	0.2	0.3	0.4	0.3	0.4
trans-Pinocarveol	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.1	t	0.1	0.1
trans-Verbenol*	1.2	1.8	1.3	2.0	1.2	2.4	2.0	2.2	1.7	2.0	1.2
Neral	0.1	t	0.1	0.1	t	—	t	—	0.1	t	t
α-Terpineol	1.0	1.0	0.9	1.2	0.7	0.9	1.1	1.2	0.9	0.6	0.5
α-Terpenyl acetate	0.3	0.3	0.4	0.3	0.3	0.4	0.2	0.4	0.3	0.2	0.3
Borneol*	29.0	28.0	26.0	23.0	15.0	32.0	20.1	18.6	25.8	23.1	25.4
Verbenone	0.1	0.1	t	0.2	0.2	0.2	0.1	t	0.1	0.1	0.2
Bornyl butyrate	0.1	0.1	0.1	0.1	—	0.1	t	t	0.1	—	t
Bornyl isovalerate	t	t	t	t	t	0.1	t	t	t	t	0.1
Myrtenol	t	t	t	t	—	t	t	—	t	—	t
Geranyl isobutyrate	t	t	t	0.1	t	t	t	t	t	t	t
Geranyl propionate	t	t	t	t	t	t	t	t	t	t	t
trans-Carveol	t	t	0.1	t	0.1	0.1	0.1	0.1	t	0.1	0.2
Geraniol	t	t	—	—	t	—	t	—	—	—	—
p-Cymen-8-ol	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Geranyl butyrate	t	t	t	t	—	t	t	—	t	t	—
Thymol	t	t	t	0.1	t	t	0.5	0.1	0.1	t	—
Carvacrol	—	—	t	t	—	t	0.1	t	t	—	—
Sesquiterpene hydrocarbons	2.5	2.3	1.6	1.7	2.8	1.5	2.7	1.4	1.6	1.0	1.8
β-Bourbonene	t	t	t	t	t	t	t	t	t	t	—
α-Gurjunene	0.1	0.1	t	0.1	0.1	t	0.1	t	0.1	0.1	t
β-Caryophyllene*	1.8	1.6	0.9	1.2	2.1	1.1	1.9	1.0	0.9	0.6	1.0
Alloaromadendrene	0.1	0.2	0.1	0.2	0.2	0.1	0.4	t	0.3	t	0.4
α-Humulene	0.2	0.1	0.2	t	0.1	0.1	t	0.1	t	t	0.1
D-Germacrene	0.1	0.2	0.2	0.1	t	t	t	t	0.1	t	0.1
Bicyclogermacrene	t	t	t	t	0.1	t	0.1	t	t	t	t
γ-Cadinene	t	t	t	t	0.1	t	t	t	t	t	t
Oxygenated sesquiterpenes	2.6	3.0	2.2	1.9	4.9	3.6	5.1	2.6	2.2	2.7	1.1
Isocaryophyllene oxide	—	t	—	t	0.1	t	t	t	—	t	—
β-Caryophyllene oxide	0.4	0.3	0.2	0.3	0.8	0.2	0.8	0.5	0.2	0.1	0.2
Ledol	0.1	0.1	0.1	0.1	0.4	0.1	0.4	0.1	0.3	0.1	0.1
β-Elemol*	1.0	1.8	1.1	1.0	1.9	2.3	1.8	0.8	0.4	1.9	0.4
Viridiflorol	0.7	0.4	0.4	0.1	0.9	0.2	0.8	0.3	0.9	0.1	0.2
10-epi-γ-Eudesmol	t	t	t	t	0.2	0.1	0.3	0.3	t	0.1	—
Spathulenol	t	—	t	—	t	0.1	t	0.2	t	—	t
γ-Eudesmol	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.1	t
α-Eudesmol	0.1	t	t	0.1	0.2	0.2	0.2	0.1	0.1	0.1	t
β-Eudesmol	t	t	t	0.1	0.2	0.2	0.2	0.1	0.1	0.1	t
Intermedeol	—	t	—	—	t	—	0.3	0.1	t	t	t
Others	0.2	0.1	0.2	0.1	0.8	0.2	0.4	0.4	0.1	0.1	0.2
3-Octanone	t	t	t	t	0.2	0.1	0.1	0.2	t	t	t
3-Octenyl acetate	t	—	—	—	0.2	t	0.1	t	—	—	t
3-Octanol	t	t	t	t	0.2	t	0.1	0.1	t	t	t
1-Octen-3-ol	t	t	0.1	t	0.2	t	0.1	0.1	t	t	0.1
Total identified:	97.2	95.9	96.2	94.8	97.3	94.7	94.7	96.4	94.9	97.4	94.4

t: Traces ($\leqslant 0.05\%$).

*Constituents selected for multivariate analysis.

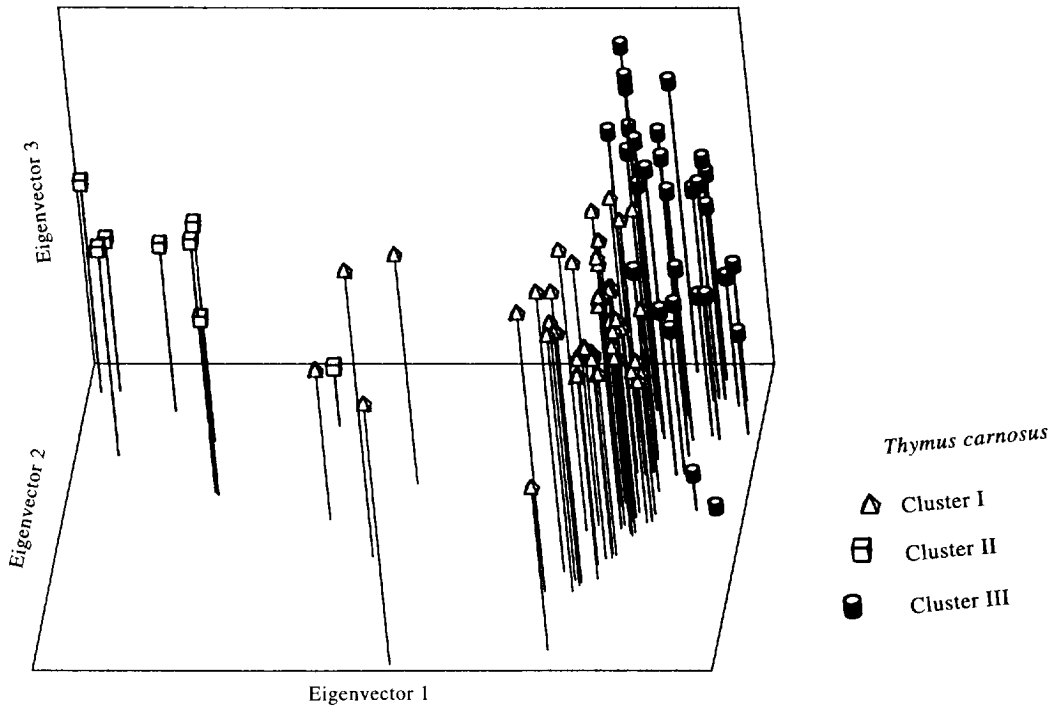

Fig. 1. Relative position of samples in the space defined by the first three Principal Components.

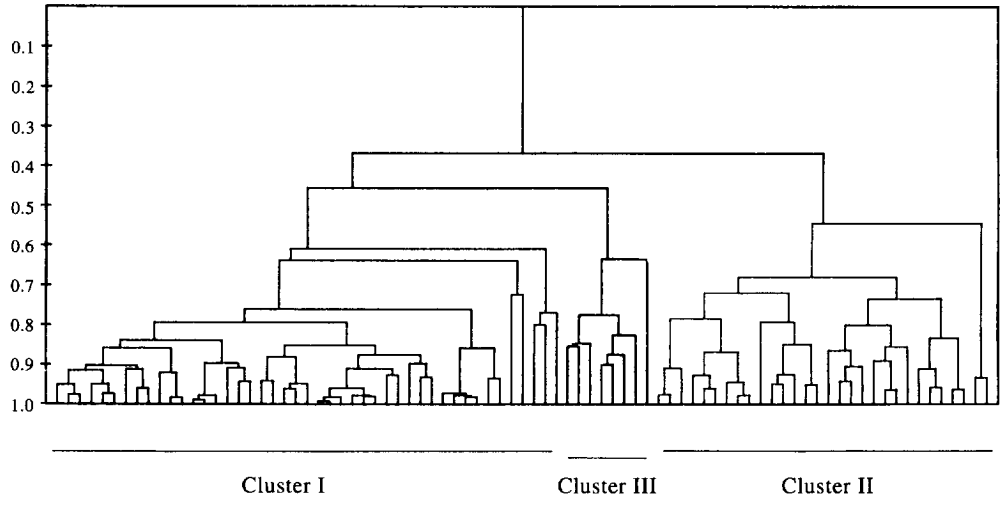

Fig. 2. Two-dimensional dendrogram obtained in the Cluster Analysis of the essential oils of individual plants of *Thymus carnosus*. Horizontal: samples analysed; vertical: differentiation level between samples.

essential oils of representative samples of each population.

EXPERIMENTAL

Plant material. Aerial parts of *T. carnosus* Boiss. were collected at the flowering stage in June–July of 1992 and 1993 in 11 different localities in Portugal, Ilha da Culatra (A), Praia da Manta Rota (B), Ilha da Armona (C), Praia das Júlias (D), Praia Verde (I) (Algarve), Apostiça (E), Fonte da Telha (G), Costa da Caparica (H) (Estremadura), Comporta (F), Tróia (J) and Lagoa de Melides (K) (Baixo Alentejo). Voucher specimens of each sample are deposited in the Herbarium of the Instituto Botanico of the University of Coimbra. In order to study the variability of essential oil composition, individual plants from populations A–F were collected in each place at the same time.

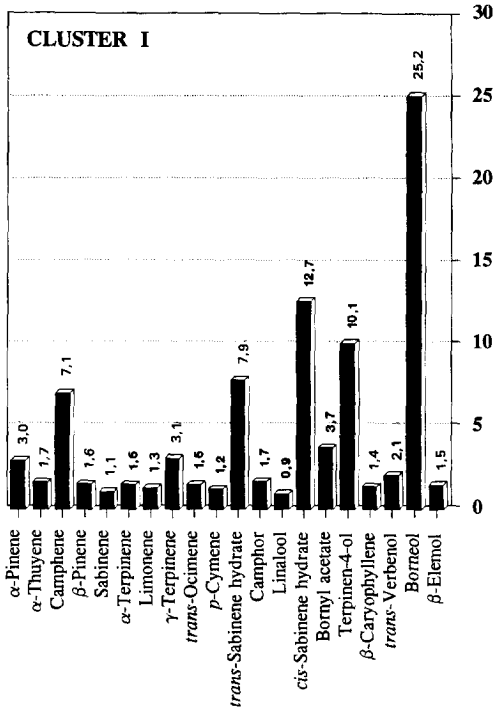

Fig. 3. Mean chemical composition of essential oil of cluster I. Vertical: mean percentage in essential oil.

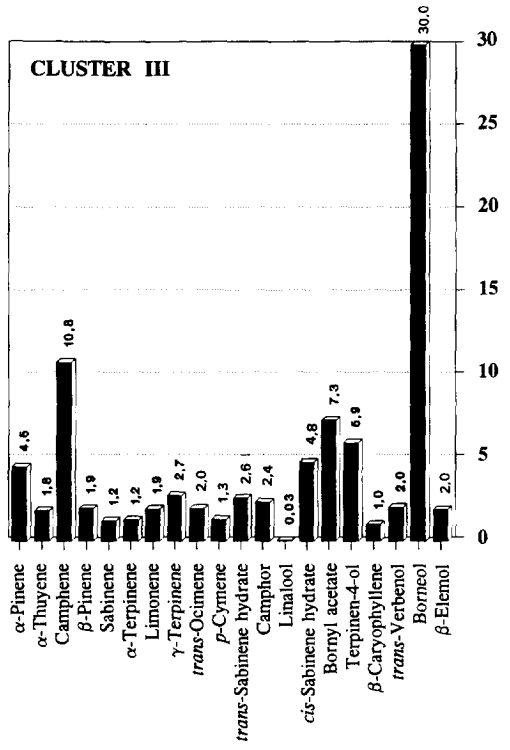

Fig. 5. Mean chemical composition of essential oil of cluster III. Vertical: mean percentage of essential oil.

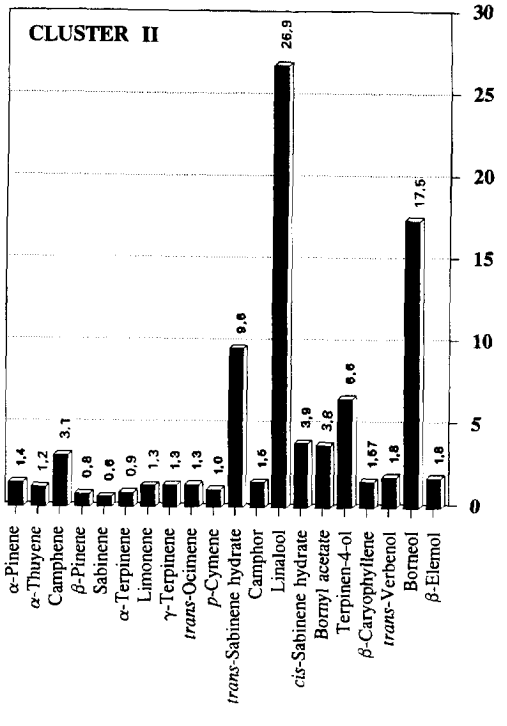

Fig. 4. Mean chemical composition of essential oil of cluster II. Vertical: mean percentage in essential oil.

Analysis of essential oils. Essential oil contents of air-dried plant material were determined according to the European Pharmacopoeia method [15]. Analysis of volatile oils obtained by hydrodistillation was carried out by FID GC and GC-MS using fused silica capillary columns with 2 different stationary phases. Analytical conditions for FID GC were as previously described [16]. GC-MS were obtained with a computerized system coupled to a mass selective detector, using Supelcowax 10 and SE-30 fused silica capillary columns. Analytical conditions were, inj. 250°, interface 280°, oven prog. from 80° to 220° at 6° min^{-1}, He flow rate 1 ml min^{-1} and split 1:60. NMR were recorded at 200 MHz for ^1H and 50 MHz for ^{13}C, in $CDCl_3$, with all shifts ref. to int. TMS. ^{13}C NMR were recorded with the following parameters: pulse width (PW) 3.2 msec, acquisition time 1.3 sec for 32 K data table with spectral width (SW) of 250 ppm. The number of accumulated scans was 10000 for each sample of essential oil (70 mg in 0.5 ml $CDCl_3$). ^{13}C NMR were recorded with CPD mode decoupling and a digital resolution of 0.763 Hz pt^{-1}. Exponential multiplication of the free induction decay with a line broadening of 1 Hz was used before Fourier transformation. Identification of components was made on the basis of their RI, with ref. to a homologous series of fatty acid Me esters, and their MS, which were compared with lit. data and authentic samples [17, 18]. Compounds with a percentage equal to or higher than 1% were also identified by ^{13}C NMR.

Infraspecific variability. The essential oil of each individual plant, obtained by hydrodistillation, was analysed by GC on the 2 stationary phases mentioned above. Identification of components was made by comparison with the chromatograms of the essential oil of the population collected at the same place. When necessary, identification was improved by GC-MS analysis. From all the volatile constituents, those which showed a percentage equal to or higher than 2% (20 variables × 85 individuals = 1700 data) were selected to be included in the multivariate analysis. The selected constituents are given in Table 1. All data were processed by PARVUS [19] and ESTATS [20] chemometric packages. From PCA, correlation matrix, eigenvalue and eigenvector evaluation showed that the whole data set could be projected in the space defined by the 3 first principal components retaining a significant percentage of the total variation. Projection coordinates of each individual in that reduced space ('score') were evaluated for their posterior graphical presentation. Cluster Analysis was also applied to the study of the similarity of individuals on the basis of constituent distribution. Euclidean distance between samples was used as an index of such a similarity and clustering was performed according to the weighted average linkage method.

REFERENCES

1. Proença da Cunha, A. and Salgueiro, L. (1991) *J. Ess. Oil Res.* **3**, 409.
2. Salgueiro, L. (1992) *Flav. Frag. J.* **7**, 159.
3. Adzet, T., Cañigueral, S., Gabaldá, N., Ibáñez, C., Tomás, X. and Vila, R. (1991) *Phytochemistry* **30**, 2289.
4. Cañigueral, S., Vila, R., Vicario, G., Tomás, X. and Adzet, T. (1994) *Biochem. Syst. Ecol.* **22**, 307.
5. Bernardini, A., Corticchiato, M. and Casanova, J. (1992) *Riv. Ital. EPPOS* Special number 664.
6. Stahl-Biskup, E. (1991) *J. Ess. Oil Res.* **3**, 61.
7. Velasco-Negueruela, A. and Pérez-Alonso, M. J. (1990) *Bot. Complutensis* **16**, 91.
8. Marhuenda, E. and Alarcón de la Lastra, C. (1986) *Fitoterapia* **57**, 448.
9. Fernandes Costa, A. (1975) *Elementos da Flora Aromatica*, Junta de Investigaçoes Cientificas do Ultramar, Lisboa.
10. Corticchiato, M. and Casanova, J. (1990) *Analusis* **20**, 51.
11. Tomi, F., Terriaga, I., Bradesi, P. and Casanova, J. (1993) *Riv. Ital. Eppos* Special number 245.
12. Formácek, V. and Kubeczka, K. H. (1982) *Essential Oils Analysis by Capillary Gas Cromatography and Carbon-13 Spectroscopy*, John Wiley & Sons, Chichester.
13. Jolliffe, I. T. (1986) *Principal Components Analysis*. Springer, New York.
14. Masart, D. L. and Kaufman, L. (1983) *The Interpretation of Analytical Chemical Data by The Use of Cluster Analysis*. J. Wiley, New York.
15. Conseil de l'Europe (1983) *Pharmacopée Européenne*, Vol. 1, Maisonneuve S. A., Sainte Ruffine.
16. Adzet, T., Vila, R., Ibáñez, C. and Cañigueral, S. (1989) *Flav. Frag. J.* **4**, 133.
17. Stenhagen, E., Abrahamson, S. and McLafferty, F. W. (1984) *Registry of Mass Spectral Data*, John Wiley & Sons, New York.
18. Central Institute for Nutrition and Food Research. (1980) *Compilation of Mass Spectra of Volatile Compounds in Food (T.N.O.)*, Utrecht.
19. Forina, M., Leardi, R., Armanino, C. and Lanteri, S. (1988) *PARVUS: An Extendable Package of Programs for Data Exploration*. Elsevier, Amsterdam.
20. Tomàs, X., Rius, J., Obiols, J. and Sol, A. (1988) *J. Chemometrics* **3**, 139.