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Stereospecific analysis of the triacylglycerol fraction and linear discriminant analysis in a climatic differentiation of Umbrian extra-virgin olive oils

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Abstract

Stereospecific analysis of the triacylglycerol fraction is an important tool for the characterisation of extra-virgin olive oils. The composition in the *sn*-1, *sn*-2 and *sn*-3 positions of triacylglycerols of extra-virgin olive oils produced in years 1992–1994 in areas characterised by increasing environmental severity (groups 1 and 2, respectively) is considered. This analytical method is based on chromatographic techniques, coupled with enzymatic ones. Linear discriminant analysis (LDA) has been applied successively for the differentiation and classification of the two groups for each year according to the positional distribution of fatty acids (palmitic, palmitoleic, stearic, oleic and linoleic acids) in the glycerol backbone. Even though the data were not very numerous, LDA has allowed a partial or total separation of scores along the discriminant eigenvector according to the climatic groups.

Keywords: Olive oil; Linear discriminant analysis; Chemometrics; Fatty acids; Triacylglycerols

1. Introduction

Because of their characteristics, Umbrian extra-virgin olive oils are considered to be among the high quality oils produced in Italy. To establish relationships between environmental conditions and composition, extensive chemical analysis and sensory evaluation of these oils is an important tool for their characterisation according to their origin [1–6].

Besides sites of origin and thus climatic conditions, olive oil characteristics can depend on various other factors, such as cultivar, picking method and technological processes (NCF=non-climatic factors). All these factors can also explain a certain variability for products from the same area.

Chemometric techniques are of great significance in the study of complex matrices for the characterisation and classification of olive oils. Among multivariate methods, linear discriminant analysis (LDA) is considered to be an important classical parametric tool for grouping samples when the sample allocation is known beforehand. The main purpose is to estimate an adequate linear function by means of which it is possible to classify cases of unknown

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origin to one group or to the other, successively. In the literature there are various applications of discriminant analysis for the geographical differentiation and the classification of olive oils with regard to their fatty acid and sterol composition [2,4–6].

This work was undertaken to verify a possible group separation, using the results of stereospecific analysis of the triacylglycerol fraction (TAG), of extra-virgin olive oils from areas of Umbria with different climatic conditions. The procedure used, reported in Refs. [7–9], allows the positional distribution of fatty acids in triacylglycerol molecules to be determined.

2. Experimental

2.1. Sampling

The samples examined were from different areas in Umbria and were assigned to different climatic groups established by comparison of the climatic diagrams or climograms with a reference diagram [10]. The climograms employed were obtained from the mean of the twenty-year monthly averages of rainfall and temperature. The climatic area 1 was characterised by mild climatic conditions, whereas in the second climatic area more severe conditions were registered.

The extra-virgin olive oils from the two areas were produced in three years (1992, 1993 and 1994). For each year, the number of samples of the two groups was asymmetric, except for 1993. In fact, in 1992 $n_1=8$, $n_2=13$ ($N=21$); in 1993 $n_1=12$, $n_2=14$ ($N=26$) and in 1994 $n_1=5$, $n_2=14$ ($N=19$).

The drupes were processed for oil extraction immediately after the harvest. The NCF did not substantially influence the sets of samples considered.

2.2. Chemicals

Organic solvents of analytical reagent grade were purchased from BDH (Poole, UK), together with most of the salts used. Silica gel precoated plates (20×20 cm, 250-μm layer thickness) and ATP disodium salt were supplied by Sigma (St. Louis, MO, USA); magnesium turnings and ethylbromide were obtained from Aldrich (Milwaukee, WI, USA). Lipase from hog pancreas (3.6 U/mg) and cardiolipin disodium salt (5 mg/ml) in methanol were supplied by Fluka (Bucks, Switzerland). *sn*-1,2-Diacylglycerol kinase (from *E. coli*, 1 mg/ml phosphate buffer, 10.6 U/mg) was obtained from Calbiochem (San Diego, CA, USA).

2.3. GC apparatus

The chromatographic analyses were carried out with a Chrompack 9001 chromatograph (Chrompack, Middelburg, Netherlands) equipped with a split–splitless injector, a flame ionization detection (FID) system, a Supelcowax 10 column (30 m×0.25 mm I.D., 0.25 μm film thickness, Supelco, Bellefonte, PA, USA) and an EMI 80 386 computer with Mosaic integration software (Chrompack, Middelburg, Netherlands). The operating conditions were: injector temperature, 270°C; injection split ratio, 1:50; FID temperature, 270°C; carrier gas (He) flow, 2 ml/min; oven temperature program, initial 165°C, after 3 min 3 °C/min temperature gradient to 240°C.

2.4. Stereospecific analysis of triacylglycerols (TAG)

The procedure for the stereospecific analysis is reported in the literature [8,9]. In short, it is based on the isolation of the TAG fraction from extra-virgin olive oil samples by thin-layer chromatography (TLC) on SiO_2 plates. This fraction was subjected to the following steps:

1. Enzymatic hydrolysis of TAG with pancreatic lipase to obtain *sn*-2 monoacylglycerols (MAG).
2. Partial deacylation of TAG by chemical hydrolysis with Grignard reagent, ethyl magnesium bromide.
3. Separation of enantiomeric *sn*-1,2(2,3)-DAG by TLC on boric acid–SiO_2 plates from the mixture of hydrolysis products and treatment of the *sn*-1,2(2,3)-DAG fraction with *sn*-1,2-diacylglycerol kinase from *Escherichia coli* to obtain *sn*-1,2-phosphatidic acids (PA). A commercial, purified preparation of the enzyme has been used which catalyzes the stereospecific phosphorylation of *sn*-

1,2-DAG in the presence of ATP, thus simplifying and shortening the stereospecific analysis of TAG.
4. Determination of the fatty acid compositions of TAG, *sn*-2-MAG and *sn*-1,2-PA by means of high-resolution gas chromatography (HRGC) of methyl esters obtained from the cited fractions.

2.5. Linear discriminant analysis (LDA)

As the group composition was already known, LDA was applied for the two-group sample differentiation and also for the classification of each datum expressed as a discriminant score, assuming a multinormal distribution for each group.

The variables considered were the mol% concentrations of the palmitic (p), palmitoleic (po), stearic (s), oleic (o) and linoleic (l) acids in the three positions (*sn*-1, *sn*-2 and *sn*-3) of the glycerol backbone. The acronyms for the 15 variables were the following: p*sn*-1, p*sn*-2, p*sn*-3; po*sn*-1, po*sn*-2, po*sn*-3; s*sn*-1, s*sn*-2, s*sn*-3; o*sn*-1, o*sn*-2, o*sn*-3; l*sn*-1, l*sn*-2, l*sn*-3.

The variables entered in the analysis are the ones selected by means of the multiple regression method, rejecting the variables linearly associated to the others already in the equation [11]. This is the SPSS default method for discriminant analysis (SPSS, Chicago, IL, USA) and was applied to the data matrices referring to 1992, 1993 and 1994.

As the 1993 data set had the highest N and $n_1 \simeq n_2$, this two-group sample was analysed in detail by considering another well-known method for the selection of variables, the stepwise procedure based on Wilks' lambda criterion. The results obtained by the two methods were thus compared and discussed although the sample numerosity allowed only explorative consideration and classifications.

In order to evaluate the between-group separation, three explorative criteria were considered. The first one is the eigenvalue (the ratio of the between-group to within-group sums of squares) associated with the discriminant eigenvector along which the discriminant scores are projected. To estimate the discriminant power of the function employed to describe group differences, the classical Bartlett's test, an approximate χ^2 statistic, was also considered. This statistic corresponds to:

$$b = \left(N - \frac{p+g}{2} - 1\right)\ln \Lambda$$

where N stands for the number of observations, p for the number of variables, g for the number of groups and Λ represents the ratio of the within-group sum of squares to the total sum of squares, a statistic used to determine whether most of the total variability is due to the differences between the group means or to the within-group variability. The value of Λ can range between 0 and 1: $\Lambda=1$ occurs when the means of the two groups are equal, whereas $\Lambda=0$ if they differ.

The second general criterion is the discriminant score classification. If all scores are correctly classified and overlap is not appreciable, a tendency for separation seems to exist. The objective of Bayes' method applied to a two-group sample system is to estimate the posterior probability of membership, $P(G_i/D)$, of each discriminant score (D) in population G_i, according to which the scores are classified. The most-likely group membership can be compared with the actual one, so that it is possible to estimate the adequacy of the classification rule, computed using the discriminant function [11]. However, the unequal numerosity of the two-group sample, as well as an extremely low N, implies that the classification results should be considered exploratively.

A third explorative and indicative criterion is the inter-centroid distance, expressed for instance by the classical Δ^2 Mahalanobis distance which is equal to Wilks' Λ ratio in the case of a two-group sample [12].

3. Results and discussion

3.1. Univariate statistics

In Tables 1–23, the univariate statistics for the 15 variables and for each climatic group are reported.

3.2. Discriminant analysis

3.2.1. Year 1992

With the multiple regression method used for variable selection, the following variables were rejected: l*sn*-2, po*sn*-3, s*sn*-3, o*sn*-3 and l*sn*-3, whereas p*sn*-1, po*sn*-1, s*sn*-1, o*sn*-1, l*sn*-1, p*sn*-2,

Table 1
Mean (mol%) and standard deviation of each acid concentration in the three positions of TAG fraction samples for climatic group 1 and 2 (year 1992)

Variable	Climatic group			
	$n_1=8$		$n_2=13$	
	$\bar{X}$	s	$\bar{X}$	s
p*sn*-1	14.84	0.59	14.99	0.92
po*sn*-1	1.01	0.06	0.88	0.13
s*sn*-1	1.52	0.46	1.91	0.41
o*sn*-1	74.66	0.43	75.11	0.91
l*sn*-1	8.02	1.12	7.15	1.25
p*sn*-2	1.97	0.44	1.85	0.59
po*sn*-2	0.99	0.16	0.86	0.20
s*sn*-2	0.51	0.20	0.40	0.17
o*sn*-2	89.11	0.30	89.05	0.41
l*sn*-2	7.26	1.09	7.72	1.09
p*sn*-3	17.97	0.74	17.48	1.63
po*sn*-3	0.29	0.18	0.12	0.09
s*sn*-3	1.89	1.09	2.42	0.85
o*sn*-3	77.13	2.83	76.02	3.71
l*sn*-3	1.54	0.92	2.48	1.36

po*sn*-2, s*sn*-2, o*sn*-2 and p*sn*-3 were selected for further processing. As can be clearly seen, nearly all alkyl groups in the *sn*-1 and *sn*-2 positions gave a distinct discriminant power, whereas others in the *sn*-3 did not.

Table 2
Mean (mol%) and standard deviation of each acid concentration in the three positions of the TAG fraction samples for climatic group 1 and 2 (year 1993)

Variable	Climatic group			
	$n_1=12$		$n_2=14$	
	$\bar{x}$	s	$\bar{X}$	s
p*sn*-1	15.11	0.82	15.39	0.62
po*sn*-1	1.13	0.12	0.87	0.18
s*sn*-1	1.95	0.18	2.18	0.25
o*sn*-1	74.59	0.57	74.65	0.65
l*sn*-1	7.22	0.65	6.88	0.54
p*sn*-2	1.65	0.25	1.36	0.48
po*sn*-2	0.87	0.12	0.72	0.20
s*sn*-2	0.31	0.05	0.21	0.10
o*sn*-2	88.89	0.38	88.83	0.29
l*sn*-2	8.39	0.35	8.96	1.06
p*sn*-3	18.17	1.39	18.23	1.19
po*sn*-3	0.65	0.12	0.36	0.13
s*sn*-3	3.08	0.27	3.44	0.73
o*sn*-3	74.93	2.44	72.84	3.07
l*sn*-3	1.83	0.69	3.39	1.20

Table 3
Mean (mol%) and standard deviation of each acid concentration in the three positions of TAG fraction samples for climatic group 1 and 2 (year 1994)

Variable	Climatic group			
	$n_1=5$		$n_2=14$	
	$\bar{X}$	s	$\bar{X}$	s
p*sn*-1	14.72	0.84	15.69	0.46
po*sn*-1	1.10	0.12	0.92	0.13
s*sn*-1	1.98	0.19	2.12	0.18
o*sn*-1	74.54	0.61	74.12	0.45
l*sn*-1	7.68	0.61	7.13	0.54
p*sn*-2	1.36	0.17	1.09	0.29
po*sn*-2	0.76	0.15	0.74	0.14
s*sn*-2	0.26	0.06	0.10	0.08
o*sn*-2	89.02	0.40	88.64	0.20
l*sn*-2	8.70	0.32	9.46	0.33
p*sn*-3	17.80	1.47	19.09	0.92
po*sn*-3	0.72	0.15	0.50	0.12
s*sn*-3	3.32	0.23	3.68	0.26
o*sn*-3	75.26	2.52	71.15	1.04
l*sn*-3	1.66	0.80	3.64	0.35

As far as the canonical discriminant vector was concerned, only one was obviously allowed. The corresponding eigenvalue was not very high (1.32) with a medium canonical correlation (0.75) and a Wilks' Λ (0.43) too high to be considered as significant in the two-group separation indicating therefore a probable mixing. The Bartlett approximate χ^2 (11.8 for 10 degrees of freedom) was very far from the usual significance, it's value being only 0.30.

Although the total numerosity was extremely low, the following standardised canonical function coefficients were calculated: p*sn*-1 (7.77), po*sn*-1 (−1.10), s*sn*-1 (0.52), o*sn*-1 (−1.15), l*sn*-1 (3.05), p*sn*-2 (0.16), po*sn*-2 (2.47), s*sn*-2 (−1.82), o*sn*-2 (1.60), p*sn*-3 (−7.67).

The correlations between each variable and the canonical discriminant eigenvector were, in general, low and were distributed as follows (in size order): po*sn*-1 (−0.52), s*sn*-1 (0.39), po*sn*-3 (−0.39), l*sn*-3 (0.37), l*sn*-1 (−0.32), po*sn*-2 (−0.30), s*sn*-3 (0.29), s*sn*-2 (−0.27), o*sn*-1 (0.26), l*sn*-2 (0.21), p*sn*-3 (−0.16), o*sn*-3 (−0.16), p*sn*-2 (−0.10), p*sn*-1 (0.08), o*sn*-2 (−0.07). Although the significance of each correlation was not considered here, this sequence of values is very important because it tends to define each variable according to its discriminant power. Among the $p=15$ variables considered here, the most important was the palmitoleic group in the *sn*-1 position with a negative medium correlation. This variable also had a high F value ($F=6.82$) with a low significance (0.017). Another group of variables (s*sn*-1, po*sn*-3, l*sn*-3) had weak positive correlations that could perhaps be considered.

In Fig. 1, the distribution of the discriminant scores for the two groups is reported together with the corresponding centroid position in the discriminant space ($c_1=-2.73$, $c_2=2.34$). As can be seen immediately, a slight intersection was clearly appreciable between the tail of the group 1 and the front of the group 2 distribution.

Score intermixing was also confirmed by Bayes' classificaton method. The posterior probabilities for the two misclassified cases belonging to the other group were 0.83 and 0.80, respectively. As a consequence of this intermixing, the final classification, expressed as the percentage of oil samples correctly classified, was about 90%.

3.2.2. Year 1993

Using the multiple regression method for the selection of variables, all variables entered in the analysis, except palmitic and oleic, both in *sn*-3. The discriminating eigenvalue was 6.90 and Bartlett's χ^2 value corresponds to 36.17 with $d.f=13$.

The following standardised canonical function coefficients were calculated: p*sn*-1 (−4.07), po*sn*-1 (−0.90), s*sn*-1 (−0.42), o*sn*-1 (2.22), l*sn*-1 (4.08), p*sn*-2 (2.48), po*sn*-2 (0.97), s*sn*-2 (−2.18), o*sn*-2 (−1.12), l*sn*-2 (−17.11), po*sn*-3 (3.41), s*sn*−3 (6.08), l*sn*-3 (14.97).

The correlations between the selected variables and the canonical discriminant eigenvector were distributed as follows (in size order): po*sn*-3 (−0.45), po*sn*-1 (−0.33), l*sn*-3 (0.31), s*sn*-2 (−0.25), s*sn*-1 (0.21), po*sn*-2 (−0.17), p*sn*-2 (−0.14), o*sn*-3 (−0.14), l*sn*-2 (0.14), s*sn*−3 (0.13), l*sn*-1 (−0.12), p*sn*-1 (0.08), o*sn*−2 (-0.04), o*sn*−1 (0.02), p*sn*-3 (0.00). The most discriminating variable is palmitoleic acid in position 3 with a negative medium correlation. This variable showed also the highest significative F value of 32.90, ($P=0.0000$).

In Fig. 2, the combined discriminant score distribution for the two groups is presented. The data size for group 1 ($n_1=12$) was too low and probably created an anomalous contour score area, whereas the group 2 scores seem to have a tendency for a normal distribution. The complete separation of the two-group scores was also confirmed by Bayes' procedure. As the percentage of cases classified

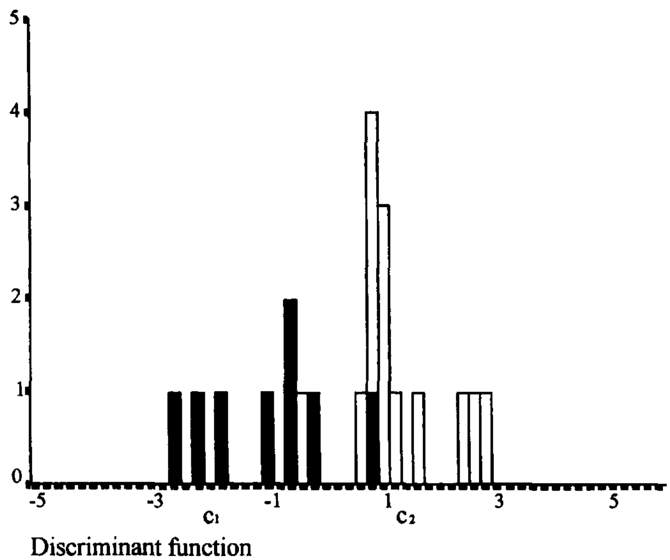

Fig. 1. Histogram of the discriminant scores belonging to the two climatic groups ($n_1=8$ black bars, $n_2=13$ white bars) in 1992, obtained with LDA based on multiple linear correlation selection of variables.

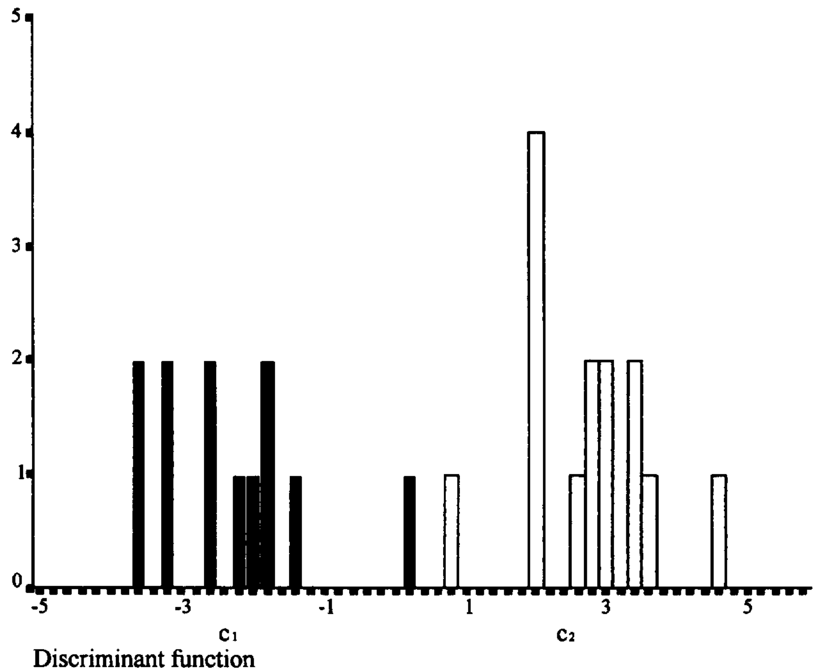

Fig. 2. Histogram of the discriminant scores, belonging to the two climatic groups ($n_1=12$ black bars, $n_2=14$ white bars) in 1993, obtained with LDA based on multiple linear correlation selection of variables.

correctly was 100%, no score overlap occurred between the two groups, whose centroids were computed as -2.73 for the climatic group 1 and 2.34 for group 2.

For the 1993 data set another variable selection algorithm was also performed by employing the classical stepwise Wilks' Λ. Adopting the criterion, the variable was entered in the analysis results in the minimum Wilks' Λ for the discriminant function. In this two-group sample the first variable entered in the model was po*sn*-3 with a $\Lambda=0.42$. In the second step, the variable s*sn*-2 was included for its value of $\Lambda=0.30$. At the third step, po*sn*-2 was the last variable entered with $\Lambda=0.24$. Thus, only three variables out of 15 were selected by means of the Wilks' Λ criterion.

The eigenvalue associated with the discriminant eigenvector was $\lambda_1=3.095$. The approximate χ^2 value was 31.72 with *d.f.*=3 ($P=0.0000$).

The standardised canonical discriminant function coefficients were as follows: po*sn*-2 (−0.84), s*sn*-2 (1.22), po*sn*−3 (1.56).

The ordered sequence of correlations between each discriminating variable and the canonical discriminant vector was as follows: po*sn*-3 (0.67), po*sn*-1 (0.45), l*sn*−3 (−0.40), s*sn*-2 (0.37), p*sn*-2 (0.30), po*sn*-2 (0.26), l*sn*-2 (−0.24), o*sn*-3 (0.20), s*sn*-1 (−0.18), s*sn*-3 (−0.14), p*sn*-1 (−0.06), l*sn*-1 (−0.06), o*sn*-1 (0.05), p*sn*−3 (−0.03), o*sn*-2 (0.02). From this sequence, three groups of correlations could be identified. The first one formed by po*sn*-3 showed a distinct medium correlation value; the second corresponding to a group characterised by low correlations, in which po*sn*-1, l*sn*-3 and s*sn*-2 variables were included; the last group included variables giving a very low contribution, confirming the results obtained by means of Wilks' Λ procedure for the selection of variables.

In Fig. 3, the histogram of the discriminant scores for the two groups is displayed. From this plot, the presence of a group 2 case among the scores of the other group is visually appreciable. The location of the group centroids is also indicated ($c_1=1.83$, $c_2=-1.56$). The discriminant score intersection was also demonstrated by Bayes' rule. The amount of grouped cases correctly classified was in fact about 96%.

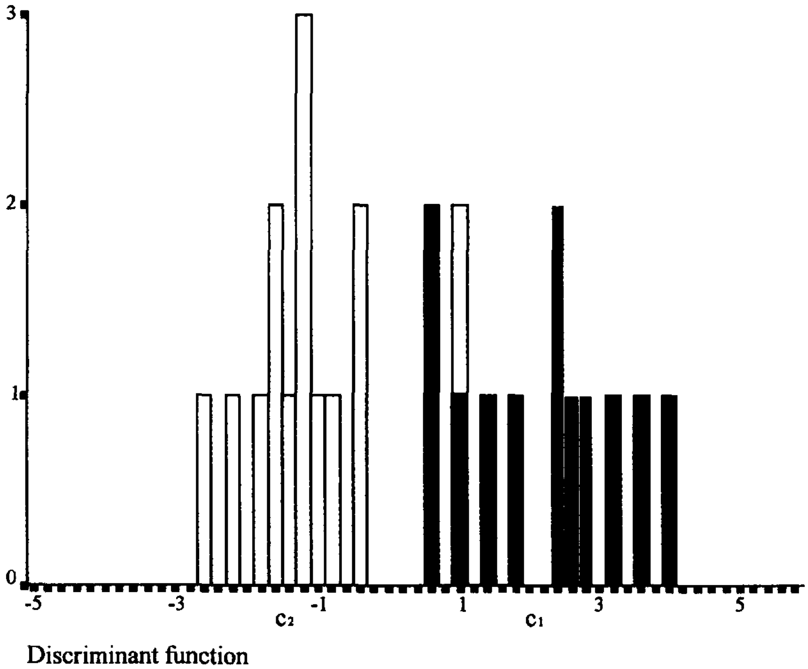

Fig. 3. Histogram of the discriminant scores, belonging to the two climatic groups ($n_1=12$ black bars, $n_2=14$ white bars) in 1993, obtained with LDA based on Wilks' selection of variables.

Therefore, when results of multiple regression selection are compared with those selected step-wise by means of Wilks' Λ method, the latter method appears to be less differentiating than the former, at least in this two-group sample.

3.2.3. Year 1994

When the multiple regression was applied, all variables were entered in the analysis, except palmitic, stearic and oleic, all in the *sn*-3 position. The eigenvalue of the eigenvector was 13.23, with a Λ value of 0.07 and a $\chi^2=29.21$ with $d.f.=12$ ($P=0.0037$).

The following standardised canonical function coefficients were calculated: p*sn*-1 (3.90), po*sn*-1 (−1.73), s*sn*-1 (6.45), o*sn*-1 (4.14), l*sn*-1 (8.08), p*sn*-2 (1.20), po*sn*-2 (4.47), s*sn*-2 (4.36), o*sn*-2 (−1.64), l*sn*-2 (4.75), po*sn*-3 (0.08), l*sn*-3 (−3.05).

The ordered sequence of correlations between each variable and the canonical discriminant vector was as follows: l*sn*-3 (−0.51), o*sn*-3 (0.34), l*sn*-2 (−0.30), s*sn*-2 (0.28), s*sn*-3 (−0.23), po*sn*-3 (0.22), p*sn*-1 (−0.22), o*sn*-2 (0.19), po*sn*-1 (-0.18), p*sn*-3 (−0.16), p*sn*-2 (0.13), l*sn*-1 (-0.13), o*sn*-1 (0.11), s*sn*-1 (−0.10), po*sn*-2 (0.02). The most discriminating variable, linoleic acid in position 3, had the highest significant F value (58.7) also.

A total separation between the two-group scores was observed, as can be estimated from the centroid interdistance ($c_1=5.76$, $c_2=-2.06$), superior to the distance seen for the 1993 data set.

In Fig. 4, the distribution of standardised scores on the discriminant eigenvector is reported, pointing out the total score separation. The location of the centroids for each group is also displayed.

4. Conclusion

In conclusion, the combination of stereospecific TAG analysis with exploratory multivariate linear discriminant procedures allowed a partial or a total separation of Umbrian extra-virgin olive oils according to their climatic characteristics.

In all the years and in the two-group sample considered in this preliminary research, the score

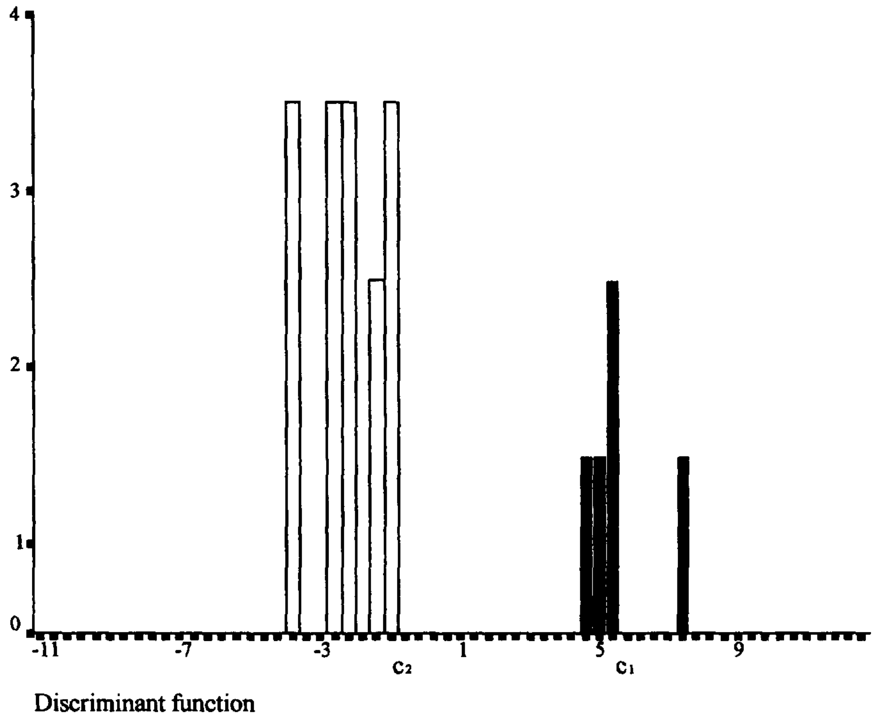

Fig. 4. Histogram of the discriminant scores, belonging to the two climatic groups ($n_1 = 5$ black bars, $n_2 = 14$ white bars) in 1994, obtained with LDA based on multiple linear correlation selection of variables.

separation tended to be complete, except in 1992. In this year, a score intermixing of one group was observed among the scores of the other group, just in correspondence with the maximum of the distribution. This particular intermixing was perhaps not attributable to a misclassification of the olive oil sample as far as climatic afference is concerned, but to an extension of the forward tail of the distribution.

As far as the comparison of inter-centroid absolute distances (determined by the multiple regression variable selector method) for each year was concerned, in year 1992 $|c_1 - c_2| = 2.25$, in year 1993 $|c_1 - c_2| = 5.06$ and in year 1994 $|c_1 - c_2| = 7.81$. Therefore, on the basis of the available data, an increasing inter-centroid distance appears from 1992 to 1994. However, further statistical research in this area is still in progress.

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