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Characterisation of 19 almond cultivars on the basis of their free amino acids composition

L. Helena Seron,[a] E. Garrigós Poveda,[b] M. S. Prats Moya,[b] M. L. Martín Carratalá,[b] V. Berenguer-Navarro[b] & N. Grané-Teruel[b]*

[a]Departamento de Quimica, Centro Ciencias Exatas e Tecnol., Univ. Federal de Sao Carlos., Caixa Postal 676, Sao Carlos, SP Brazil
[b]Departamento de Química Analítica, Universidad Alicante. P.O. Box 99, 03080 Alicante, Spain

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Several multivariate techniques (ANOVA, principal components analysis, cluster, linear discriminant function) were applied to the chromatographic data on free amino acids for a set of 19 almond cultivars. It is concluded that the cultivars studied have significantly different free amino acid patterns. The information provided by multivariate techniques using amino acids is complementary to that obtained from fatty acid composition. Spanish cultivars can be successfully classified as a single class different from the rest. Cultivars *Texas*, *Genco* and *Wawona* have such peculiar patterns that each of them can be considered as a consistent class. Cultivars *Titan* and *Non Pareil* associate either with the Spanish cultivars or with the American in function of amino acids processed. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Multivariate techniques have been proved to be an interesting set of powerful tools to analyse and recognise hidden patterns in complex matrices of experimental data, and in this way to characterise similar objects. These techniques are of paramount importance, especially in food chemistry, since they can be used to distinguish or classify samples according to their geographical origin and varieties. Actually, multivariate techniques have been successfully applied to discriminate alcoholic distillates (Ortiz *et al.*, 1993), wines (Shimoda *et al.*, 1993; Martín *et al.*, 1996) coffee (Tomás and Molins, 1990), tea (Varela *et al.*, 1996), orange juice (Robards and Antolovich, 1995) and apple juice (Pilando and Wrolstad, 1992). These techniques are also recommended for selecting reference materials in food analysis (Pennickx *et al.*, 1996).

Amino acids are a rich fraction of many natural foods, and sometimes this has been exploited for differentiation purposes. In a previous paper (Prats and Berenguer, 1994), the ability of free amino acids to establish significant differences within a limited number of almond cultivars from the same region has been reported. The aim of the present paper is to verify the extent of this statement using a set of 19 selected and well-defined almond cultivars from very different origin, but cultivated in the same experimental field and under the same conditions, in order to exclude accidental sources of differentiation.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Samples

The set studied consisted of the following 19 cultivars: eight Spanish (*Malagueña* MA, *Peraleja* PE, *Atocha* AT, *Del Cid* DC, *Desmayo Largueta* DL, *Ramillete* RA, *Marcona* MR, and a *hybrid* CE obtained at CEBAS (Centro de Edafología y Biología Aplicada del Segura, Murcia), four American (*Texas* TE, *Non Pareil* NP, *Titan* TI, *Wawona* WA), three Italian (*Genco* GE, *Tuono* TU, *Cristomorto* CR), one Australian (*Chellaston* CH), one Tunisian (*Achaak* AC), one from the Caucasian region (*Primorskyi* PR), and one French (*Ferragnes* FE). The trees corresponding to the abovementioned Spanish cultivars (generally very diversified) were selected and accepted as the most authentic representatives of these cultivars some years ago by a technical committee (Felipe *et al.*, 1984). Such selected cultivars have been replicated in other experimental research fields in Spain. All the samples for the present study are a mixture of almonds taken from different trees grown under the same conditions in CEBAS (Centro de Edafología y Biología Aplicada del Segura) experimental fields. All

the almonds belong to the 1993 harvest, and since then had been carefully stored in a refrigerator after grinding, drying and defatting.

Reagents

Methanol, tetrahydrofuran and acetonitrile were HPLC gradient grade and were employed as supplied. Amino acids, mercaptoethanol, *o*-phthaldialdehyde, internal standards (norvaline and taurine) were of analytical grade, from Sigma (St Louis, MO, USA). Salts for buffer solutions and for adjusting ionic strength were likewise of analytical grade, from Merck (Darmstadt, Germany). The water used was of high purity from a Milli-Q (Millipore) system.

Standard stock solutions of amino acids were about 10^{-2} M, made up to 10^{-1} M in HCl, and stored in a refrigerator at about 4°C. Working standards were freshly prepared daily by serial dilution with water.

A solution for derivatising amino acids was prepared dissolving 50 mg of *o*-phthaldialdehyde (OPA) in 4.5 ml of methanol, and adding 50 μl of 3-mercaptopropionic acid, together with 0.5 ml of 1.0 M sodium borate (pH 9.5). The reagent mixture was kept in the refrigerator at 4°C, and was freshly prepared every week.

Apparatus

The chromatograph used was a 'Waters Multisolvent HPLC system', equipped with two peristaltic pumps and a controller Waters 600E. The injection valve was a Rheodyne model 7125 with a 20 μl loop. Detection of OPA derivatives was carried out with a fluorescence detector at an excitation wavelength of 330 nm and an emission wavelength of 450 nm, using a Waters 474 detector, provided with a flow cell of 5 μl to optimise resolution of chromatographic peaks.

The chromatographic column was a packed Waters Accq (type RP bounded silica C18). The run of chromatograms and the processing of chromatographic data were performed with computer assistance, using the Waters program Baseline 810.

Chromatographic method

Separation of amino acids was achieved after a pre-column derivatisation with *o*-phthaldialdehyde. This method was selected because of its good resolution and sensibility. Extraction of amino acids, derivatisation to OPA-derivatives and chromatographic procedure were carried out as described elsewhere (Prats and Berenguer, 1994).

Thus, an aqueous solution A (buffer phosphate solution 0.02 M pH 6.4, containing 2% of tetrahydrofuran, and adjusted to an ionic strength 0.08 with $NaNO_3$) and an aqueous-organic solution B (buffer phosphate 0.02 M pH 6.4: methanol: acetonitrile, 50: 35: 15) were used. The gradient program was slightly modified. From a starting mixture 5% in B, elution began with a linear ramp to 10% in B for 2 min, then the composition was held at 10% in B for 6 min; it was linearly ramped to 80% in B for 30 min, followed by another linear ramp to 100% in B for 3 min; it was held at 100% for 3 min, and finally returned to the starting point in 4 min.

Amino acids determined in order of increasing t_R are: aspartic acid (asp), glutamic acid (glu), asparagine (asn), serine (ser), glutamine (gln), glycine (gly), threonine (thr), histidine (his), alanine (ala), arginine (arg), tyrosine (tyr), valine (val), methionine (met), phenylalanine (phe), isoleucine (ile), and leucine (leu).

Statistics

Several statistical methods in SPSS package (Release 6.0.1; SPSS Inc., II, 1994) were used for the treatment of data: one-way ANOVA, principal components (PCA), hierarchical cluster analysis (CA) and linear discriminant analysis (LDA). Details for all these methods are given elsewhere in a similar application to fatty acids composition of the same set of almond cultivars (García-López *et al.*, 1996).

RESULTS AND DISCUSSION

Mean values of amino acid contents in each cultivar from four independent analysis are shown in Table 1. Standard deviations of the mean values are never above 10%. Exceptionally, only three values for cultivar DC are averaged, since the fourth management deviates significantly from the rest. Mathematical algorithms have been applied to the individual data from all the determinations made for each sample, not to the mean values given in the table. In the case of the DC cultivar, for convenience of statistical treatment, a fourth value was used as the mean of the three significant ones. Glutamic and aspartic acids are not processed separately but as a whole, because of limited resolution of their chromatographic peaks in almond samples.

A preliminary test for the ability of each amino acid to differentiate cultivars is conducted by analysis of variance. Comparison of individual amino acid contents in all possible pairs of cultivars allows arrangement of amino acids according to their discriminant power, given by their F ratio. They decrease in the following way: leu (338) > val (318) > ala (213) > ile (194) > arg (158) > phe (118) > asn (116) > thr (108) > gln (91) > met (88) > ser (86) > tyr (82) > gly (76) > his (70) > > asp + glu (27).

Clearly, all amino acids contribute, although at a different extent to differentiate (or associate) cultivars, thus, multivariate analysis is likely to be convenient.

By applying cluster analysis to all the amino acids, dendogram 1 arises (Fig. 1), where five groups are differentiated at a dissimilarity level between 10 and 15. There is a sharp differentiation between the first group, consisting mainly of Spanish cultivars, and the remainder.

Table 1. Free amino acids composition (in mg per 100 g) of different cultivars (averages from four independent determinations)

Cultivars	A+G*	asn	ser	gln	gly	thr	his	ala	arg	tyr	val	met	phe	ile	leu
Achaak	260	212	12.0	25.1	3.5	13.2	17.7	24.1	48.4	8.5	13.1	6.1	15.3	14.0	6.3
Atocha	330	168	21.8	23.0	5.4	17.1	29.4	33.5	93.0	11.5	23.3	5.1	24.8	18.0	14.3
Clon Cebas	378	221	22.3	33.2	4.9	14.7	34.4	33.3	75.8	14.1	28.2	10.0	22.3	21.7	16.7
Chellaston	310	292	32.6	37.1	5.0	9.4	27.2	20.5	52.4	9.1	19.4	7.8	21.0	15.9	10.9
Cristomorto	268	98	26.3	21.8	4.3	20.3	27.8	9.9	97.9	7.0	22.5	3.2	22.1	20.6	20.0
Del Cid	233	270	25.8	18.9	3.8	28.5	27.8	27.9	129	10.4	25.6	3.6	28.0	18.1	12.1
D. Largueta	281	260	19.5	28.4	4.2	19.3	26.4	33.4	82.5	7.4	19.3	6.6	18.7	17.6	12.8
Ferragnes	306	231	40.7	27.0	5.0	19.1	29.0	15.3	107	7.2	32.3	6.8	22.5	27.1	30.6
Genco	439	333	26.8	25.7	6.9	34.9	40.1	36.2	185	13.5	53.5	7.0	44.0	37.1	31.1
Malagueña	310	128	20.4	25.7	2.0	12.8	20.0	6.5	43.8	8.0	12.4	3.2	15.6	12.6	7.8
Marcona	341	283	20.8	16.0	3.9	17.8	16.4	18.2	54.4	6.9	14.3	2.4	14.0	13.0	7.4
N. Pareil	348	270	26.4	24.8	9.2	22.7	44.4	25.1	99.9	13.6	28.7	10.9	30.5	22.0	15.7
Peraleja	199	229	17.3	52.9	3.9	16.4	18.4	65.9	76.5	10.5	15.2	3.1	21.4	12.2	12.6
Primorsky	344	371	28.6	31.8	8.0	25.7	37.6	57.2	148	13.2	39.6	7.4	36.9	33.5	24.9
Ramillete	293	263	19.3	66.1	3.2	18.5	25.1	34.2	83.8	6.4	17.2	6.1	13.5	15.4	11.2
Texas	540	275	56.4	26.0	9.7	19.5	49.3	15.9	121	10.3	47.6	9.7	36.6	37.4	20.4
Titan	356	278	22.4	48.6	5.1	21.0	37.8	27.9	139	11.5	31.4	5.7	24.3	25.7	19.4
Tuono	278	206	23.9	46.1	6.4	29.2	35.2	57.7	111	16.9	27.0	9.5	32.7	23.7	24.9
Wawona	295	641	22.1	61.7	6.4	27.4	37.3	64.9	218	11.2	38.2	4.6	28.8	30.8	19.6

*,asp + glu.

Among these an association is formed by PR, TU and NP, while cultivars GE, WA, and especially TE, can be considered as rather singular.

In the first group American cultivar TI somehow appears exceptionally associated with the Spanish ones, while the cultivar NP, which a previous partial study associated with Spanish cultivars (Prats and Berenguer, 1994) associates now with the American ones. It is interesting to observe a migration of these two cultivars from one group to another, when cluster analysis is again applied to all amino acids but (glu + asp), or to the first eight amino acids with higher F ratio. In the first case, only TI leaves the large group of cultivars; in the second case both TI and NP migrate into it, although at a different level. These migrations can be observed in dendograms 2 and 3 (Figs 2 and 3). Nevertheless, except for this point, all three dendograms confirm the fundamental association between Spanish cultivars, as different from the rest.

By application of analysis of principal components (PCA) to the eight more significant amino acids, three new variables are obtained. It is found that the variance explained by these three principal components is 55.8, 15.5 and 10.4%, respectively, so that the first three components accumulate 81.7% of the total variance. The amino acids that contribute most to the first PC are val (weighing factor, wf = 0.940), ile (wf = 0.938), his (wf = 0.923) and phe (wf = 0.923). The second PC is mainly determined by ala (wf = 0.788) and ser (wf = −0.641) and the third PC by gln (wf = 0.577), met (wf = 0.569) and tyr (wf = 0.521).

Figure 4 shows a projection of scores for each cultivar in the reduced space determined by the first three PC, wherein the associations suggested by dendogram 3 are encircled. Cultivars TI and NP appear in an intermediate area, which explains the ability of these cultivars to migrate from one group to another.

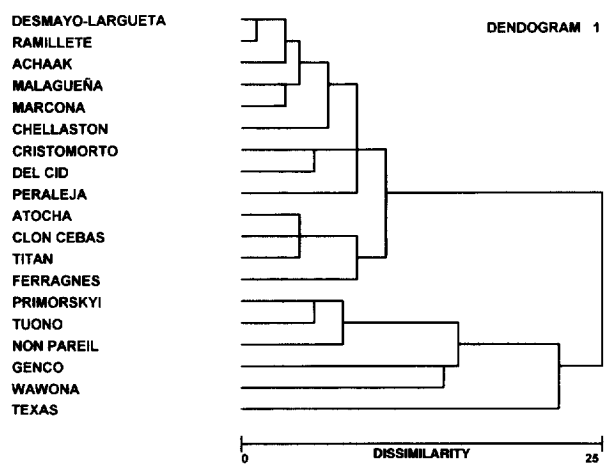


Fig. 1. Dendogram from all the amino acids.

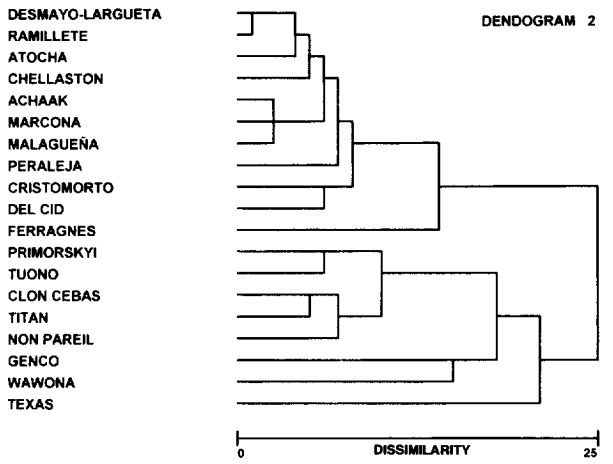


Fig. 2. Dendogram from all the amino acids except aspartic and glutamic acids.

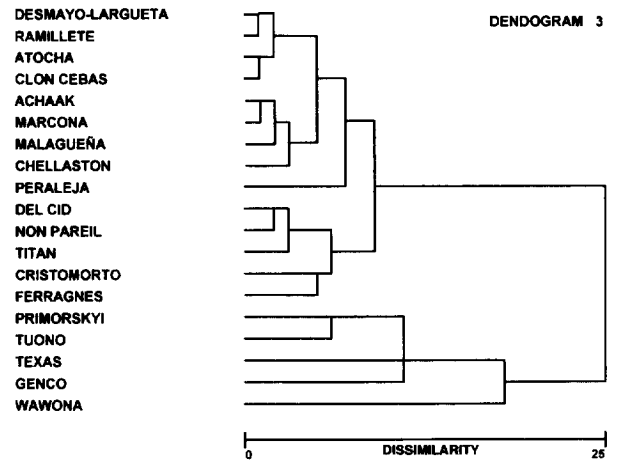


Fig. 3. Dendogram from the eight amino acids with the highest F ratios.

In comparing the migrations observed in dendograms 2 and 3 with the amino acids considered in their deduction, it follows that the amino acids which can determine these changes are glutamine, methionine, serine, glycine, tyrosine, or histidine, as they are the only ones not processed in dendogram 3. Now the unique amino acid in analysis of variance which associates TI and NP, and only with the American cultivars, is glutamine. It can be concluded that inclusion (or not) of glutamine in the amino acid set determines the association of TI and NP with American or Spanish cultivars.

Discriminant analysis has been applied to individual values of amino acids assuming there to be six different classes, three consisting each of a single cultivar (TE, GE and WA), an intermediate (TU+PR), and a large one, encompassing the Spanish cultivars, with which cultivars TI and NP are sometimes considered associated, and from which they are sometimes excluded (to be associated then with TU and PR). Now, a completely successful assignment of cultivars to their class is always achieved. In particular, discriminant functions can be found able either to associate TI and NP with Spanish cultivars or to differentiate them from these. Projections of cultivars scores on the three determined discriminant functions are

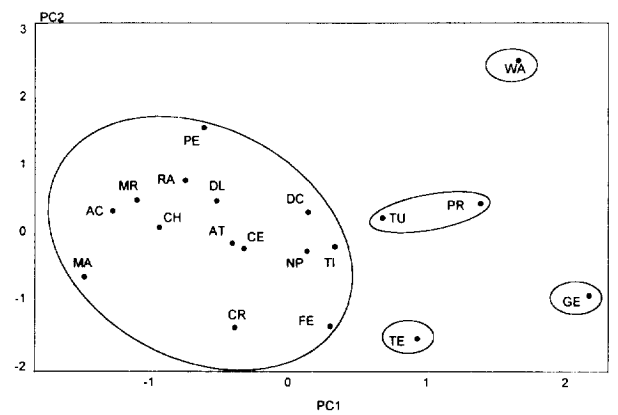
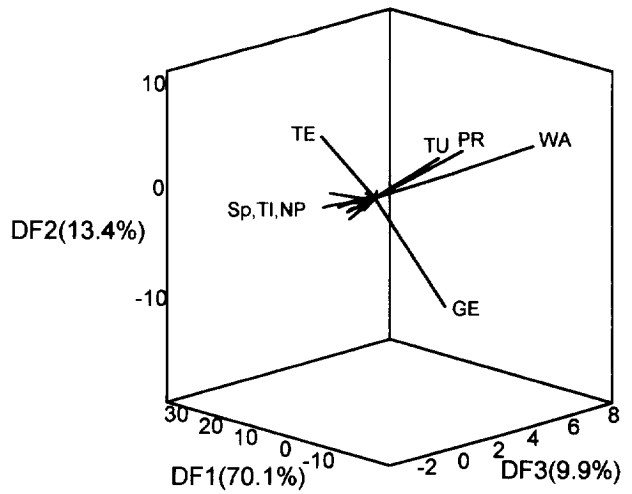


Fig. 4. Projections of cultivar scores on the space determined by PCs.



Spanish cultivars (Sp)

Fig. 5. Projections of cultivar scores on the space determined by DFs when cultivars TI and NP are grouped with the Spanish ones.

shown in Figs 5 and 6, wherein strong association between Spanish cultivars and the versatility of cultivars TI and NP is apparent. In these analyses neither glycine nor arginine have a tolerance level high enough to be significant in discriminant analysis. When TI and NP are associated with Spanish cultivars, the variance explained by the first discriminant functions are 70.09, 13.36 and 9.94%, respectively while, when they are associated with PR and TU (that is, nearer the American cultivars), the variance explained is 56.37, 24.38 and 12.32%, respectively . In either alternative, the most significant amino acid for the first discriminant function is leucine. For the second discriminant function, the amino acids isoleucine and valine are important when TI and NP are associated with Spanish cultivars, while glutamine determines

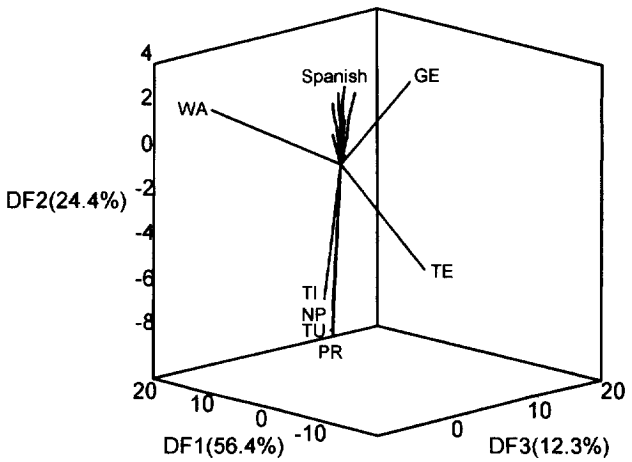


Fig. 6. Projections of cultivar scores on the space determined by DFs when cultivars TI and NP are grouped with TU and PR.

this function when they are differentiated from the Spanish ones.

CONCLUSIONS

Multivariate techniques applied to the amino acid composition of almond cultivars allow classification of Spanish cultivars as a consistent group, characterised by proper discriminant functions. Cultivars TE, GE and WA appear rather singular, and each can be considered as an independent class. Among Spanish cultivars, MR reveals some peculiar features. Cultivars TI and NP can be associated, for convenience, with the Spanish ones or brought near to the American ones, according to the amino acids processed, and, therefore, they can constitute a boundary class in this respect.

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