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Statistical comparative study of free amino acid HPLC data from a selected almond set

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Abstract

This paper compares the free amino acid profiles of five agriculturally important almond cultivars grown in four different geographical areas and conditions, discussing the relative influence of locality and cultivar in this chemical fraction. Some amino acids do indeed seem to be associated with the different areas and others with the cultivars. Nevertheless, multivariate statistical techniques reveal the ability of amino acids to associate cultivars, independently of locality, in two groups. The first group includes the Spanish cultivars Marcona and Desmayo-Largueta, and the second one brings together Cristomorto, Ferragnes and Ferraduel cultivars, which are genetically related.

1. Introduction

Comparative studies on the chemical composition of almond cultivars, grown in Spain are scarce (Gomis et al., 1990; Calull et al., 1990). Previous studies in this Department (Prats and Berenguer, 1994; Seron et al., 1997) have shown that multivariate statistical techniques can reveal some ability of free amino acids in almonds to differentiate cultivars or groups of cultivars cultivated in the same field. The aim of this paper is to study the influence of locality in the amino acid composition of almonds, in order to test the consistency of this conclusion. For this purpose, we apply the same statistical techniques to several samples of five cultivars cultivated in four different localities. All the samples are considered different at the beginning of the study, and we checked whether the samples of the same cultivar associate or not, independently of the locality.

The selected cultivars, except Cristomorto, are commercially very important in Spain, and known in many countries. Their cultivation is extended in very different Spanish regions, and therefore they are suitable for studying the influence of external conditions. Moreover, it is known that Ferragnes and Ferraduel are genetically related to Cristomorto, and for this reason they are all especially fitted to offer evidence of characteristic properties of cultivars. The origins of Marcona and Desmayo-Largueta are unknown, and it is generally assumed that they are not monoclonal. Nevertheless, they have excellent organoleptic properties, specific morphological characteristics and in previous studies (Prats and Berenguer, 1994; Seron et al., 1997) have always shown distinguishing features in their composition compared with the other Mediterranean cultivars.

2. Materials and methods

2.1. Samples

The set of almonds considered consists of three samples of five different cultivars (Desmayo-Largueta, Cristomorto, Ferragnes, Ferraduel and Marcona), from four different Spanish localities: Alcañiz (A) in Teruel, Castalla (C) in Alicante, Reus (R) in Tarragona and Santomera (S) in Murcia. Castalla, and especially Alcañiz, are cold and high regions; in contrast Reus and Santomera have a typical Mediterranean climate. The localities are far enough from each other and the agricultural conditions are not the same, so significant differences are expected among them due to the soils and cultivation systems. All of the samples belong to the 1995 harvest.

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After blanching, the seeds were ground and the fraction passing through a 1 mm light sieve was dried at 60°C, stored in a desiccator, and finally submitted to fat extraction and chromatographic analysis.

2.2. Chemicals

Methanol, tetrahydrofuran and acetonitrile were all of HPLC gradient grade, from Lab Scan (Unit T26 Stillorgan Ind, Park, Co. Dublin, Ireland). Amino acids, 3-mercapto-propionic acid, o-phthalaldehyde (OPA) and amino acid internal standards (norvaline and taurine) were of analytical grade from Sigma (St Louis, MO, USA). HPLC-quality water was from a MilliQ system (Millipore, Bedford, MA, USA). Salts for buffer solutions and for adjusting ionic strength were likewise of analytical grade, from Merck (Darmstadt, Germany).

Amino acid standard stock solutions of about 10^{-2} M were prepared in HCl (1:10) and stored in a refrigerator below 4°C. Working standards were prepared daily by serial dilution with water.

The amino acid derivatizating solution was obtained by adding 50 µl of 3-mercapto-propionic acid and 0.5 ml of 1.0 M sodium borate (pH = 9.5) to 50 mg o-phthalaldehyde previously dissolved in 4.5 ml of methanol. The reagent mixture had to be kept in a refrigerator below 4°C and be freshly prepared every week.

2.3. Apparatus

The chromatograph used was a 'Waters Multisolvent HPLC system', equipped with a double piston pump and a Waters 600 E controller. The injection valve was a Rheodyne model 7125 with a 20 µl loop. Detection of OPA-derivatives was carried out fluorimetrically at an excitation wavelength of 340 mn and emission wavelength of 425 nm, using a Waters 474 detector, provided with a flow cell of 5 µl to optimise resolution of chromatographic peaks.

The column selected was a packed Waters AccQ.Tag type (Type RP bonded silica C18). The run of chromatograms and the processing of chromatographic data were performed with the aid of a computer, using the Waters program 'Baseline 810'.

A 'Selecta-P' centrifuge and a 'SBS.A-06' magnetic stirrer were used for the extraction of the amino acids. Absorbance measurements were carried out with a Hitachi U-2000 spectropholometer at a wavelength of 336 nm.

2.4. Amino acid extraction

The extraction solvent was a methanol:water (10:2) mixture, containing the internal standards (norvaline and taurine).

About 0.2 grams of defatted sample (exactly weighed) was stirred for 15 min with 15 ml of the solvent. Afterwards, the sample was centrifuged for 15 min at 4500 r.p.m. and then, the liquid was decanted and filtered through a 0.45 µm filter. From this filtrate, a sample was taken and submitted to chromatography. The whole process was repeated until total removal of amino acids (3 times). The recovery of amino acids in the first extraction was approximately constant, about 85%, as proved by absorption measurements.

2.5. Chromatographic conditions

Separation of amino acids was achieved after a pre-column derivatization with o-phthalaldehyde. This method was chosen for its good resolution and high sensitivity (Godel et al., 1984, 1992). Derivatization and chromatographic procedures were carried out following the indications previously described (Seron et al., 1997).

All the analyses were done at room temperature with a flow rate of 1 ml min^{-1}. A gradient from a binary eluent was employed for the separation of the OPA-derivatives, in which solvent A was a phosphate buffer solution, pH = 6.4 (adjusted with KOH), with 2% of tetrahydrofuran and solvent B was a mixture of the phosphate buffer, methanol and acetonitrile (50:35:15).

The optimum gradient programme found starts with a linear ramp from 5% to 10% in B for 2 min, then the composition is held at 10% in B for 8 min; after that was a linear ramp to 80% in B for 30 min, and next another linear ramp to 100% in B for 3 min. This proportion is maintained for 3 min, and finally a ramp to 5% again for 9 min, that is 5% in B. The gradient program appears drawn over the chromatogram in Fig. 1.

Amino acids, determined in order of increasing retention time, are: aspartic acid (ASP), glutamic acid (GLU), asparagine (ASN), serine (SER), glutamine (GLN), glycine (GLY), threonine (THR), histidine

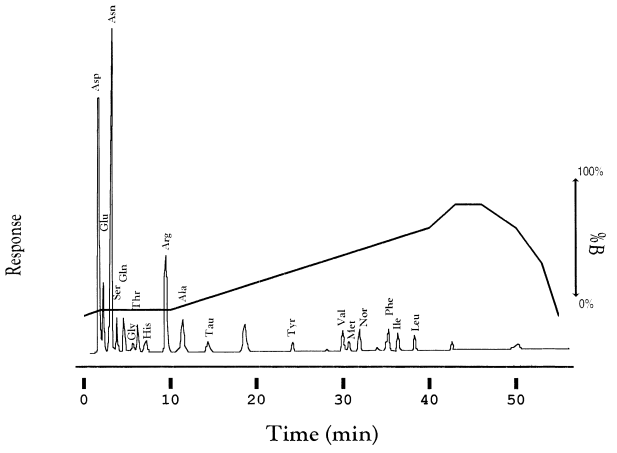

Fig. 1. Typical chromatogram of free amino acids in almonds with their elution gradient.

(HIS), arginine (ARG), alanine (ALA), tyrosine (TYR), valine (VAL), metheonine (MET), phenylalanine (PBE), isoleucine (ILE) and leucine (LEU).

2.6. Statistical data processing

Several statistical methods in SPSS (Release 6.0.1, 1994) package were used for the treatment of data: one-way ANOVA, principal component analysis (PCA), hierarchical cluster analysis (CA) and linear discriminant analysis (LDA). Details for all these methods were given in a similar study applied to the fatty acid composition (Garcia et al., 1996).

3. Results and discussion

120 chromatographic determinations corresponding to the 60 samples in the whole were carried out by HPLC. 16 amino acids were identified in all the samples (Fig. 1). The concentration in, percentage of each amino acid, was determined by means of the internal standard method.

Table 1 shows the mean values from six determinations of each one. Standard deviations of the means were always under 6%, except for aspartic and glutamic acids, which are poorly resolved and not precisely quantified, probably due to irregularities at the beginning of the chromatogram. Reproducibility of the addition of both peaks was not much better; that is why these two variables were dropped in the statistical process. Therefore, the number of variables was reduced from 16 to 14.

Even though, in the Table 1 and in the graphics, the mean values of replicates and samples are shown, for the sake of clarity, mathematical algorithms were applied to the averages of the two replicates of each sample.

A preliminary test for determining the ability of each amino acid to differentiate almond cultivars, was conducted by analysis of variance, whereby mean values for each amino acid are compared in all possible pairs of cultivar samples.

Amino acids serine and asparagine, with the highest F-ratio, present a great discriminant power among cultivars. On the other hand, arginine was not useful to establish differences among the cultivars studied because of its low F-ratio value.

This conclusion can be corroborated by representing mean values for each amino acid against cultivation locality (Fig. 2). The arginine graph allows differences among localities to be visualised, whereas the serine graph indicates differences among cultivars.

The dendrogram (Fig. 3) shows the results obtained by applying Cluster Analysis. In this figure Marcona and Desmayo-Largueta cultivars appear associated, while Ferragnes, Ferraduel and Cristomorto cultivars, form another group. With only a few exceptions, two blocks are apparent in the dendogram. At a dissimilarity level of ten, all samples are correctly classified according to the two mentioned blocks. As can be seen, the majority of exceptions refer to samples from one locality (Santomera) and can be attributed to particular conditions.

Significant data from the Principal Component Analysis (PCA) are shown in Tables 2 and 3. Only the

Table 1
Mean values of each amino acid in percentage (n = 6, from 3 samples and 2 replicates of each one)

	A+G	ASN	SER	GLN	GLY	THR	HIS	ARG	ALA	TYR	VAL	MET	PHE	ILE	LEU
CR(A)	63.9	11.6	4.35	2.51	0.41	2.41	3.82	1.45	4.66	0.36	1.26	0.13	1.37	1.11	0.66
DL(A)	60.0	18.1	1.98	2.19	0.28	1.81	2.75	3.11	3.41	0.54	1.38	0.58	1.84	1.36	0.65
FD(A)	54.8	16.3	3.16	2.04	0.45	2.07	3.56	3.70	4.18	0.56	2.74	0.51	2.85	2.07	0.99
FE(A)	57.7	21.1	3.19	2.67	0.31	1.60	2.47	2.81	4.41	0.22	1.03	0.11	1.03	0.86	0.52
MR(A)	59.9	22.3	1.77	2.96	0.34	1.66	1.96	2.92	2.94	0.28	0.71	0.13	1.14	0.60	0.42
CR(C)	58.1	18.1	2.12	1.87	0.40	2.01	2.67	0.26	4.20	0.67	2.46	0.53	2.94	2.34	1.36
DL(C)	63.0	18.6	1.84	2.03	0.28	1.68	2.65	0.33	2.49	0.53	1.52	0.48	2.06	1.72	0.76
FD(C)	57.0	17.4	2.35	1.80	0.42	3.13	3.17	0.45	4.32	0.70	2.48	0.76	3.24	2.04	0.79
FE(C)	55.5	19.4	2.43	1.80	0.37	2.34	2.79	0.26	4.33	0.64	2.76	0.63	2.76	2.46	1.52
MR(C)	57.5	25.0	1.60	2.35	0.31	1.80	2.01	0.41	2.65	0.57	1.38	0.29	1.87	1.53	0.75
CR(R)	53.8	17.4	2.75	2.31	0.44	2.10	2.99	4.31	4.59	0.63	2.54	0.67	2.32	1.98	1.14
DL(R)	56.3	19.5	1.45	1.96	0.31	2.75	3.03	4.20	3.43	0.74	1.50	0.85	1.72	1.56	0.78
FD(R)	51.2	17.6	3.26	2.03	0.44	3.08	3.48	2.65	4.09	0.72	3.16	0.91	3.21	2.59	1.60
FE(R)	52.5	18.0	2.54	1.39	0.26	2.71	3.23	2.42	5.92	0.75	2.84	0.53	2.71	2.42	1.84
MR(R)	59.0	21.8	1.86	2.22	0.35	1.93	1.82	2.86	2.50	0.71	1.15	0.24	1.97	1.03	0.61
CR(S)	46.4	12.2	2.48	6.47	0.67	3.00	4.46	0.97	5.81	2.35	3.38	1.36	4.81	2.63	3.03
DL(S)	43.2	22.9	2.19	3.93	0.69	3.00	3.51	1.63	5.84	2.00	2.74	0.81	3.82	1.91	1.87
FD(S)	61.1	12.6	3.42	2.35	0.41	2.30	3.67	0.18	3.54	0.67	2.52	0.48	3.35	2.07	1.44
FE(S)	41.2	14.6	2.79	7.11	0.74	3.07	4.50	1.18	4.64	2.70	3.68	1.70	5.23	2.94	3.94
MR(S)	50.9	20.9	2.06	3.72	0.61	2.59	2.95	1.69	4.44	1.43	1.90	1.08	2.98	1.49	1.24

principal components (PCs) which had an eigenvalue higher than 1 were retained. The total variance accounted for by the first three PCs is 81.2%. The loadings for each amino acid in the PCs retained and their communality values are given in Table 3. According to these data, arginine (which marks differences between localities as was mentioned above) is the amino acid with the highest weight in PC3, whereas the same amino acid scarcely has an influence in PC 1 and PC2. Since the first two PCs explain 59.4% and 13.5% of variance, respectively, (which together account, to a larger extent, for the variance explained), it is possible to establish

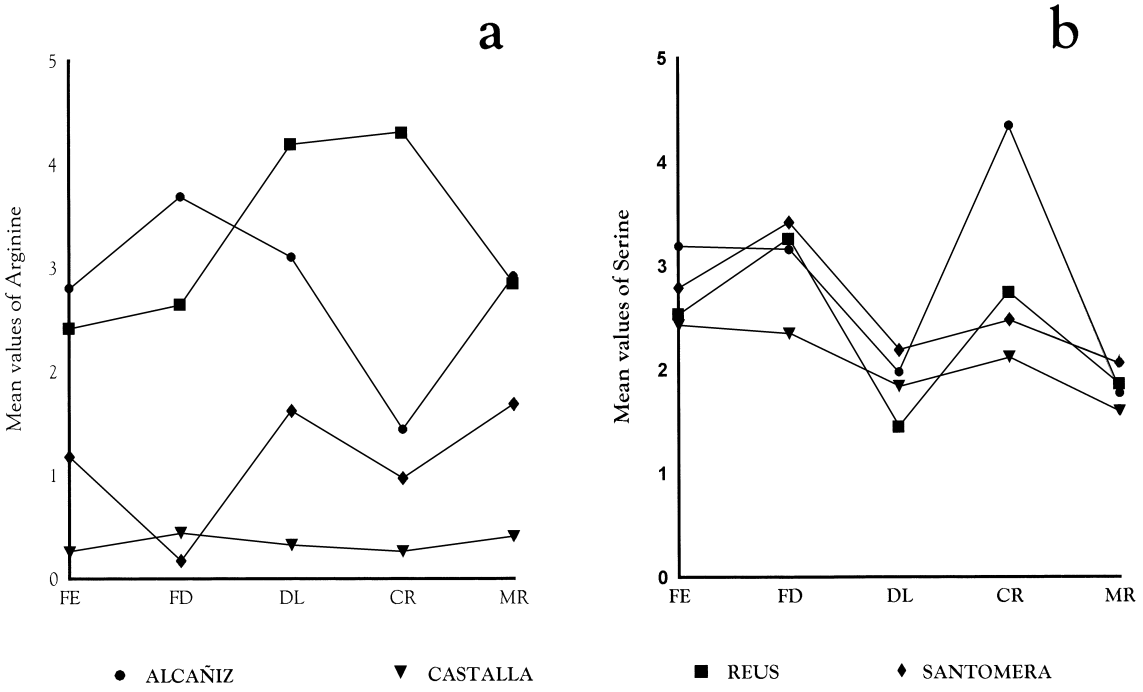

Fig. 2. Representation of the amino acid content mean values for each cultivar and locality. (a) Arginine. (b) Serine.

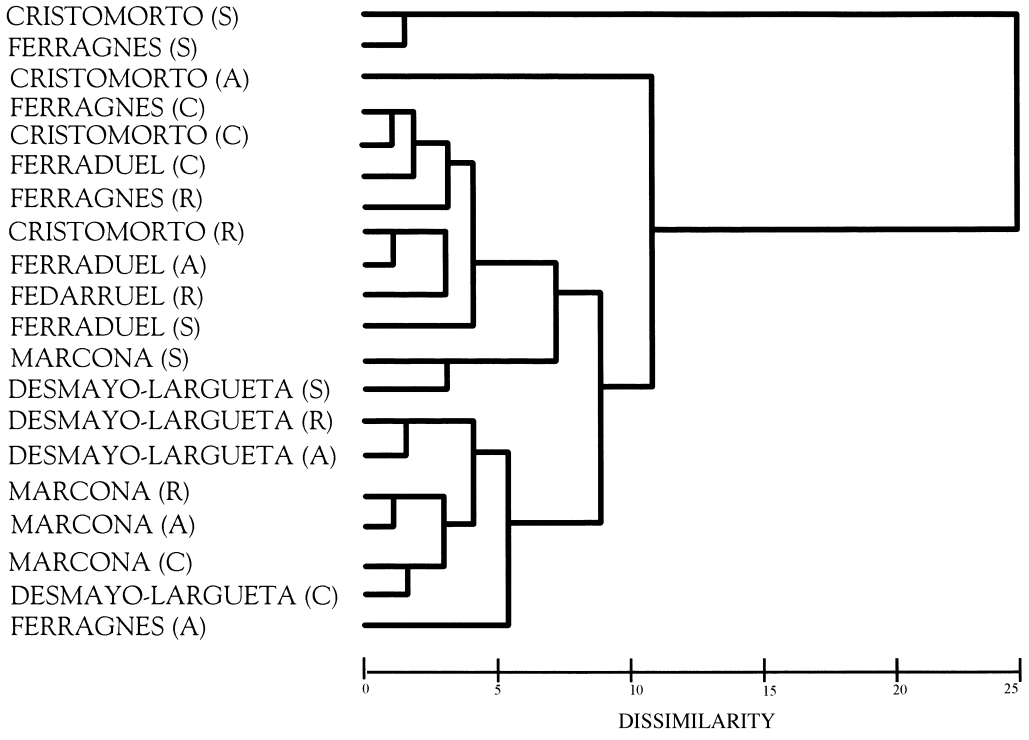

Fig. 3. Dendrogram showing the result of Cluster Analysis.

Table 2
Eigenvalues and the percentage of variance for the first three principal components

Principal component	Eigenvalue	Pct of variance	Cum Pct
1	8.35823	59.7	59.7
2	1.90388	13.6	73.3
3	1.15215	8.2	81.5

Table 3
Loadings for each amino acid studied in the first three principal components and their communality values

Variable	Principal component 1	Principal component 2	Principal component 3	Communality
Ala	0.68574	−0.26305	0.21041	0.58371
Arg	−0.27295	−0.06002	0.70515	0.57534
Asn	−0.54100	0.68064	−0.01069	0.75607
Gln	0.74015	0.33863	0.36519	0.79585
Gly	0.84122	0.22379	0.28523	0.83908
His	0.87973	−0.37518	0.13360	0.93301
Ile	0.80900	−0.09329	−0.46159	0.87627
Leu	0.93751	0.13248	−0.03564	0.89774
Met	0.88285	0.29407	0.03630	0.86722
Phe	0.94989	0.14257	−0.18241	0.95580
Ser	0.26824	−0.86665	0.17827	0.85481
Thr	0.79882	−0.03946	0.00323	0.63968
Tyr	0.88122	0.40122	0.16636	0.96521
Val	0.88862	−0.12311	−0.26394	0.87447

Table 4
Correlations between discriminating variables and canonical discriminant function

Variable	Discriminant function
Ser	0.31291
Asn	−0.25688
Ile	0.20288
Val	0.19840
His	0.19213
Ala	0.18100
Leu	−0.17092
Phe	0.11272
Arg	0.11158
Thr	0.09510
Met	0.05119
Gly	0.04244
Gln	0.01850
Tyr	0.01357

differences among cultivars even though the samples studied were from different localities.

Fig. 4 shows the mean scores for the 20 samples in the reduced space formed by the first two PCs. This graph shows that the cultivars Marcona and Desmayo-Largueta have values near to −1 in PC1 and positive values in PC2, whereas the cultivars Cristomorto, Ferraduel and Ferragnes present values near to zero in PC1 and negative values in PC2.

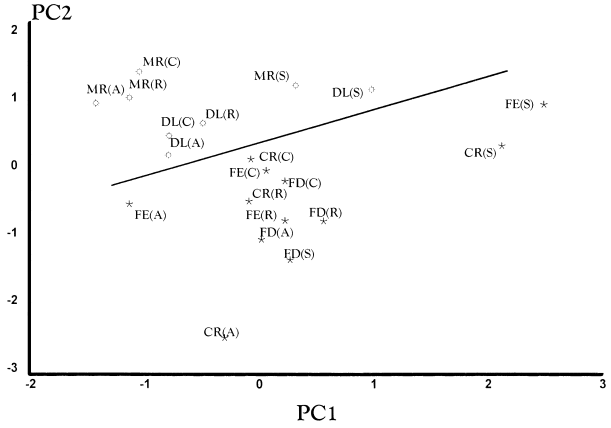

Fig. 4. Graph of the mean score for the samples projected on the reduced space by the first two principal components (PCs).

Taking into account the latter, these two groups were used when the linear discriminant analysis (LDA) was applied, with the aim of establishing the discriminant functions and the classification functions.

The correlation values obtained between the discriminant variables and the discriminant function calculated are given in Table 4. It can be seen that the amino acid serine has the biggest weight in this function. This fact was also noted in the analysis of variance. Three samples were used as the test set, a Marcona from Reus, a Ferraduel from Alcañiz and a Cristomorto from Santomera. All three, even the sample from Santomera, were successfully classified at 100%.

4. Conclusions

From a group of five cultivars (Cristomorto, Desmayo-Largueta, Ferraduel, Ferragnes and Marcona) not very different among themselves (Seron et al., 1997) and cultivated in different localities, a clear differentiation has been found, on the basis of their amino acid profiles, between two groups (a) Marcona and Desmayo-Largueta and (b) Cristomorto, Ferraduel and Ferragnes; (b) is made up of cultivars, that are genetically related. Serine and asparagine have proved to be suitable parameters for differentiating cultivars whereas arginine is more associated with localities.

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