JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 6841−6846 **6841**

Discriminating Significance of the Free Amino Acid Profile in Almond Seeds

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Known statistical techniques have been applied to the free amino acid composition of 107 samples from 10 different almond cultivars (Marcona, Desmayo-Largueta, Guara, Tuono, Ferragnes, Masbovera, Non Pareil, Titan, Texas, and Primorskyi) cultivated in seven different locations and growing conditions. It is concluded that free amino acid composition can constitute a basis for classifying and typifying these cultivars into five groups: (1) Marcona and Texas, (2) Ferragnes and Masbovera (and probably Primorskyi), (3) Tuono and Guara, (4) Non Pareil (and probably Titan), and (5) an isolated cultivar (Desmayo Largueta). As a result, an easy decision tree is proposed to discriminate the cultivar of an almond flour, as used in confectionery, if it consists of a single cultivar.

KEYWORDS: Almonds; free amino acids; multivariate techniques

INTRODUCTION

Almond trees have been cultivated in the Mediterranean basin for centuries. From there it was brought to many countries all over the world. In Spain almonds represent an important crop, with over 200 000 million tons produced annually, which sustains an active industrial sector. There is an impressive number of different known almond cultivars, even in the same country, but not all have the same market value. That is why there is considerable interest in the availability of reliable tests to ascertain the almond cultivar employed in a product, to ensure its quality and to prevent fraudulent practices. Normally, it is relatively easy to morphologically identify the fruit or seed from a cultivar, but this is not so in screening some industrial products, such as almond flour, which is frequently used in confectionery.

The mean chemical composition of almonds is well established regarding the proximate values and some particular fractions (*1*), as well as the individual components of important fractions (proteic, lipid, and hydrocarbon) for some cultivars (*2*−*5*). All of these values are very variable when taken individually, due to the influence of many external factors (*6*−*8*), but in our opinion the significance of their pattern in different cultivars has not been evaluated. Previous studies have pointed out that fatty acids (*9*, *10*), tryacylglycerols (*11*), and probably also the free amino acid composition (*12*, *13*) of different cultivars grown under the same experimental conditions show some differences, as revealed by multivariate methods, that can be used to classify or typify them. In fact, the free amino acid fraction was suspected to be a discriminator among almond cultivars in a previous work (*14*). The aim of this work was to test whether the free amino acid composition of almond seeds is a stable enough parameter to allow differentiation of almond cultivars, independent of growing location, or if, on the contrary, the growing conditions and the site of cultivation have such an influence as to obliterate the possible discriminating significance of this chemical fraction.

With this purpose, 10 different almond cultivars were studied. The samples were obtained from seven locations with different growing conditions and climates in order to represent, as much as possible, the variability of chemical data.

MATERIALS AND METHODS

Samples. The cultivars (**Table 1**) were grown in the following different geographical areas: Maria (Almeria, ALM), La Puebla de Don Fabrique (Granada, GR), Santomera and Cehegín (Murcia, MUR), Castalla and Bacarot (Alicante, ALI), Mas Valero, Mas Bove, and Mora d'Ebre (Tarragona, TAR), and Aula Dei (Zaragoza, ZAR), all in Spain, and Avignon (AVIG), France. These locations differ significantly in climatological conditions (**Table 2**). Granada and Zaragoza are interior regions with continental weather, while the others localities situated near the coast have typical Mediterranean climates, although under different moisture and/or irrigation conditions. The total number of samples studied was 107.

Almond seeds were blanched and ground in an electric grinder, and afterward the fat was extracted in a homemade apparatus which works in the same way as the commercial Sohxtec (J.P. Selecta S.A., Barcelona, Spain). The extraction was carried out at 60 °C using cellulose thimbles, 24 mm × 80 mm (Whatman International Ltd., Maidstone, England), as sample holders and a mixture of hexane with diethyl ether (1:1) as solvent. The total extraction time was 2.25 h, including the time needed for the evaporation of the organic solvent.

Reagents. Methanol, tetrahydrofuran, and acetonitrile were all of HPLC gradient grade, from Lab Scan (Unit T26, Stillorgan Ind. Park Co., Dublin, Ireland). All amino acids, 3-mercaptopropionic acid, and *o*-phthalaldehyde (OPA) were of analytical grade from Sigma (St Louis,

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10.1021/jf020108t CCC: $22.00
Published on Web 10/10/2002

Table 1. Almond Samples Included in This Study

cultivar name	abbreviation	hybrid	no. of samples included in the study	no. of samples and site of cultivation
Desmayo Largueta	DL	unknown	16	1 Alicante, 3 Almería, 1 Avignon, 3 Granada, 4 Murcia, 2 Tarragona, 2 Zaragoza
Ferragnes	FE	Ai × Cristomorto	16	1 Alicante, 3 Almería, 1 Avignon, 3 Granada, 3 Murcia, 3 Tarragona, 2 Zaragoza
Guara	GU	unknown	13	1 Alicante, 3 Almería, 1 Avignon, 3 Granada, 2 Murcia, 1 Tarragona, 2 Zaragoza
Marcona	MR	unknown	17	2 Alicante, 3 Almería, 1 Avignon, 3 Granada, 4 Murcia, 2 Tarragona, 2 Zaragoza
Masbovera	MS	Primorskyi × Cristomorto	10	1 Avignon, 4 Murcia, 3 Tarragona, 2 Zaragoza
Non Pareil	NP	unknown	8	3 Almería, 2 Avignon, 1 Tarragona, 2 Zaragoza
Primorskyi	PR	Princesse2077 × Nikitskyi53	4	1 Avignon, 1 Murcia, 2 Zaragoza
Texas	TE	unknown	8	1 Alicante, 3 Almería, 1 Avignon, 1 Tarragona, 2 Zaragoza
Titan	TI	unknown	4	2 Murcia, 2 Zaragoza
Tuono	TU	unknown	11	3 Almería, 1 Avignon, 3 Granada, 1 Murcia, 1 Tarragona, 2 Zaragoza

Table 2. External Growing Conditions of the Locations Where Samples Were Cultivated

site of cultivation	altitude (m)	rainfall[a] (mm/m^2)	temp[b] (°C)	growing conditions
Maria (Almería)	1150	340	17.1–5.8	nonirrigated
La Puebla (Granada)	1100	337	21.8–7.3	nonirrigated
Santomera (Murcia)	130	305	24.2–11.1	nonirrigated
Cehegín (Murcia)	500	367	22.0–9.9	nonirrigated
Castalla (Alicante)	600	340	20.7–9.5	nonirrigated
Bacarot (Alicante)	65	305	23.4–11.2	nonirrigated
Mas Valero (Tarragona)	50	518	20.1–11.9	nonirrigated
Mora d'Ebre	65	518	20.1–11.9	nonirrigated
Mas Bové	117	518	20.1–11.9	nonirrigated
Aula Dei (Zaragoza)	nd[c]	340	20.0–10.0	irrigated
Avignon (France)	nd	700	19.3–9.8	irrigated

[a] Average rainfall per year. [b] Maximum and minimum temperatures. [c] nd, no data.

MO). HPLC-quality water was obtained from a Milli-Q system (Millipore, Bedford, MA). Salts for buffer solutions (KH_2PO_4, K_2HPO_4, and H_3BO_3) were of analytical grade from Merck (Darmstadt, Germany). Hexane and diethyl ether were from Prolabo (Fontenay S., France).

Amino acid standard solutions of about 10^{-2} M were prepared in 0.1 M HCl from a commercial stock solution, reference AA-S-18, from Sigma and stored in a refrigerator below 4 °C. Working standards were prepared every week by dilution with water in the range of concentration between 5 and 40 ppm.

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

Instrumentation. The free amino acid content was determined using a Waters Multisolvent HPLC system, equipped with a double piston pump and a Waters 600E controller. Detection of OPA derivatives was carried out fluorimetrically at an excitation wavelength of 340 nm and an emission wavelength of 425 nm, using a Waters 474 detector provided with a flow cell of 5 μL to optimize resolution of chromatographic peaks.

The selected column for the separation of the free amino acids was a Waters AccQ.Tag, 3.9 mm × 150 mm (Waters, Milford, MA). The chromatograms were run and the data processed with the program Baseline 810 (Waters).

Sample Preparation. About 0.2 g of defatted sample was stirred for 15 min with 10 mL of a mixture of methanol and water (80:20) containing the internal standards (norvaline and taurine); the sample was then centrifuged for 15 min at 4500 rpm, and the liquid was decanted. The whole process was repeated two more times. The three aliquots were combined to obtained the amino acid almond extract.

Derivatization was carried out by mixing and shaking, for 1 min in an Ependorf tube, 0.2 g of the amino acid extract together with 0.2 g of boric buffer (pH 9.5), 0.6 g of water, and 50 μL of the derivatizating solution of *o*-phthalaldehyde.

Chromatographic Conditions. The chromatographic procedure was essentially the same as that previously described (*12*, *13*). Twenty microliters of the derivatizated solution was filtered through a 0.45 μm Millipore Millex filter (Millipore, Bedford, MA) and analyzed by HPLC at room temperature with a flow rate of 1 mL/min. A gradient elution was achieved by using two solvents: solvent A, phosphate buffer solution (pH 6.4) plus 1% of tetrahydrofuran; solvent B, a mixture of phosphate buffer, methanol, and acetonitrile (50:35:15).

The gradient started with a linear ramp from 5% to 10% in solvent B for 2 min, and then the composition was held at 10% in solvent B for 8 min; after that, a linear ramp to 80% in solvent B followed for 30 min, and next another linear ramp to 100% in solvent B for 3 min. This proportion was maintained for 3 min, followed by a final ramp to 5% solvent B for 9 min, to recover the initial chromatographic conditions.

Norvaline and taurine were used as internal standards because the authors verified that almond samples did not contain any of them and also because they appeared totally resolved from the rest of the amino acids in the chromatogram. Taurine was used as an internal standard for the amino acids that eluted before 10 min and norvaline for the rest of the amino acids considered.

Statistical Data Treatment. Experimental data were processed with the aid of the SPSS statistical package, version 10.0 (*15*). An analysis of variance was applied using the Tukey-b test to determine the least significant difference among means at the level of 0.05. Principal component analysis (*16*) was employed to visualized possible differences or similarities among the cultivars. The number of the components to be retained was selected by using the Scree test and Kaiser criteria. Cluster analysis was carried out by applying the Ward method for agglomeration, with the square of Euclidean distance as the criterion of proximity (*17*). A linear discriminant analysis was conducted stepwise by employing Wilks's λ statistics for variable selection (*18*). In all cases, the algorithms used were applied to the mean values obtained from three replications for each sample.

RESULTS AND DISCUSSION

The amino acids identified and quantified in the chromatograms in increasing order of retention time were aspartic acid (Asp), glutamic acid (Glu) + asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), threonine (Thr), hystidine (Hys), arginine (Arg), alanine (Ala), taurine (Tau), tyrosine (Tyr),

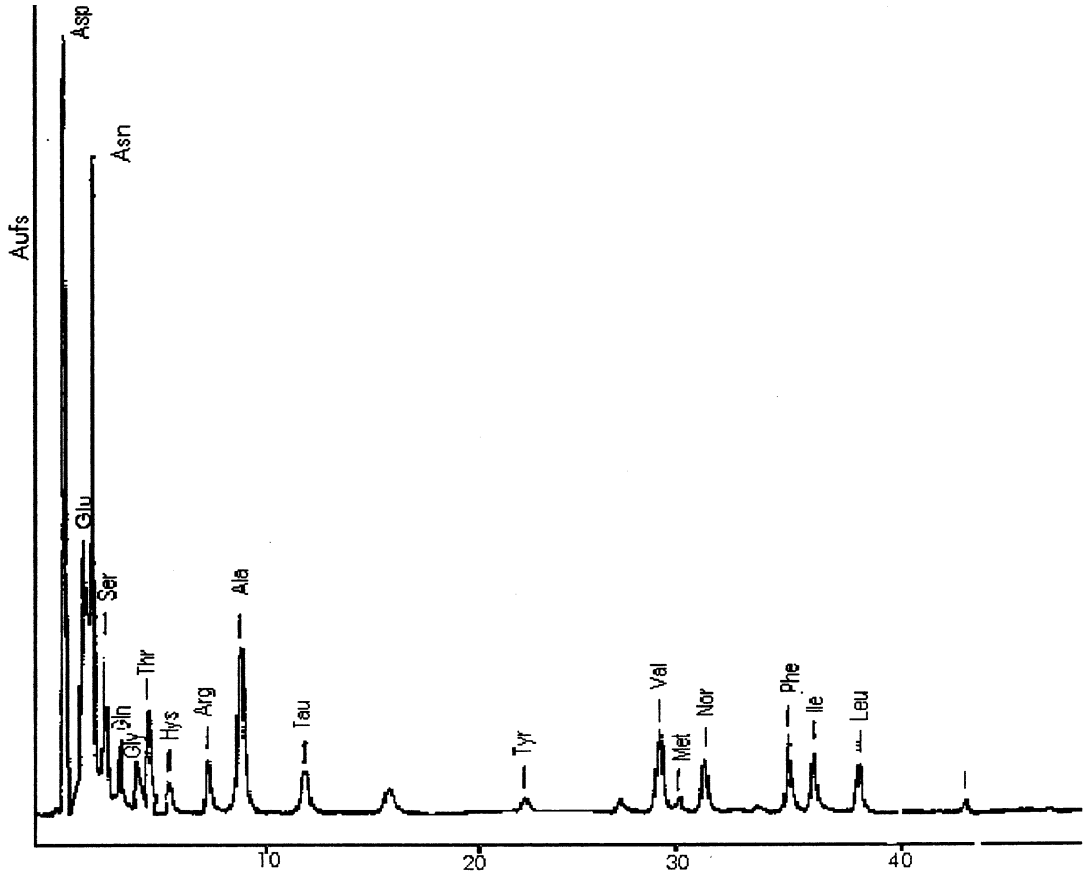

Figure 1. Chromatogram obtained when a Marcona sample was analyzed.

Table 3. Free Amino Acid Mean Values (mg per 100 g of Dry Sample) and Relative Standard Deviations of All Samples Belonging to the Same Almond Cultivar Grown in Different Localities[a]

cultivar		Asp	Asn+Glu	Ser	Gln	Gly	Thr	His	Arg	Ala	Tyr	Val	Met	Phe	Ile	Leu
DL	mean	28.31^{b,c}	34.97^{b}	6.03^{b,c}	4.38^{b,c}	0.91^{b}	5.00^{f}	0.98^{a}	3.44^{a}	3.87^{a}	0.55^{b,c}	2.75^{a,b}	0.37^{a}	3.93^{c}	2.64^{b,c}	1.69^{b,c}
	%rsd	6.13	6.64	7.11	11.88	11.25	13.65	7.76	15.92	14.24	11.75	14.21	19.11	11.22	15.32	16.45
FE	mean	24.02^{a,b}	36.38^{b}	5.39^{b,c}	3.03^{a}	0.90^{b}	3.99^{d,e}	1.60^{b}	4.73^{a,b,c}	7.45^{c}	0.45^{a,b}	3.28^{b,c}	0.46^{a,b}	3.39^{b,c}	2.70^{c}	2.15^{b,c}
	%rsd	6.97	8.18	12.24	22.67	8.82	12.54	26.10	16.55	12.31	13.51	18.55	18.68	15.72	15.22	16.42
GU	mean	31.12^{c}	31.90^{a,b}	5.39^{b,c}	4.83^{c}	0.97^{b}	3.70^{d}	1.24^{a,b}	6.08^{b,c,d,e}	3.94^{a}	0.57^{c}	2.44^{a}	0.41^{a,b}	2.96^{c}	2.09^{a,b}	1.80^{b}
	%rsd	7.25	9.02	25.55	13.59	9.01	17.08	10.54	19.94	36.32	16.34	11.47	14.52	13.02	20.50	7.06
MR	mean	24.54^{a,b}	48.12^{c}	2.96^{a}	2.71^{a}	0.70^{a}	2.61^{a,b,c}	0.86^{a}	4.83^{a,b,c,d}	4.02^{a}	0.44^{a,b}	2.14^{a}	0.36^{a}	2.99^{b,c}	1.75^{a}	1.19^{a}
	%rsd	8.79	7.09	21.28	27.32	19.46	27.08	42.98	45.85	19.33	15.74	24.17	35.46	15.93	28.19	31.60
MS	mean	27.57^{b,c}	31.64^{a,b}	4.99^{b}	3.07^{a}	1.06^{b}	4.55^{d,e}	1.55^{b}	3.28^{a}	7.95^{c}	0.58^{c,d}	3.87^{c}	0.41^{a,b}	4.22^{c}	3.07^{c}	2.64^{d}
	%rsd	16.19	14.54	21.40	23.55	7.78	16.67	16.71	23.91	6.90	19.12	11.89	50.59	23.62	14.71	16.03
NP	mean	29.45^{c}	33.34^{b}	6.71^{c}	4.36^{b,c}	0.93^{b}	3.19^{a,b}	1.45^{b}	3.85^{a,b}	4.66^{a,b}	0.45^{a,b}	3.45^{c}	0.34^{a}	2.93^{a,b}	2.91^{c}	1.98^{b,c}
	%rsd	7.67	3.23	14.72	22.69	9.71	20.95	6.27	45.86	37.38	15.37	6.02	18.86	36.53	6.12	12.05
PR	mean	32.10^{c}	31.17^{a,b}	5.68^{b,c}	3.32^{a,b}	1.34^{c}	2.44^{a,b}	1.25^{a,b}	3.45^{a}	5.68^{b}	0.59^{c,d}	3.34^{b,c}	0.36^{a}	4.17^{a,b}	2.83^{c}	2.28^{c,d}
	%rsd	19.84	22.74	25.01	21.86	11.88	10.79	10.20	6.85	12.19	10.85	13.38	20.26	15.33	21.15	30.24
TE	mean	21.33^{a}	43.73^{c}	5.30^{b,c}	3.08^{a}	0.91^{b}	2.16^{a}	1.41^{b}	7.28^{d,e}	4.63^{a,b}	0.37^{a}	2.30^{a}	0.37^{a}	2.60^{a,b}	1.91^{a}	1.10^{a}
	%rsd	18.46	10.69	24.36	24.23	18.65	19.97	13.37	29.64	29.25	11.15	12.94	31.93	20.33	15.84	9.42
TI	mean	30.17^{c}	31.06^{a,b}	4.90^{b}	2.94^{a}	1.06^{b}	2.75^{a,b,c}	1.26^{a,b}	7.18^{c,d,e}	3.60^{a}	0.63^{d}	4.17^{d}	0.55^{b}	3.87^{c}	3.78^{c}	2.11^{b,c}
	%rsd	22.10	26.01	42.74	22.40	20.83	16.81	21.58	14.81	5.55	19.23	5.90	30.14	16.45	12.99	10.03
TU	mean	30.56^{c}	27.82^{a}	6.19^{b,c}	4.68^{c}	0.94^{b}	3.46^{c,d}	1.54^{b}	8.36^{e}	5.22^{a,b}	0.50^{b,c}	2.12^{a}	0.32^{a}	2.41^{a}	2.08^{a,b}	1.84^{b,c}
	%rsd	12.59	10.23	9.75	30.88	18.92	11.19	25.92	34.63	28.64	12.47	19.17	18.29	16.74	21.58	14.57

[a] Values in the same column with different superscript letters (a–f) are significantly different at $p < 0.05$.

valine (Val), methionine (Met), norvaline (Nor), phenylalanine (Phe), isoleucine (Ile), and leucine (Leu).

In comparison to a previous work (*13*), better resolution of Asp and Glu plus Gly, Tyr, and Hys was attained, presumably due to a slight modification in the composition of one of the solvents (solvent A), but, on the other hand, Glu and Asp coeluted, as shown in **Figure 1**. All samples were analyzed in triplicate, and the relative standard deviations were lower than 5% in all cases, showing good reproducibility of the chromatographic method.

In **Table 3** are shown the free amino acid mean values and relative standard deviations for all samples belonging to the same almond cultivar (10 values for each amino acid) grown in the localities included in the analysis, but they are not the ones used in the statistical procedure, in which the mean values for the three replications of 107 samples analyzed were used (107

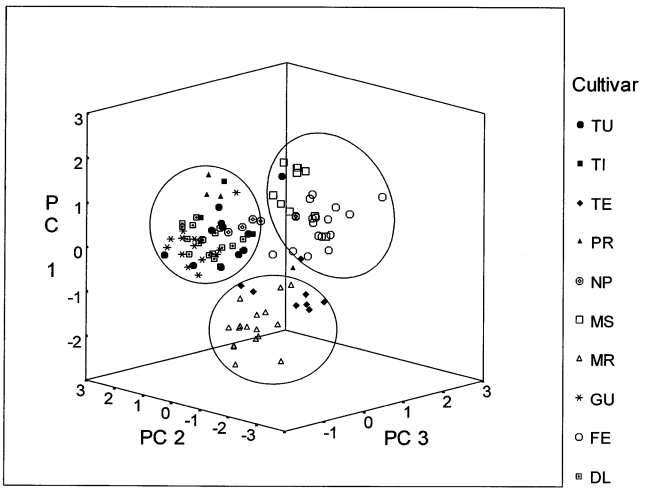

Figure 2. Score representation of the 107 samples in the reduced space established by the first three principal components.

values for each amino acid). From the table it can be observed that three major amino acids (Asp, Glu, and Asn) are found in the almonds, representing more than 60% of the total content of free amino acids. Ser, Gln, Thr, Arg, Ala, Phe, Val, Ile, and Leu amount to less than 6% each. The remaining free amino acids, Gly, His, Tyr, and Met, amount to less than 1% each. The high relative standard deviations observed highlight important variations in composition of samples of a given cultivar from location to location, for example, the composition of Ser in Titan cultivars or Met in Masbovera cultivars.

The total free amino acid contents for all cultivars are similar, ranging from 98.08 to 100.45 mg/100 g dry weight. In addition, differences in individual amino acid compositions seem to be small. However, the potential significance of amino acids to characterize the different cultivars, as such, can be demonstrated by applying a one-way ANOVA to the mean values for each amino acid in all possible pairs of cultivars, regardless of the site of cultivation, or in all pairs of sites of cultivation, regardless of the cultivar.

When cultivars are compared, some amino acid contents appear significantly different and consequently establish classes, which are represented in **Table 3** in superscript letters. (It follows, for example, that Asn + Glu and Leu contents are significantly different for the cultivars Marcona and Texas compared to the eight remaining ones, and that Ala content in Ferragnes and Masbovera cultivars is significantly different from the rest).

In contrast, when all amino acids from different sites of cultivation are compared, regardless of cultivars, no significant differences are observed, suggesting that the cultivar variable has a stronger influence on the free amino acid profile than the variable site and way of cultivation, or at least enough of an influence to be apparent regardless of the weight the external conditions clearly have.

Multiple regression techniques, studying the amino acid patterns of different cultivars, have proved to be very useful in supporting the discriminative significance of this fraction as a whole. Principal component analysis allows us to visualize, when the first three principal components are represented (**Figure 2**), some similarities between the cultivars Marcona and Texas and between the cultivars Masbovera and Ferragnes cultivated in different localities, while the rest of the cultivars appear rather undistinguished. Similarly, cluster analysis is a convenient way to classify all the cultivars on the basis of a

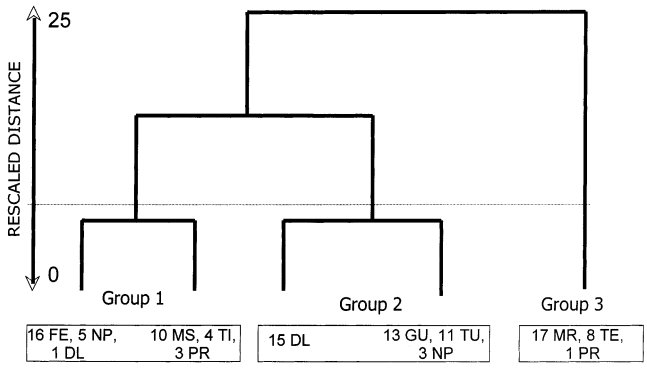

Figure 3. Dendrogram obtained after applying a cluster analysis to the sample set.

nearness criterion. In the summary resultant dendrogram shown in **Figure 3**, at a rescale distance of 10, three groups of samples can be distinguished. The first one encompasses all Ferragnes and Masbovera samples, together with some samples of Titan, Non Pareil, and Primorskyi. The second group contains all samples of the cultivar Desmayo Largueta, Guara, and Tuono and three samples of the cultivar Non Pareil. All samples of the cultivars Marcona and Texas and one sample of the cultivar Primorskyi form the third group.

Based on the previous information, a linear discriminant analysis demonstrates the ability of the amino acid fraction to find some classification functions that can be used to assign unknown samples to previously established groups. These groups were (1) Desmayo Largueta, (2) Ferragnes and Masbovera, (3) Guara and Tuono, (4) Marcona and Texas, and (5) Non Pareil. Titan and Primorskyi cultivars were not included in the calculation of the discriminant functions because of the small number of samples used.

Four discriminant functions were obtained by using the variable selection rule for minimizing Wilks's λ. The variance explained by each discriminant function was 48.4%, 27.6%, 17.5%, and 6.5%, respectively. The variables Gly and Arg were excluded from the calculation because they presented an F to enter lower than 1.000 (0.711 and 0.362).

To validate the proposed model, 10 different test sets (constituted randomly by approximately 25% of the samples) were used as unknown samples to establish a classification of them in one of the groups previously mentioned. The predicted model was applied to each sample of the test set, obtaining a correct classification of the samples in the groups in more than 98% of the cases.

Even though it is quite difficult to compare these results with the ones obtained in previous works because the cultivars analyzed are not the same, the authors find a good agreement between some of the results obtained in ref *13* and the present work. In both studies, Ser, Asn, and Glu are shown to be essential variables for differentiating cultivars, whereas Arg and Gly are more associated with the localities of cultivation.

As a whole, the statistical approaches have proved to be very useful to reveal concealed similarities and dissimilarities between related cultivars. Our findings demonstrate the subtle robustness of the chemical composition of natural goods, which sometimes emerges even in presumably different external conditions. The associations established on the basis of amino acid composition are congruent with known genetic relationships or could be proposed as heuristic hypotheses in agronomical research. The relationship between Ferragnes, Masbovera, and Primorskyi cultivars can be justified because Ferragnes and Primorskyi have Cristomorto as a common ancestor, and the cultivar Masbovera

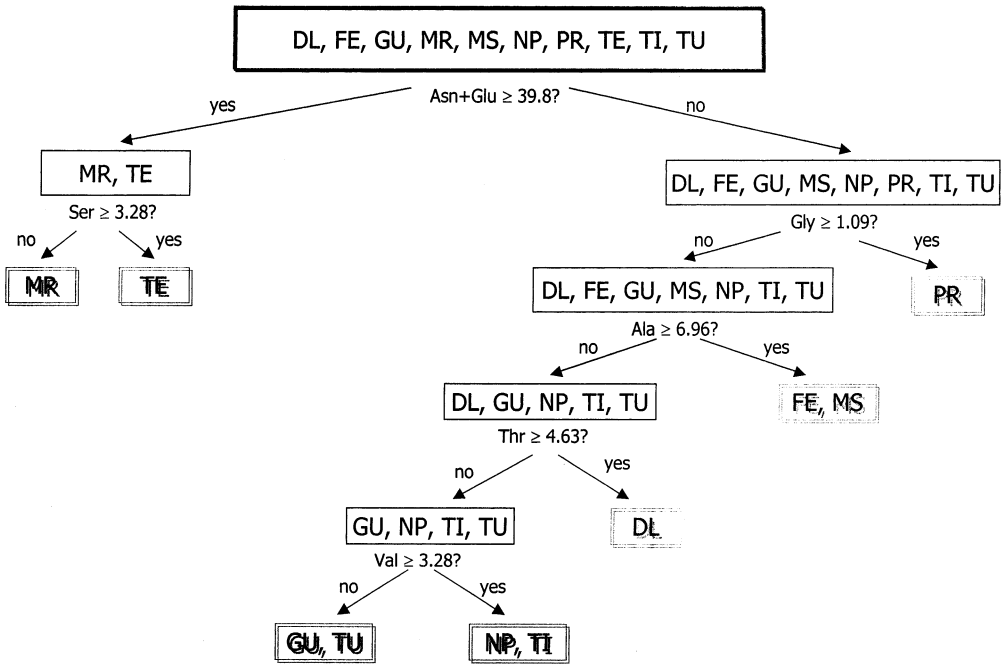

Figure 4. Decision tree that allows classification of the cultivars studied on the basis of the composition of some amino acids.

comes from Primorskyi cultivar. Tuono is suggested to be probably genetically related to the Guara cultivar. The origin of the cultivar Texas (known also as Mission) is unknown, but its classification along with Marcona could point out a common ancestor, quite possible if Spanish missionaries brought the almond culture to America; a common origin is also probable for the two American cultivars, Titan and Non Pareil. Finally, cultivar Desmayo Largueta, extensive in Spain, appears as a unique cultivar, in terms of the amino acid composition.

The statistical techniques used in this study are quite difficult to use in common practice, but they ensure the value of simple practical guidelines to determine the cultivar from which a product is made. The mean values of some amino acids are so different, as the Tukey-b test reveals, that they can be employed to elaborate a decision tree for discriminating the cultivars studied in a simple way. In **Figure 4**, a scheme (yes/no) is proposed that can be followed in order to decide what almond cultivar is the constituent of a ground almond in a sample, as an almond flour, supposing that it consists of a single cultivar. A cultivar is identified as soon as a question in the tree is answered with a "yes". The interest of this scheme is related to its being based to a great extent on amino acids that elute early (up to 2 min in the case of Marcona) in the chromatographic run, and therefore it allows the chromatographic analysis time to be reduced significantly. Accordingly, a Marcona cultivar, whose identification is of great concern for the manufacturers and customers in Spain, can be identified very easily if the asparagine + glutamic acid content accounts for more than 40 mg/100 g of dry seed and the serine value is less than 3.28. It can rapidly be excluded if these questions are answered with a "no". More or less easily, other cultivars can similarly be identified. The screening of mixtures is naturally more complex and is contemplated as a future research goal of our research program.

ABBREVIATIONS USED

DL, Desmayo Largueta; FE, Ferragnes; GU, Guara; MR, Marcona; MS, Masbovera; NP, Non Pareil; PR, Primorskyi; TE, Texas; TI, Titan; TU, Tuono; ALM, Almería; AVIG, Avignon; GR, Granada; MUR, Murcia; ALI, Alicante; TAR, Tarragona; ZAR, Zaragoza.

ACKNOWLEDGMENT

We thank the following for providing us with the almond samples: Federico Dicenta Lopez-Higuera (CEBAS, Murcia), Rafael Socias i Company (SIA-Zaragoza), Philippe Froment (INRA-Avignon), Antonio Sanchez Navarro (La Puebla de Don Fabrique), Agustín Navarro Muñoz (Los Velez- Almería), and Francisco Vargas García (IRTA, Tarragona). We also thank Angel Berenguer for his help in the revision of the English version of the manuscript.

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Received for review January 29, 2002. Revised manuscript received July 16, 2002. Accepted July 19, 2002.

JF020108T