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Changes in Carbohydrate and Protein Content and Composition of Developing Almond Seeds

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Water-soluble sugars, sugar alcohols, cell wall constituents, protein, total amino acids, and free amino acids were determined in developing almond kernels (*Prunus amygdalus*) at 3-week intervals from March until harvest. A sharp drop of sugars (from 60 to 5–6% dry weight) was observed in a first stage. Reducing sugars decreased from initial high percentages to a final trace level. Changes in hemicellulose, cellulose, and lignin were also studied; the results suggest that these constituents are employed as a carbohydrate source during a period. Protein content increased steadily up to harvest, more markedly during a 20-day period. Significant variations of amino acid content were detected. The content of free amino acids is considerable in developing almonds, while ripe kernels contain insignificant amounts.

The changes in oil content and fatty acid composition of almond kernels during development to maturity were previously reported (Munshi et al., 1982; Munshi and Sukhija, 1984; Soler et al., 1988). Oil is the major fraction of this fruit (50–65% dry weight).

In this second and final paper on the evolution of the main organic constituents during active growth of the almonds, we report the protein and carbohydrate changes.

The content and composition of these fractions in ripe almond are well-known. Water-soluble sugars range from 3 to 8% dry weight; sucrose and raffinose are the main constituents (Vidal Valverde et al., 1979; Saura-Calixto et al., 1984). The content of cell wall components, hemicellulose, cellulose, and lignin, varies between 3 and 6% dry weight (Saura-Calixto et al., 1983; Lopez-Andreu et al., 1985).

On the other hand, the protein fraction (18–25% dry weight) contains a high percentage of essential amino acids, and lysine is the limiting amino acid. The free amino acid content is very low (Nassar et al., 1977; Riquelme, 1982; Saura-Calixto et al., 1982).

The study of composition changes in developing almond should provide information useful for understanding the physiological and morphological processes throughout maturation. This is the objective of the present work.

EXPERIMENTAL SECTION

Sampling. The samples used corresponded to the Pons variety, cultivated on the Spanish island of Mallorca. Samples were collected from 20 selected trees at approximately 3-week intervals, from March to September. The weight of one kernel was determined as an average of the weight of 30 kernels.

To quantitate the data, fruit set (time 0) was conventionally considered when the fruits presented the following dimensions: length 1.1 cm, width 0.9 cm, thickness 1.0 cm.

After hulls, shells, and tegument were removed, the fruits were dried and homogenized. The oil was extracted with diethyl ether on a Soxhlet extractor over 18 h. The defatted samples used for analyses are described below.

Analytical Procedures. *Soluble Sugars.* Soluble sugars were determined on extracts of 80% ethanol. Munson-Walker (Lee, 1978) and Haas (Snell and Ettre, 1973) methods were employed to quantify reducing and total sugars, respectively.

Sugar and sugar-alcohol composition was determined by gas–liquid chromatography. The solvent was removed by vacuum distillation to yield dry residues. The procedure of Sweeley et

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Table I. Changes in Moisture Content, Fresh Weight, and Dry Weight of a Kernel[a]

DAFS (1983)	moisture, %	fresh wt, g	dry wt, g	DAFS (1984)	moisture, %	fresh wt, g	dry wt, g
0	96.4 ± 0.4	0.61 ± 0.05	0.022 ± 0.001				
21	95.4 ± 0.4	0.82 ± 0.04	0.038 ± 0.002	23	94.2 ± 0.4	0.72 ± 0.04	0.042 ± 0.002
42	94.4 ± 0.1	1.30 ± 0.07	0.073 ± 0.003	40	93.3 ± 0.1	1.21 ± 0.07	0.081 ± 0.004
63	91.2 ± 0.2	1.22 ± 0.06	0.107 ± 0.005	64	91.1 ± 0.1	1.23 ± 0.07	0.102 ± 0.006
84	80.6 ± 0.8	1.73 ± 0.08	0.332 ± 0.015	85	78.2 ± 0.5	1.52 ± 0.08	0.331 ± 0.017
105	57.3 ± 0.9	1.81 ± 0.09	0.773 ± 0.038	105	58.4 ± 0.8	1.72 ± 0.08	0.716 ± 0.033
126	43.2 ± 0.3	1.74 ± 0.07	0.988 ± 0.039	126	46.3 ± 0.1	1.63 ± 0.06	0.875 ± 0.032
156	5.0 ± 0.1	1.51 ± 0.06	1.432 ± 0.057	156	17.3 ± 0.2	1.52 ± 0.05	1.257 ± 0.041
				173	5.8 ± 0.2	1.44 ± 0.05	1.356 ± 0.047

[a] Average value ± SD. Number of determinations 4.

al. (1963) and the conditions described by Laker (1980) and Zweig and Sherma (1982) were followed to prepare trimethylsilyl derivatives. Anhydrous pyridine (1 mL), hexamethyldisilazane (0.6 mL), and trimethylchlorosilane (0.4 mL) were added to dry samples and the mixtures shaken occasionally. Derivatization occurs in 1 h at room temperature. Similar treatment was carried out with 10 mg of each standard with different standard mixtures, previously equilibrated for 24 h, in aqueous solution, which was then evaporated at 40 °C under vacuum.

Identifications were made by comparing the retention time of samples and standards. Quantitative analyses were performed by comparing the peak-corrected areas. Several mixtures of standards were prepared to determine the response factors.

Gas chromatography was performed on a Model Sigma 3B Perkin-Elmer chromatograph equipped with a Sigma 10B station data integrator and a flame ionization detector, using a stainless steel column (3 m × $^1/_8$ in.) packed with 3% SE-30 on Supelcoport 80/100. Experimental conditions: temperature program, 140–290 °C, 2.5 °C/min; carrier gas flow, 23 mL/min; injector and detector temperatures, 300 °C; sample size, 0.2–0.3 μL.

Cell Wall Components. Samples weighing 3.0 g were treated with 300 mL of neutral detergent solution (sodium lauryl sulfate at pH 7.0) for 1 h at boiling to obtain neutral detergent fiber (NDF) (Van Soest and Wine, 1967). Hemicellulose was determined as weight loss of the NDF residue when treated with acid detergent (cetylmethylammonium bromide in 0.5 M H_2SO_4) for 1 h at boiling (Van Soest, 1963; Van Soest and Robertson, 1979). The residue was treated with $KMnO_4$ solution and 72% H_2SO_4 to determine cellulose and lignin contents (Van Soest and Wine, 1968).

Protein and Amino Acids. Protein solution of dried and defatted samples were obtained with 0.5 N NaOH (Saura-Calixto et al., 1982). Protein contents were determined by Gornall–Biuret (Gornall et al., 1949) and Lowry (Lowry et al., 1951) spectrophotometric methods. Egg albumin was used as standard. Nitrogen content was determined by the Kjeldahl method.

The hydrolysis of protein was carried out on 50–60 mg of defatted samples by heating with 6 N HCl containing 4% thioglycolic acid for 21 h at 110 °C in a nitrogen atmosphere. Amino acids were determined with a Beckman 119C automatic analyzer using a column of AA-20 resin. Buffered solutions of pH 3.25, 4.12, and 6.40 at 50 °C were used for elution. Sample size was 50 μL. The ninhydrin reaction was used for all amino acids, taking readings at 570 nm for all amino acids, except for proline which was at 440 nm.

Free amino acids were extracted with 150 mL of a methanol–chloroform–water (60:25:15) mixture (Young, 1974). After the solvent was removed, samples were dissolved with a pH 2.2 buffer and analyzed with an automatic analyzer. Some aliquots of free amino acids were hydrolyzed under the same conditions as protein and then analyzed.

RESULTS AND DISCUSSION

The moisture and the fresh and dry weights of the almond kernels change markedly during the period studied. Data corresponding to two different years are reported in Table I.

From 0 to 63–64 days after fruit set (DAFS) the moisture content is over 90%; this stage is followed by a period (65–105 DAFS) of dry matter accumulation—mainly protein (7–17%) and oil (10–50%) (Soler et al., 1988).

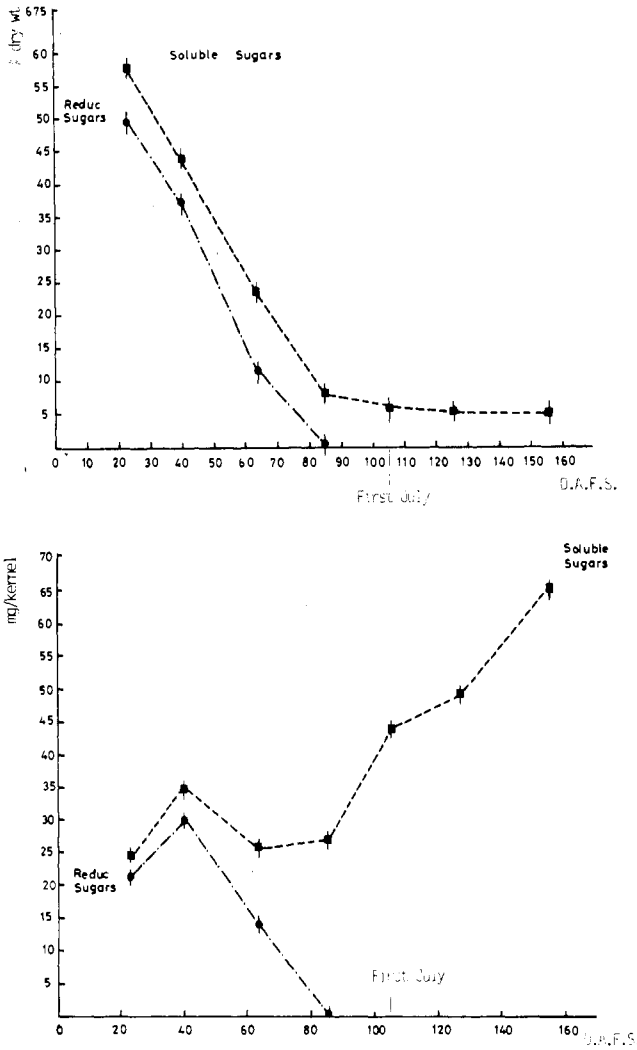

Figure 1. Changes in total soluble sugars and reducing sugars contents of developing almonds (crop 1983).

Later, the moisture continues decreasing at a higher rate than the dry matter accumulation and, subsequently, the fresh weight of the kernels diminishes.

The results corresponding to the variation of the different substances considered in this work will be referred to percent dry weight, percent fresh weight, or grams/average weight of one kernel. Data of Table I can be taken into account to transfer the results included in the tables and figures of this section to the other possible forms.

Water-Soluble Sugars and Sugar Alcohols. Figure 1 shows the changes in water-soluble sugar content, determined spectrophotometrically. A sharp drop (from 60 to 5–6% dry weight) was observed from the beginning until about 90 DAFS. A marked increase of oil and protein content was observed about 75 DAFS (Soler, 1986; Soler et al., 1988). Nevertheless, the total sugar content, referred

Table II. Compositions of Soluble Sugars and Sugar-Alcohols, as Percentages, in Almond Kernels at Different Stages[a]

DAFS	2-desoxyribose	fructose	glucose	sorbitol	inositol	sucrose	raffinose
23	9.1 ± 0.2	14.5 ± 0.4	59.2 ± 1.2	9.4 ± 0.2	4.4 ± 0.1	3.4 ± 0.0_5	not detected
40	11.9 ± 0.3	14.2 ± 0.3	60.2 ± 1.4	6.7 ± 0.1	5.9 ± 0.1	1.1 ± 0.0_5	not detected
64	3.3 ± 0.0_5	15.7 ± 0.4	28.1 ± 0.6	3.5 ± 0.1	11.6 ± 0.3	38.2 ± 1.7	not detected
85	6.9 ± 0.2	tr	tr	tr	51.7 ± 1.9	41.4 ± 1.5	not detected
105	3.7 ± 0.1	tr	tr	tr	5.4 ± 0.2	90.1 ± 3.3	0.8 ± 0.0_5
126	2.9 ± 0.1	tr	tr	tr	8.0 ± 0.3	75.0 ± 2.7	14.1 ± 0.4
156	tr	tr	tr	tr	0.8 ± 0.0_5	93.7 ± 3.8	5.5 ± 0.2

[a] Average value ± SD. Number of determinations 4.

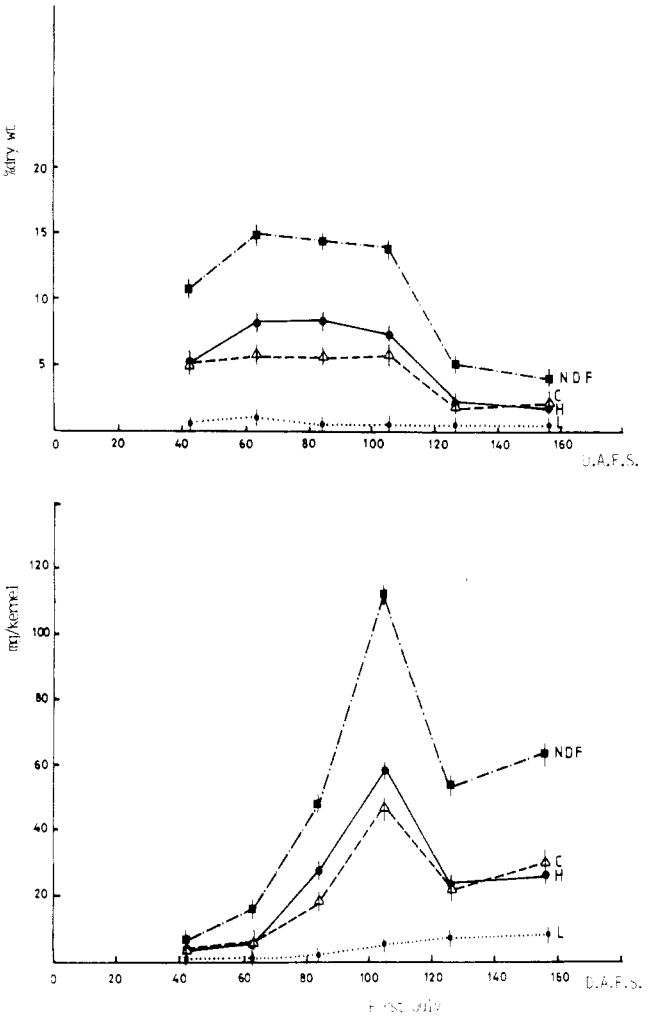

Figure 2. Changes in cell wall components of developing almonds (1983). Key: C, cellulose; H, hemicellulose; L, lignin.

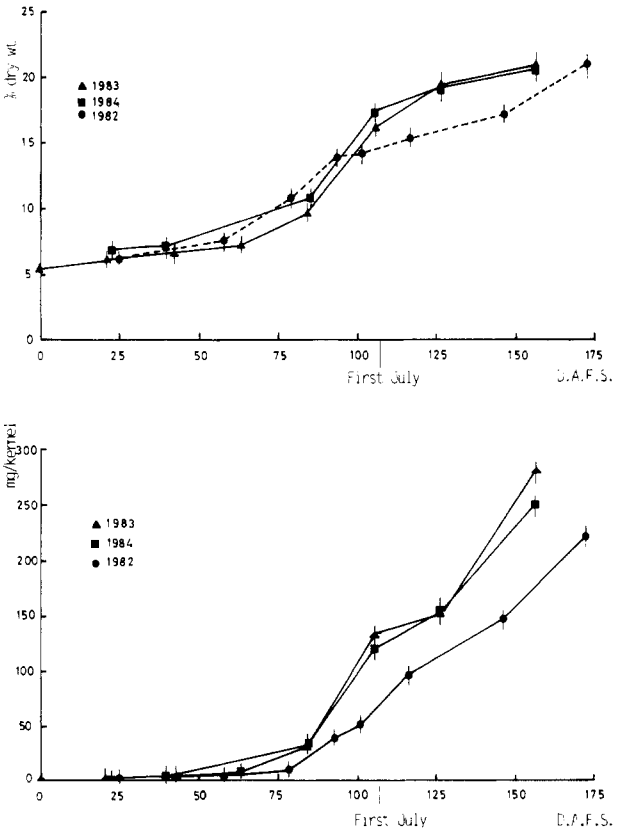

Figure 3. Changes in protein content of developing almonds (1982–1984).

to one kernel, was nearly constant until 90 DAFS. From then, an appreciable increase was observed (Figure 1).

The composition of the sugar fraction at different stages, determined by GLC, is shown in Table II. Traces of xylose and galactose are detected in initial stages. Concerning reducing sugars, high percentages are initially detected and then decrease to trace levels (Figure 1; Table II). Just the opposite happens with sucrose. This suggests that the sucrose transported from leaves is transformed to reducing sugars into the kernels, to provide suitable matter to synthesize other components. Finally, soluble sugars are stored as sucrose and raffinose (see Table II), the only quantitatively important components of sugar fraction in ripe almond.

Conversion of D-glucose into inositol is the only established route yet known for biosynthesis of inositol from carbohydrates (Loewis and Dickinson, 1980). In a first stage, the inositol content in kernels present a sharp rise, coinciding with a marked decrease of glucose content, declining thereafter until harvest. On the other hand, the highest value of inositol was reached 85 days after fruit set, just when storage of lipids started to be appreciable (Soler et al., 1988). (Inositol is a compound related with phospholipids.)

Cell Wall Components. Variations of total cell wall components (as neutral detergent fiber), hemicellulose, cellulose, and lignin are shown in Figure 2. NDF content (referenced to 100 g of dry weight) is notably stabilized during a long intermediate period (58–105 days after fruit set) or increase unitl that date, if we refer the results to one kernel. Then, NDF decreases rapidly for about 1 month, just when protein and oil increase appreciably, which requires carbohydrates. Nevertheless, the sugar content does not decreases (see Figure 1). This suggests that, during this period, cell wall components are employed as a carbohydrate source, together with the sucrose transported from leaves into the kernels. It is in agreement with the fact that hemicellulose and cellulose follow the same tendency as NDF while lignin does not. Thus, the lignin to cellulose ratio increases gradually during the last stage of maturation. As is known, the secondary cell wall, rich in lignin, is formed from enlargement of the primary cell wall (Lai and Sarkanen, 1971).

Protein. Changes in the protein content of developing almonds, corresponding to the 3 years studied, are shown

Table III. Total Amino Acid Composition during Development (mg/100 g of Dry Matter)[a]

amino acid	23	40	85	105	126	156
isoleucine	263 ± 13	238 ± 10	672 ± 40	674 ± 41	695 ± 35	690 ± 27
leucine	433 ± 22	300 ± 12	1279 ± 77	1046 ± 84	1434 ± 77	1420 ± 57
lysine	132 ± 6	98 ± 4	626 ± 38	548 ± 30	476 ± 24	468 ± 19
methionine	203 ± 6	294 ± 12	318 ± 23	164 ± 12	160 ± 8	170 ± 6
cystine	250 ± 20	101 ± 4	519 ± 41	740 ± 59	330 ± 20	300 ± 11
phenylalanine	415 ± 21	309 ± 12	980 ± 59	1185 ± 71	946 ± 50	929 ± 37
tyrosine	364 ± 18	343 ± 14	695 ± 42	799 ± 48	702 ± 36	696 ± 28
threonine	336 ± 17	255 ± 10	704 ± 42	636 ± 38	570 ± 29	561 ± 22
valine	396 ± 20	327 ± 13	791 ± 47	838 ± 50	700 ± 36	628 ± 25
essential amino acids	2792	2265	6584	6630	6013	5862
arginine	1069 ± 53	1860 ± 74	3029 ± 182	2308 ± 138	2280 ± 115	2270 ± 91
histidine	142 ± 7	73 ± 3	551 ± 33	475 ± 29	479 ± 26	477 ± 19
alanine	427 ± 21	357 ± 14	1130 ± 68	1046 ± 63	1051 ± 62	1047 ± 42
aspartic acid	2131 ± 99	2464 ± 98	4192 ± 252	2460 ± 148	2290 ± 116	2267 ± 87
glutamic acid	1477 ± 74	1538 ± 61	4521 ± 271	5389 ± 323	5200 ± 268	5145 ± 206
glycine	333 ± 17	220 ± 9	997 ± 60	1152 ± 69	1230 ± 79	1246 ± 48
proline	420 ± 21	324 ± 13	984 ± 57	983 ± 60	804 ± 44	790 ± 31
hydroxyproline	tr	tr	tr			
serine	429 ± 28	283 ± 19	1118 ± 67	969 ± 58	922 ± 47	916 ± 37
nonessential amino acids	6428	7119	16522	14782	14256	14158
total amino acids	9220	9384	23106	21412	20269	20020

[a] Values are means ± SD.

Table IV. Free Amino Acid Composition during Development (mg/100 g of Dry Material)

amino acid	23	40	85	105	126	156
isoleucine	150 ± 8	103 ± 6	179 ± 9	49 ± 2	18.2 ± 0.9	3.1 ± 0.2
leucine	136 ± 7	98 ± 5	248 ± 12	56 ± 2	201 ± 1	3.0 ± 0.2
lysine			134 ± 6	41 ± 2	15.5 ± 0.8	1.2 ± 0.1
methionine			103 ± 5	26 ± 1	9.5 ± 0.7	1.0 ± 0.1
cystine						
phenylalanine	199 ± 10	136 ± 8	623 ± 32	193 ± 10	57.1 ± 3.1	10.0 ± 0.5
tyrosine	202 ± 11	105 ± 9	138 ± 6	42 ± 2	9.1 ± 0.5	1.1 ± 0.1
threonine + serine	214 ± 13	225 ± 15	2092 ± 97	653 ± 33	55 ± 3	31 ± 2
valine	107 ± 6	136 ± 7	204 ± 11	55 ± 3	11.3 ± 0.6	5.2 ± 0.3
essential amino acids	1008	803	3721	1115	196	56
arginine	19 ± 1	25 ± 1	880 ± 43	675 ± 34	66 ± 3	32 ± 2
histidine			217 ± 10	44 ± 2	7.1 ± 0.3	2.1 ± 0.1
alanine	68 ± 3	97 ± 5	851 ± 40	334 ± 16	42 ± 2	9.2 ± 0.5
aspartic acid	86 ± 5	148 ± 8	218 ± 11	92 ± 4	25 ± 1	19 ± 1
glutamic acid			850 ± 42	719 ± 37	94 ± 5	46 ± 2
glycine	16 ± 0	29 ± 2	230 ± 11	46 ± 2	7.2 ± 0.4	1.1 ± 0.1
proline	72 ± 3	108 ± 5				25 ± 1
hydroxyproline	61 ± 3	69 ± 3	tr			
nonessential amino acids	321	476	3246	1910	241	134
total amino acids	1329	1279	6967	3025	437	190

[a] Values are mean ± SD.

in Figure 3. Protein content increased steady up to harvest. The increase was more marked between mid-June and beginning-July. This period (85–105 DAFS in 1983 and 1984) corresponds to the highest protein synthesis activity, and it coincides with a rapid decline of water soluble reducing sugars. About 30% total protein is accumulated in 3 weeks (85–105 DAFS).

In 1982, the increase started some days earlier and was more gradual probably because of exceptional climatic conditions.

Total Amino Acids. The highest content of total amino acids is reached 85 days after fruit set. Then, a slight decline is observed until the end of the process (see Table III). This evolution is correlated with that corresponding to protein. In a first stage (0–85 DAFS), amino acids are acumulated and then, in a second stage, the protein synthesis notably increases.

Kjeldahl nitrogen contents at 8.5 and 156 DAFS were 3.98 and 3.36 g/100 g of dry matter, respectively. The nitrogen content at 85 DAFS is higher that the value expected from the protein content determined spectrophotometrically (see Figure 3). There is a good agreement between nitrogen and protein contents in the ripe almond. The total amino acids content is higher than the total protein content in the period from 85 to 105 DAFS because of the presence of free amino acids. The part of amino acids not dedicated to protein synthesis must follow other biochemical pathways to non-protein-containing nitrogen compounds (Barcelô et al., 1985).

Glutamic acid, aspartic acid (including glutamine and asparagine), and arginine are the major constituents at all the dates considered, while sulfur amino acids—methionine and cystine—and lysine and histidine are present the lowest content. The highest contents of total amino acids were detected at intermediate stages (85–105 DAFS).

On the other hand, the total amino acid composition—as percentage of protein content—does not present important differences in the period studied.

Free Amino Acids. Ripe almonds contain insignificant amounts of free amino acids (Nassar et al., 1977; Cañellas, 1986). However, the content is considerable in developing fruits, as can be deduced by substracting protein (Figure 3) from total amino acid content (Table III). In order to obtain experimental data, analyses of free amino acids were performed. The results are listed in Table IV. The values are lower than the data obtained by difference mainly because of the presence of five nonidentified peaks. These peaks were quantitatively important at initial stages, and

they must correspond to nonprotein amino acids. This fact was previously reported. Marcy et al. (1981) detected two unknown peaks in muscadine grapes. Young (1974) reported five nonprotein amino acids in inmature peanuts, identifying two of them (γ-methyleneglutamine and γ-methyleneglutamic acid).

The unknown peaks of free amino acid chromatograms do not appear in the chromatograms corresponding to total amino acids of the same date. That is to say, they should correspond to derivatives that yield known amino acids after acid hydrolysis. To check this fact, some extracts of free amino acids were hydrolyzed and then analyzed. Most of the unknown peaks disappeared after hydrolysis, while significant amounts of glutamic acid not detected before hydrolysis appeared.

The compositions of free amino acid fractions present important changes during development and, logically, the contents decrease because of their utilization for synthesis. The highest content of each amino acid was observed at 85 DAFS. Lysine, methionine, histidine, and glutamic acid were not found at initial developing stages. Hydroxyproline was detected only initially, and cystine was not detected during all the process.

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