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Original paper

Determination of amino acids in ripening apples by high performance liquid chromatography

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Entwicklung des Gehaltes an Aminosäuren in Äpfeln während des Reifungsprozesses mit Hochleistungsflüssigchromatographie

Zusammenfassung. Die Hochleistungsflüssigchromatographie wurde angewendet, um Reifungsprozesse zu untersuchen mit dem Ziel, das Wissen über die Biochemie des Reifungsprozesses zu verbessern, einerseits um das Reifungsoptimum zu erkennen und andererseits um die mögliche Bedeutung des Gehalts an Aminosäuren der verschiedenen Apfelsorten für die daraus hergestellten Säfte und Gärungsgetränke festzustellen.

Summary. High performance liquid chromatography (HPLC) was used to monitor amino acid changes in ripening apples in order to increase knowledge of the biochemistry of this type of fruit with a view to determining the optimum ripening time and evaluating the potential influence of the amino acid content of the different apple varieties studied on the juices and fermented beverages made from them.

Introduction

Cultivation of different apple varieties with suitable sensory features for the manufacture of juices, ciders, vinegars, spirits, liquors and appetizers, among others, is currently under extensive development on account of its socioeconomic significance. The nutritional value and sensory properties of apples are currently assessed by the use of objective methods of analysis applied to their major constituents (organic acids, sugars, phenols, amino acids, etc.), all of which are responsible to a greater or lesser extent for the particular features of the fruit.

The analytical determination of free amino acids in fruits in general, and in apples in particular, is becoming increasingly important for a number of reasons. First, the concentration of amino acids in the fruit varies significantly as a result of metabolic changes during growth and ripening. This can be exploited to determine the optimum ripening time provided major changes take place near that time. In such a case, this would provide an alternative or complement to current commonly used parameters such as ethylene concentration, sugar/organic acid ratios and starch index. Second, amino acids in fruits determine the quality of the products that are obtained from them. Thus, they take an active part in the Maillard reaction and in browning processes after the enzymatic oxidation of polyphenols [1, 2], which determine the sensory quality of such products as juices and fruit concentrates. Likewise, these compounds play a major role in the manufacture of fermented beverages, in which they are used as a source of assimilable nitrogen for yeasts. Finally, we should note that amino acid profiles vary from fruit to fruit and between fruits of the same type but of different origin, so they can be used to characterize fruit juices and fermented beverages and to check for fraudulent mixing or adulteration [3, 4].

In this work we studied changes in the major amino acids of ripening apples in order to increase knowledge of the biochemistry of this fruit with the aim of developing a means of determining more objectively the optimum ripening time according to the intended use of the fruit. We also evaluated the potential influence of amino acid contents of apples on the quality of juices and ciders obtained from them. For this purpose, we determined amino acids in four apple varieties by reversed-phase liquid chromatography, using *o*-phtaldialdehyde (OPA) as a pre-column derivatizing reagent for primary amino acids.

Materials and methods

Sample preparation. Apple extracts were obtained according to Richmond et al. [5] modified as described elsewhere [6] in order to ensure the extraction of other apple constituents such as sugars, organic acids and phenols.

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Table 1

Solvent	Time (min)										
	0	1	2	10	15	20	25	30	45	55	70
% A	97	97	85	85	75	75	50	50	0	0	97
% B	3	3	15	15	25	25	50	50	100	100	3

The derivatization procedure comprised the following steps: 100 µl of each apple extract was filtered through 0.45-µm Millex HV membrane (Millipore), mixed with 100 µl of the derivatizing solution and diluted to 1 ml with water. The solution was then mixed thoroughly and allowed to stand at room temperature for at least 1 min, after which an aliquot of 20 µl was injected into the HPLC system. The *o*-phtaldialdehyde/2-mercaptoethanol (OPA) derivatizing solution was prepared according to Hill et al. [7], by adding 100 µl of 2-mercaptoethanol and 1 ml of 0.4 mol borate buffer, pH 10 (adjusted with NaOH), to 100 mg OPA dissolved in 9 ml methanol. The solutions were mixed and stored in the dark at 4 °C for 24 h prior to use.

Apparatus. A Bromma LKB (Sweden) high performance liquid chromatograph equipped with two model 2150 pumps, a model 2152 controller, a Rheodyne 7125 injector (20 µl loop volume) and a Shimadzu (Kyoto, Japan) RF-535 fluorescence spectrophotometer furnished with a 12-µl flow-cell were used for the analysis of amino acids. Data were obtained by a Shimadzu C-R3A chromato-integrator. The OPA-derivatives were monitored at 340 and 425 nm as excitation and emission wavelengths, respetively. The chromatographic column used was a Spherisorb Tracer, Spain ODS-2 model (150 × 4.6 mm ID, 5 µm particle size) and was kept at 30° C throughout the experiments. Elution was performed in gradient mode by using two solvent mixtures. Solvent A consisted of 10^{-2} mol sodium dihydrogen orthophosphate (pH 6.8) of ionic strength 0.08 mol (adjusted with sodium nitrate) and 1% v/v tetrahydrofuran; solvent B was methanol. The gradient programme used was as follows (Table 1).

The flow rate was 1.3 ml/min and the column was equilibrated for 15 min before each fresh sample was injected. The solvents were vacuum-filtered and degassed with helium. Under these conditions, the average variation in the retention time of the 18 amino acids assayed was 0.6% as the coefficient of variation, and the average recoveries ranged between 91% and 109%, thus confirming that the applied procedure was accurate enough for the analysis of these substances.

Results and discussion

Amino acids were determined and their changes monitored in four apple varieties grown in Northern Spain, selected on the basis of their industrial significance to the manufacture of juices and ciders, and of their sensory properties: Collaos (mild-sharp), Meana (bitter-sharp), Picona Rayada (bitter-sweet) and Raxao (sharp).

For reference, the optimum ripening time of each variety was determined from its sugar, major organic acid, polyphenol and total nitrogen contents and their changes, in addition to the starch index. According to these data, the optimum harvesting dates were determined to be: Collaos (11–20 December), Meana (11–20 November), Picona Rayada and Raxao (1–10 November).

Figure 1 shows the chromatogram obtained for each of the four apple varieties studied in their optimum ripe state. As can be seen, there were clear differences between the amino acid profiles of the different varieties. In three of them (Collaos, Picona Rayada and Raxao), asparagine (Asn) was the major amino acid in the ripe fruit. However, in Meana Asn occurred at its minimum concentration at this point, even though it was the major amino acid at any other time throughout the process, and aspartic acids (Asp) was prevalent, a behaviour observed in both vintages studied. Likewise, the largest difference in the serine (Ser) content was found in Raxao, which featured concentration levels well below those of the other three varieties. Other differences involved minor amino acids. Thus, Collaos featured high relative contents of glutamine (Glu), phenylalanine (Phe) and valine (Val), while Picona Rayada contained the largest amounts of alanine (Ala).

Table 2 lists the average free amino acid composition of the different apple varieties studied during the ripening of two consecutive vintages (1987 and 1988). As can be seen, Asn, Asp, Glu and Ser are the major amino acids in the four varieties and account for roughly 80% of all amino acid nitrogen. A second group is made up of glycine (Gly), threonine (Thr), γ-aminobutyric acid (GABA), phenylalanine (Phe) and valine (Val), even though some of these can be included in the first group (e.g. Phe in Collaos). The other amino acids, viz. glutamine (Gln), histidine (His), homoserine (Hse), arginine (Arg), β-alanine (β-Ala), alanine (Ala), tyrosine (Tyr), isoleucine (Ile) and leucine (Leu) were found in quite small amounts (see Table 2).

Table 2. Average contribution (%) of each amino acid to amino nitrogen throughout ripening of four asturian apple varieties

Amino acids	Collaos	Meana	Picona Rayada	Raxao
Asp	11.4	10.0	19.1	7.2
Glu	13.6	9.4	8.5	5.5
Asn	51.4	54.0	46.8	65.1
Ser	4.3	4.7	5.6	3.3
Gln	1.9	2.9	0.9	1.2
His	1.3	0.7	0.9	0.7
Hse	0.9	1.6	2.4	0.8
Gly	1.0	1.4	1.5	0.6
Thr	0.6	1.3	2.7	0.6
Arg	1.2	0.6	0.4	0.8
β-Ala	0.6	0.5	0.3	0.6
Ala	0.8	2.2	0.4	1.0
GABA	0.5	1.9	2.6	0.8
Tyr	0.5	1.1	1.5	0.4
Val	1.8	1.7	1.5	0.9
Phe	6.7	2.2	0.1	2.1
Ile	0.6	1.9	1.1	0.1
Leu	0.7	1.9	1.3	0.3

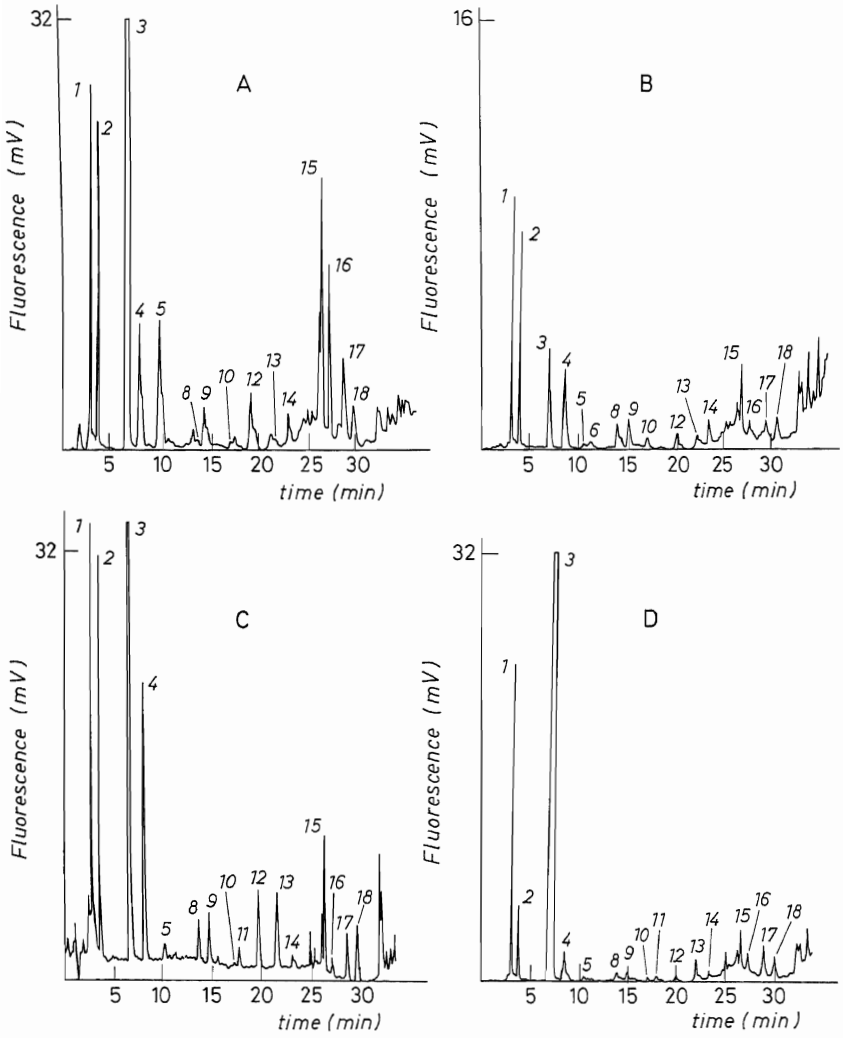

Fig. 1 A–D. Amino acid profiles at the estimated optimum ripening date for the four apple varieties studied. **A** Collaos; **B** Meana; **C** Picona Rayada; **D** Raxao. Asp (*1*), Glu (*2*), Asn (*3*), Ser (*4*), Gln (*5*), His (*6*), Hse (*7*), Gly (*8*), Thr (*9*), Arg (*10*), β-Ala (*11*), Ala (*12*), GABA (*13*), Tyr (*14*), Val (*15*), Phe (*16*), Ile (*17*), Leu (*18*)

Asn was the major amino acid occurring in the highest proportions in the four apple varieties, in which its concentration gradually decreased throughout ripening (Fig. 2A). On the basis of its properties [8] and the increased metabolic activity during apple ripening [9] we may ascribe Asn a significant proteosynthetic activity by deamination and transamination to yield the nitrogen required for the synthesis of proteins, which would account for its gradual disappearance throughout ripening. However, one should also bear in mind that during ripening ethylene and cyanide-containing compounds such as β-cyanoalanine, a precursor of Asn [10] are also produced. This may increase the Asn content at late stages of ripening, thus accounting for the relative maxima in the Asn concentrations detected in the different varieties and especially markedly in Collaos, Meana and Raxao. The subsequent decrease in the Asn concentration observed at the final stage of ripening may arise from the activity of asparaginase, which results in the accumulation of Asp (see Collaos variety in Fig. 2B). Picona Rayada and Raxao behave similarly in this respect, while Meana is somewhat divergent in its behaviour.

As noted earlier, Ser is another major apple amino acid. Figure 3A reflects the changes in Ser throughout ripening in the four apple varieties studied. It is quite significant that the four varieties yield their maximum Ser concentrations by the end of ripening (about 10 days before the respective estimated ripening dates). This fact may be related to the role that Ser plays as a bridge between proteins of the primary cell wall and arabinogalactans. It would be likely that any softening of the primary cell wall may be detected by an increase in the quantity of free Ser.

The Phe concentration is related to the accumulation of antocyanin dyes [11] and therefore, the Phe content should decrease as antocyanin compounds accumulate. Indeed, the Phe concentration decreased by the end of ripening in all four varieties studied.

Tyr, His, and Arg occur as minor amino acids in apples. However, their presence in apple juice may have significant technological and toxicological repercussions, because they are precursors of the biogenic amines tiramine, histamine and urethane, found in alcoholic beverages [12–15].

In Meana, the Tyr and Arg contents exhibited a maximum prior to the optimum ripening date and after the optimum date, respectively, which confirms the advisability of collecting the fruit on their estimated optimum da-

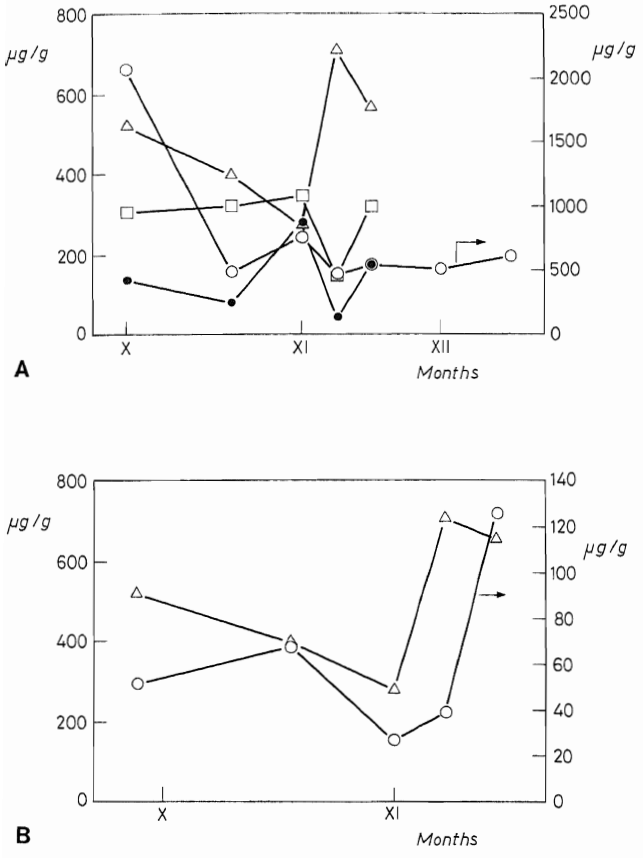

Fig. 2. **A** Variation of the Asn content of the four apple varieties throughout ripening. ○ Collaos; △ Raxao; □ Picona Rayada; ● Meana. **B** Variation of the Asn △ and Asp ○ contents of Collaos throughout ripening

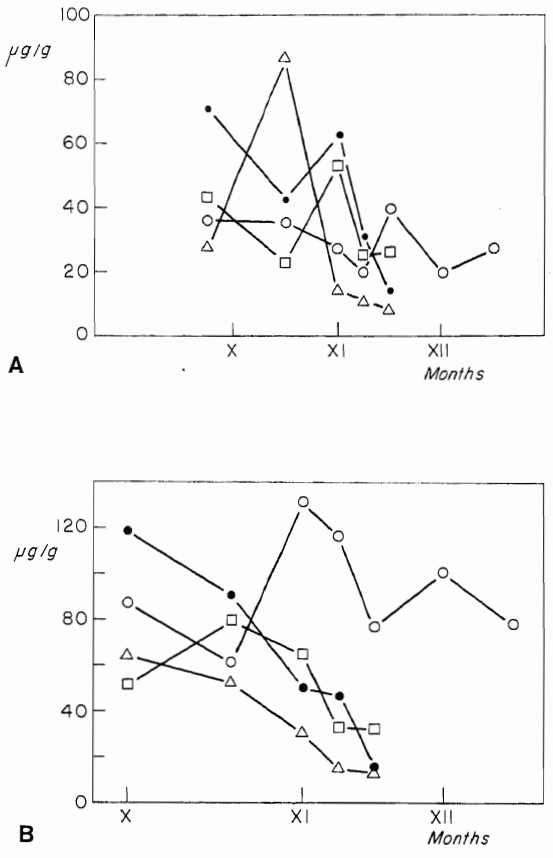

Fig. 3. **A** Variation of the Ser contents throughout ripening: ● Meana; □ Picona Rayada; ○ Collaos; △ Raxao. **B** Variation of the Glu contents throughout ripening: ● Meana; ○ Collaos; △ Raxao; □ Picona Rayada

tes. It should be noted that the maximal concentrations of these amino acids found in the four varieties were quite low (ca. 15 μg/g for Arg and His, and 25 μg/g for Tyr), and they decreased as the final stage of ripening approached.

There are significant differences between the estimated optimum collection dates for the four varieties studied as regards the total α-amino nitrogen content. Thus, Collaos featured the highest Asn, Asp, and Glu contents. The later tended to decrease throughout ripening in the four varieties, with an outstanding maximum in the concentration of Collaos near complete ripening (Fig. 3 B). Such differences may affect development of the fermentation process in the manufacture of cider, particularly as regards the fermentation rate, but also the amino acid fraction is closely related to the production of higher alcohols via keto acids and, especially, Glu, which is related to the synthesis of succinic acid [16], γ-lactones and esters [17]. Likewise, Asn, Glu, and Gln are reportedly degraded rapidly during storage of apple concentrates as a result of the Maillard reaction [18], and thus detract from the product quality.

From the nutritional point of view, there are also some differences between the four varieties as regards the content of some essential amino acids on the optimum ripening date. In this respect it should be pointed out that Picona Rayada contained the highest concentrations of Thr, while Collaos and Meana featured higher concentrations of Val and Phe, and Leu respectively.

In conclusion, we studied the content of 18 amino acids of four Asturian apple varieties throughout ripening and found Asn, Asp, and Glu and Ser to be the major amino acids in the four varieties, plus Phe in some of them. The Asn and Phe concentrations in the fruits were found to decrease at the end of ripening in the four varieties. The former decrease can be ascribed to the marked proteosynthetic activity in apples during ripening, while the latter can be attributed to the synthesis of antocyanin dyes and phenols. Ser was the only amino acid undergoing any significant concentration changes at the estimated optimum ripening date, so its level can be used as a complementary parameter for the determination of such dates. There were significant quantitative differences in the total amino acid fraction between the four apple varieties studied, which emphasizes the significance of the variety or varieties chosen for the manufacture of different derivatives.

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