

Determination of Amino Acids in Apple Extracts by High Performance Liquid Chromatography

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Key Words

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Summary

A rapid and sensitive method for the simultaneous determination of primary amino acids in apple is described. After sample preparation, amino acids were derivatized with o-phthaldialdehyde/2-mercaptoethanol and separated on a reversed phase column with a gradient of phosphate buffer-tetrahydrofuran-methanol as the mobile phase. Detection was carried out with a fluorescence detector at excitation and emission wavelengths of 340 nm and 425 nm respectively. Recovery studies showed good results for all substances (91–109 %) (with coefficients of variation ranging, from 0.1 to 9.0 %). This method was applied to the monitoring of amino acids during the ripening of apples.

Introduction

Among the different substances that constitute fruits and vegetables, amino acids are becoming increasingly important and, for various reasons, their analytical determination is becoming by the day, more and more necessary.

Leaving aside their nutritive value, the concentration of different amino acids during the ripening of fruits, and in particular of apples, changes significantly, as has been demonstrated by thin layer chromatography [1]. This can be used as an indicator in determining the time of harvesting as an alternative to the criteria currently used, namely ethylene concentration, sugar/organic acid ratio, etc.

Of further interest is the fact that amino acid profiles differ not only among different fruits but also among

fruits of different origins which makes possible, from measurement of free amino acids, the detection of mixtures and adulteration of fruit juices [2] and their industrial derivatives: ciders, wines, vinegars, etc., as each product has a characteristic profile which is altered by adulteration. Furthermore, amino acids play an important part in the nutrition of the micro-organism that are found in apple juices. During fermentation and other forms of microbial activity many changes occur among the concentrations of the amino acids. They tend to disappear and later to reappear in smaller quantities, but in a greater variety, as a result of yeast excretion and autolysis. They also influence the content of volatile compounds (higher alcohols and esters) which may cause flavour changes in secondary taste.

From the analytical point of view, amino acid analysis is an important technique with many applications in medicine and biochemistry, such as in the determination of peptide sequencing, the primary structure of proteins, etc., in which an accurate method for amino acid determination is required.

The classical method of amino acid analysis, first proposed by Moore and Stein [3], involves separation by ion-exchange chromatography followed by post-column derivatization with ninhydrin, but the lack of sensitivity led to the development of other, more sensitive, derivatizing reagents.

A general alternative is offered by pre-column derivatization, which gives derivatives that can be separated on a reversed-phase high performance liquid chromatographic column with a shorter analysis time and higher sensitivity. Typical reagents for pre-column derivatization are dansyl chloride [4, 5] phenylisothiocyanate (PITC) [6, 7] and o-phthaldialdehyde (OPA) [8–10]. All these reagents present advantages and limitations. Dansyl chloride forms fluorescent derivatives with primary and secondary amino acids. This reaction is rather time-consuming and, in addition, the reagent is, itself, fluorescent. However, it has been used for the determination of free amino acids in grape juices and wines [11, 12]. The phenylisothiocyanate reaction was frequently employed for the determination of peptide sequencing

[13] and it has had many applications in food analysis [14, 15]. However, the derivatization procedure is rather complex compared with other procedures. *O*-phthaldialdehyde reacts, in the presence of a thiol reagent, within a short time with primary amino acids to form highly fluorescent isoindole products, although secondary amino acids do not react. This fact is not a problem in our present application because secondary amino acids, such as proline, are present in very small quantities in apples. OPA has been employed for the determination of free amino acids in grape juices and wines [16], and in beer [17, 18].

In this paper, the use of *o*-phthaldialdehyde as a pre-column derivatizing reagent for primary amino acids has been evaluated. An attempt has been made to improve the analytical technique from the point of view of separation and flexibility. In addition, its applicability in the amino acid analysis of three apple varieties of Northern Spain, chosen for their industrial importance in the making of ciders and apple juices, in five ripening stages, has been investigated with a view to establishing the physiological maturity of the fruit.

Experimental

The instrument used was a LKB (Bromma, Sweden) Liquid Chromatograph. The chromatograph was equipped with two Model 2150 pumps and a Model 2152 controller for the generation of elution gradients, a Rheodyne Model 7125 injection valve with a 20 μ L loop, and a Shimadzu RF-535 fluorescence detector fitted a 12 μ L flow cell. Chromatograms were plotted and integrated by means of a Shimadzu C-R3A integrator.

The OPA derivatives were monitored at an excitation wavelength of 340 nm and an emission wavelength of 425 nm.

Separations were carried out on a 15 cm \times 4.6 mm i.d. column packed with 5 μ m Spherisorb ODS connected to a 3 cm \times 4.6 mm i.d. guard column packed with the same material (particle size 40 μ m).

The column temperature was maintained at 30 °C by placing the column in a thermostatted bath regulated by recirculated water.

Gradients were performed between two solvent mixtures. Solvent A was composed of 10⁻²M dihydrogen orthophosphate (pH 6.8) treated with sodium nitrate until its ionic strength was 0.08 M, and tetrahydrofuran at 1 % v/v. Solvent B was methanol. Before being used both solvents were vacuum-filtered and degassed with helium.

Methanol and tetrahydrofuran were of HPLC grade and were employed as supplied. Analytical grade amino acids, *o*-phthaldialdehyde and 2-mercaptoethanol were purchased from Sigma Chemical (St. Louis, MO, USA); sodium nitrate was from Merck (Darmstadt, FRG). High purity water was obtained with a Milli-Q (Millipore) system.

Amino acid standard solutions were prepared in 0.1 M HCl in concentrations of about 10⁻²M. The

standard solutions, stored in a refrigerator, were stable for approximately 3 weeks. Other amino acid standards were prepared by diluting aliquots of this solution with water.

The *o*-phthaldialdehyde/2-mercaptoethanol derivatizing solution was prepared according to Hill et al. [19], as follows: To 100 mg of OPA dissolved in 9 mL of methanol, 100 μ L of mercaptoethanol and 1 mL of 0.4 M borate buffer (pH adjusted to 10 with sodium hydroxide) were added. The solution was mixed and stored in the dark at 4 °C and allowed to stand for 24 h before use. Every two days, 10 μ L of 2-mercaptoethanol was added to maintain the reagent strength. With this procedure the derivatization reagent was usable for approximately two weeks without important contaminants appearing in the elution profiles.

The procedure for derivatization was as follows: 100 μ L of standards or unknown samples, filtered through a 0.45 μ m Millex HV (Millipore) membrane, were mixed with 100 μ L of the derivatizing solution and the total was diluted to 1 mL with water. The resulting solution was mixed thoroughly and allowed to remain at room temperature for at least 1 minute for the reaction to complete before analysis of 20 μ L by means of the HPLC system.

Preparation of Apple Extracts

Apple extracts were prepared as described by Richmond et al. [20] with some modifications previously described by us [21], in order to ensure the extraction of other components present in apples such as phenolic compounds.

Complete triplicate analyses were performed on all samples to allow the calculation of average deviations as a measurement of chromatographic reproducibility.

Results and Discussion

Preliminary studies showed that there are two groups of amino acids in apples. The first group consists, according to Baron et al. [1], of the following components: aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser) and glutamine (Gln). This group comprises 90 % of the total amino acids. It is also possible to include alanine (Ala) in this group [22]. The second group is composed of glycine (Gly), threonine (Thr), and γ -aminobutyric acid (GABA); and also phenylalanine (Phe), isoleucine (Ile), leucine (Leu), ornithine (Orn), β -alanine (β -Ala), lysine (Lys), histidine (His), homoserine (Hse) and arginine (Arg) in small quantities.

In accordance with previous works [8–10, 19] for the separation and determination of free amino acids, we chose high performance reversed-phase liquid chromatography. Separation was achieved by employing a 30 min gradient of solvent B over solvent A. The gradient shape was chosen to optimize the spacing of the separated peaks with a minimum analysis time.

Using previous studies as a basis, an investigation to determine the optimum conditions for the separation of the amino acids present in apple extracts was undertaken.

Phosphate and acetate buffers were tested at different concentrations and pH. The pH of the mobile phase was varied between 6.5 and 7.5, and the ionic strength between $1.25 \times 10^{-2}M$ and $8 \times 10^{-2}M$. Indications are that at pH 8 fluorescence intensity generally tends to become constant, followed by a decrease from pH 10.3 upwards [17] and it is further known that solvent pH 7.5 is harmful to the reverse phase column used. On the other hand, a decrease in the pH of the mobile phase resulted in increased instability of the OPA-amino acid adducts [23].

On the basis of resolution and peak areas, phosphate buffer and pH = 6.8 were chosen as the optimum. An ionic strength of $4.5 \times 10^{-2}M$ provides good resolution between Asp and Glu, but Arg and β -Ala co-elute. On increasing this parameter to 0.08 M a total separation for both was obtained.

With regard to the percentage of THF in the mobile phase, values between 1 and 2 % were tested, but percentages above 1 % decreased the resolution of Gln-His-Hse. 1 % THF was, therefore, chosen as optimum.

The column temperature was controlled at $30^\circ C + 1^\circ C$ and a flow of 1.30 mL/min was used for the solvent programmed system.

These optimization studies led to the selection of the following multi-step gradient elution program: 3 % solvent B for 1 min from the beginning of the program, linear step to 15 % for 1 min, isocratic elution step at 15 % for 8 min, linear step to 25 % B for 5 min, isocratic elution step at 25 % B for 5 min, linear step to 50 % B for 5 min, isocratic elution step at 50 % B for 5 min, linear step to 100 % B in 15 min, isocratic elution step at 100 % B for 10 min to purge the system prior to returning to the initial conditions for re-equilibration in 15 min.

Figure 1 demonstrates the separation achieved by RP-HPLC of a standard amino acid mixture. As can be seen, the majority of amino acids present in the mixture have a baseline separation and elute as sharp peaks. Using the system described the retention times were found to be very consistent from one chromatogram to another. Five different analyses showed that the average variation in the retention time of the 18 amino acids in the standard solution was 0.6 % cv (0.1 % to 1.5 %).

Quantitation and Recovery

A typical chromatogram of the derivatized amino acids extracted from apples, using the operating conditions specified, is shown in Figure 2. The sample peaks were identified by comparing the relative retention times of each one with those of the standard reference amino acids. 18 amino acids were present in the sample and they were all quantitatively determined.

The quantification of amino acids was achieved by using the external standard method. Standards were

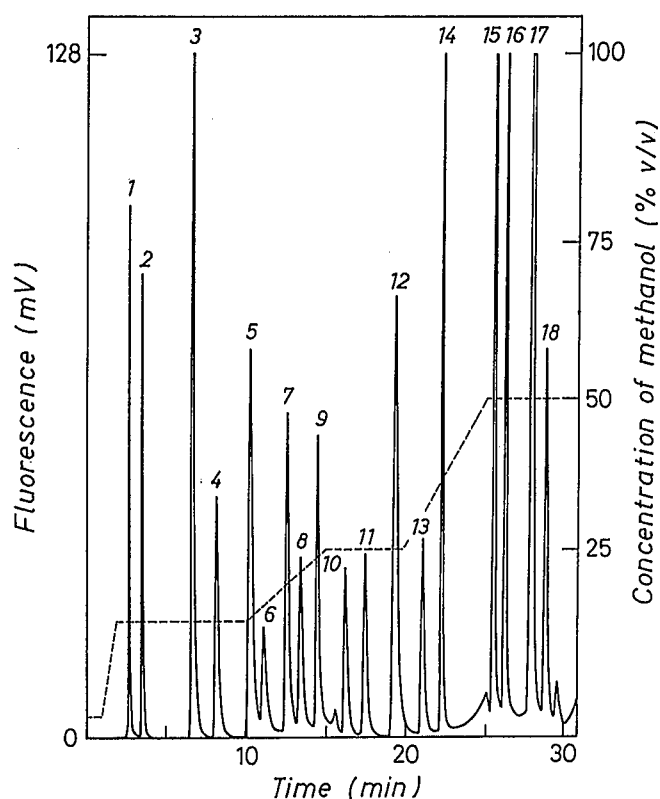


Figure 1
Separation of OPA-amino acid standards. Operating conditions: column, Spherisorb ODS (15 cm \times 4.6 cm i.d., 5 μ m particle size). For gradient program, see text. Peaks: 1 = Asp, 2 = Glu, 3 = Asn, 4 = Ser, 5 = Gln, 6 = His, 7 = Hse, 8 = Gly, 9 = Thr, 10 = Arg, 11 = β -Ala, 12 = Ala, 13 = GABA, 14 = Tyr, 15 = Val, 16 = Phe, 17 = Ile, 18 = Leu. Each peak represents 1.5 pmoles.

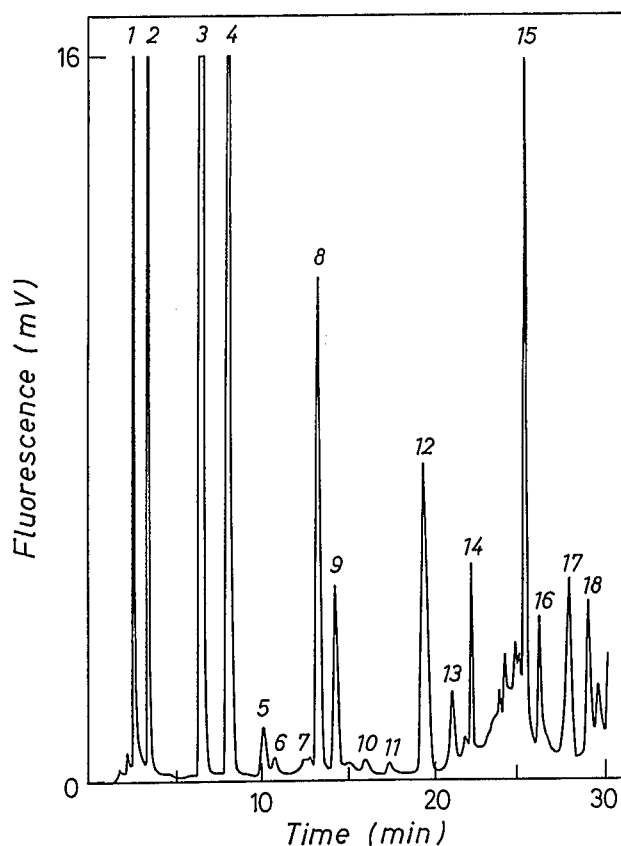


Figure 2
Typical chromatogram of OPA-amino acids in an apple extract. Column and chromatographic conditions as in Figure 1. For peak identification, see also Figure 1.

Table I Evolution of amino acids in apples during the last three months of ripening. Concentrations are given in $\mu\text{g/g}$.

		Asp	Glu	Asn	Ser	Gln	His	Hse	Gly	Thr
MEANA	29/9/87	120.8±4.5	118.2±2.2	134.2±3.1	70.6±5.1	27.6± 1.1	15.1±8.8	41.9±2.9	15.5± 7.0	19.3±1.7
	20/10/87	121.0±0.8	89.9±2.8	294.6±1.9	42.6±0.6	16.3±10.3	13.2±5.2	-	13.6± 2.5	13.6±1.8
	2/11/87	48.5±0.1	50.3±6.5	1011.0±5.6	62.1±5.9	9.9± 3.7	10.7±5.9	38.2±0.2	20.6± 0.6	11.4±5.2
	10/11/87	32.3±4.6	46.7±3.2	38.9±0.7	31.1±5.8	11.9± 3.6	-	-	10.1± 3.6	10.1±4.6
	16/11/87	16.5±4.1	15.1±5.7	179.5±0.1	14.2±5.9	8.3± 5.5	-	5.1±8.4	2.3± 8.7	3.1±2.3
RAXAO	29/9/87	51.4±4.0	64.3±0.5	517.0±3.2	27.9±2.7	10.6± 4.9	8.9±2.7	6.1±1.6	5.0± 0.7	7.8±6.9
	20/10/87	67.9±4.7	51.9±2.8	397.0±1.2	86.4±1.0	12.2± 1.3	-	8.2±5.9	3.7± 4.3	12.2±2.8
	2/11/87	26.5±3.2	30.3±2.3	279.4±3.7	14.2±9.7	9.0± 3.0	7.6±2.7	8.0±5.6	3.3± 5.8	6.6±5.9
	10/11/87	38.7±4.2	15.0±6.0	707.9±6.8	10.8±5.7	9.6± 1.3	-	6.2±0.6	3.2± 0.8	4.2±2.8
	16/11/87	125.4±0.4	12.9±1.2	565.1±3.4	7.9±7.5	6.7± 4.1	4.9±7.0	4.9±3.5	2.1± 3.7	3.0±3.3
COLLAOS	29/9/87	63.9±5.9	87.2±2.8	2064.5±1.0	35.6±4.7	3.0± 4.5	16.9±1.6	1.7±3.9	0.4±10.5	15.2±2.3
	20/10/87	109.6±5.0	60.9±4.8	482.0±2.6	35.5±5.0	2.8± 4.6	15.0±4.2	-	13.9± 3.4	15.3±7.2
	2/11/87	173.9±3.2	130.8±3.1	758.5±1.1	27.2±2.3	0.1±10.5	14.7±1.7	1.7±3.4	4.2± 5.2	15.9±5.7
	6/11/87	126.4±1.6	116.2±1.7	464.0±2.4	19.8±9.7	0.3± 6.0	12.6±0.4	1.4±1.7	5.5± 4.2	14.0±2.9
	18/11/87	102.2±1.6	76.8±1.5	533.1±2.1	39.7±6.2	-	16.4±1.5	3.9±6.4	5.2± 3.3	9.9±1.2
	1/12/87	135.9±5.6	100.0±1.6	504.1±3.1	19.9±1.9	0.7± 5.5	14.6±3.2	3.8±3.8	4.3± 2.3	8.3±4.8
	14/12/87	111.7±5.8	78.0±5.7	613.1±4.9	27.0±3.9	68.3± 3.5	11.3±7.9	1.5±2.5	3.5± 4.5	9.7±8.6

Table II Evolution of amino acids in apples during the last three months of ripening. Concentrations are given in $\mu\text{g/g}$.

		Arg	β -Ala	Ala	GABA	Tyr	Val	Phe	Ile	Leu
MEANA	29/9/87	14.7±5.3	15.5±7.5	44.6±5.6	23.0±8.0	15.9±3.7	27.2±3.2	38.9±0.8	12.1±5.4	36.6±5.4
	20/10/87	11.6±0.8	13.2±3.1	16.7±1.9	18.2±3.0	25.6±5.2	31.0±4.0	24.0±2.6	17.4±8.2	24.2±1.5
	2/11/87	11.8±5.5	12.1±1.5	27.2±3.9	15.8±4.7	12.1±2.6	23.9±3.8	15.4±9.1	20.9±5.1	25.7±5.9
	10/11/87	15.6±3.4	12.0±2.8	8.7±0.2	12.8±2.0	12.4±2.3	11.4±5.3	6.7±7.3	11.4±8.7	20.1±6.5
	16/11/87	4.8±3.1	6.3±0.9	6.0±5.0	9.1±1.2	3.4±3.4	5.1±6.3	19.9±5.5	7.1±2.1	9.1±0.5
RAXAO	29/9/87	8.9±2.8	8.4±5.0	13.4±8.6	7.3±0.7	7.3±4.7	17.9±4.5	17.9±8.0	17.3±5.9	21.1±4.3
	20/10/87	5.9±0.9	1.1±0.4	17.8±1.9	14.8±3.4	5.2±6.0	18.6±5.6	6.3±6.3	13.4±9.4	10.4±5.2
	2/11/87	7.6±1.1	7.6±5.6	5.7±5.2	10.4±4.1	5.7±5.2	6.6±1.7	6.6±0.1	10.4±5.0	12.3±5.5
	10/11/87	7.1±1.0	8.7±3.0	4.6±5.2	10.4±2.2	5.0±5.8	7.1±0.5	7.5±7.2	11.6±2.1	14.6±4.2
	16/11/87	5.5±3.3	7.0±0.6	3.3±1.5	10.3±4.8	3.3±0.4	5.2±4.7	6.7±4.2	10.9±4.9	13.4±5.6
COLLAOS	29/9/87	3.8±1.4	5.9±1.7	12.7±1.0	7.6±4.0	8.0±8.1	26.2±8.1	101.6±4.2	7.2±5.0	7.2±7.4
	20/10/87	11.1±1.9	4.9±2.0	12.5±6.5	7.7±4.1	5.6±7.4	27.1±0.1	56.7±3.7	5.2±1.9	7.0±1.0
	2/11/87	2.3±2.4	6.4±5.8	7.2±3.7	5.7±3.6	5.7±6.6	53.5±0.3	71.1±1.7	8.7±6.6	7.2±4.9
	6/11/87	3.1±4.4	5.1±1.3	7.2±6.0	7.5±3.4	5.5±9.2	25.6±6.1	45.4±3.7	12.3±5.6	9.2±6.5
	18/11/87	2.2±9.8	5.6±0.6	6.9±2.0	6.9±5.8	6.0±8.9	17.3±3.4	39.3±2.2	6.9±6.7	6.0±7.7
	1/12/87	2.7±0.9	4.7±2.5	7.3±3.9	5.3±6.4	7.8±8.0	17.3±1.7	33.9±2.2	6.9±6.7	4.7±8.2
	14/12/87	1.9±2.3	4.0±0.6	10.3±5.3	5.1±4.5	6.5±9.1	25.4±3.7	31.0±8.4	13.2±4.6	6.7±5.3

injected and the integrator response factors were computed. These were then stored in the integrator for the computation of unknown concentrations. Injection volumes of 20 μL were employed for all quantitative evaluations and the amount of amino acid was directly obtained from the data module. The data module calibration was checked regularly with standard solutions. Typical results and standard deviations from triplicate determinations on 3 varieties of apple extracts in 5 stages of maturity are presented in Tables I and II. The analytical results indicate that Gln and Ala should be included in the second group and that the majority group (Asp, Glu, Asn and Ser) comprises 80 % of the total amino acid content, and Val and Phe could be included in this group. It can be seen that the concentration of all

amino acids generally shows a steady decrease as the fruits ripen, a minimum being reached when the apples are close to ripening: early November for „Raxao“, about the second ten days of November for „Meana“, and the last days of November for „Collaos“.

This fact is important from the technological point of view when making cider or apple juices because the amino acids are the principal source of soluble nitrogen employed by the microorganisms during fermentation processes in cider making, and are involved in the non-enzymatic browning processes (Maillard reaction) that affect the quality of the concentrates and juices of the apples

Table III Recovery studies of amino acids added to apple extracts.

Compound	Amount in extract (µg/L)	Amount added (µg/L)	Found (µg/L)	% Recovery + S.D.
Asp	184.0	33.3	211.1	96.6 ± 2.1
		66.6	260.4	105.3 ± 2.2
		865.2	033.2	91.5 ± 5.6
Glu	190.4	36.8	227.4	100.0 ± 1.8
		73.6	264.0	100.0 ± 6.4
		956.2	1174.6	100.5 ± 4.0
Asn	567.6	26.4	621.2	104.8 ± 2.8
		66.1	626.1	98.7 ± 1.1
		105.7	722.5	108.7 ± 3.0
Ser	79.0	3.2	86.3	105.2 ± 3.2
		6.4	89.0	104.6 ± 5.2
		10.5	91.2	102.2 ± 3.1
Gln	11.9	0.7	12.6	100.0 ± 3.0
		1.5	13.4	100.0 ± 3.0
		3.7	15.5	99.2 ± 1.3
His	14.8	0.8	15.6	100.0 ± 4.5
		1.6	16.3	99.3 ± 3.8
		3.9	18.6	99.3 ± 4.4
Hse	2.7	0.6	3.2	96.3 ± 3.5
		1.2	3.8	96.3 ± 3.2
		3.0	5.7	100.0 ± 2.8
Gly	4.1	1.9	6.1	102.4 ± 12.0
		3.8	7.8	97.6 ± 5.0
		4.9	9.2	104.9 ± 5.5
Thr	21.1	3.0	25.1	104.7 ± 2.7
		6.0	29.0	109.0 ± 0.9
		7.7	28.7	99.5 ± 1.3
Arg	9.9	1.7	11.2	96.0 ± 0.2
		4.4	14.0	97.0 ± 0.2
		8.8	19.5	108.1 ± 3.1
β-Ala	6.5	2.2	8.1	90.8 ± 3.9
		4.5	11.2	103.1 ± 2.3
		5.8	12.4	101.5 ± 2.5
Ala	18.4	2.2	20.7	100.5 ± 3.9
		4.5	22.7	98.9 ± 4.1
		5.8	24.0	98.9 ± 1.4
GABA	17.3	2.6	19.4	97.1 ± 3.4
		5.2	22.5	100.0 ± 3.9
		6.7	24.2	101.2 ± 3.0
Tyr	8.3	4.5	12.9	101.2 ± 8.0
		9.1	17.8	104.8 ± 3.5
		11.8	19.8	96.4 ± 3.1
Val	32.2	3.5	35.5	99.4 ± 2.6
		7.0	38.8	98.8 ± 2.2
		11.7	42.6	96.0 ± 3.0
Phe	81.1	4.1	84.6	99.3 ± 3.2
		8.2	87.7	98.0 ± 3.1
		10.7	89.0	96.5 ± 1.1
Ile	10.8	3.3	14.7	105.6 ± 7.4
		6.6	17.1	97.2 ± 0.5
		8.5	19.4	100.9 ± 0.4
Leu	4.7	3.3	8.2	103.8 ± 6.5
		6.6	11.7	108.7 ± 6.6
		8.5	13.0	96.8 ± 4.8

It can clearly be seen that major amino acids, (Asn, Ser, Asp, and Glu) reach a maximum in the last stage of ripening and then decrease. This trend is clearly visible in serine evolution as is graphically illustrated by Figure 3.

This trend is similar to that observed for malic acid evolution previously reported [24].

To study the accuracy of the method, recovery experiments were performed. Known amounts of each amino acid were added to a variety of samples and the

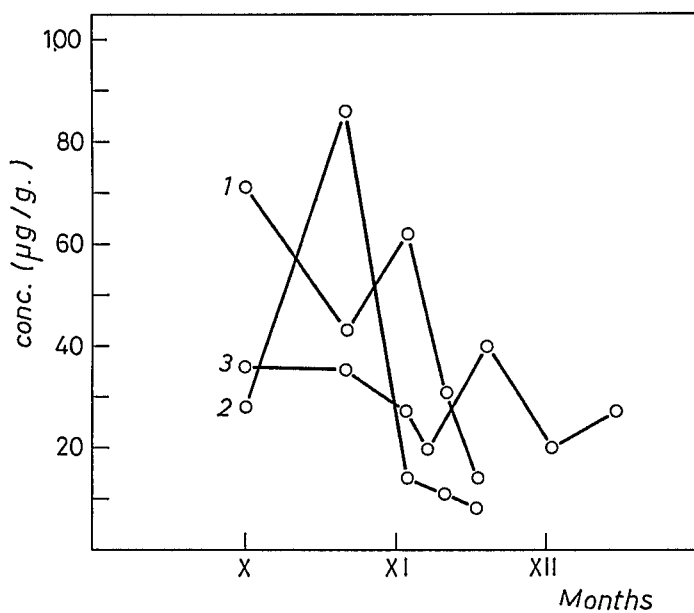


Figure 3
Evolution of Serine content in apple extracts during the last three months of ripening. 1 = Meana, 2 = Raxao, 3 = Collaos.

resulting spiked samples were subjected to the entire analytical sequence. Each solute was spiked at three different concentrations and the results for an apple extract are given in Table III. Complete triplicate analyses were performed. The average recoveries of between 91 and 109 % indicate that the method has an adequate degree of accuracy for the analysis of these substances.

Conclusions

High Performance Reversed-phase Liquid Chromatography and a pre-column derivatization that forms fluorescent adducts provides a useful tool for the efficient separation and quantification of amino acids in foods. The use of O-phthaldialdehyde 2-mercaptoethanol as a pre-column derivatizing reagent provides a rapid and sensitive method and is an attractive alternative to ion-exchange methodology.

The procedure proposed may be useful in monitoring the progression of amino acid concentrations during the ripening of the apple and could be used, together with other observations, as a means of assessing its maturity and potential behaviour in industrial processing.

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