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Retention/quantitation properties of the *o*-phthaldialdehyde–3-mercaptopropionic acid and the *o*-phthaldialdehyde–*N*-acetyl-L-cysteine amino acid derivatives in reversed-phase high-performance liquid chromatography

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Abstract

The separation/identification of 25 amino acids as their *o*-phthaldialdehyde–3-mercaptopropionic acid (OPA/MPA) and *o*-phthaldialdehyde–*N*-acetyl-L-cysteine (OPA/NAC) derivatives have been optimized [paying particular attention to those amino acids which elute with more than one derivative (glycine, histidine, γ-aminobutyric acid, β-alanine, ornithine, lysine) and that are expected to be present in apples in their free form]. Optimum separation conditions are reported on six reversed-phase columns: Nucleosil 3 and 5 μm, 150(+20 guard)×4.0 mm; Gromsil 3 μm, 150(+10 guard)×4.0 mm; Hypersil 5 μm, 150(+20 guard)×4.0 mm and 200(+20 guard)×4.0 mm; and Hypersil 3 μm, 150(+20 guard)×4.0 mm. Elutions were followed, simultaneously, with photodiode array and fluorescence detectors connected in line. Optimization studies carried out in model solutions as a function of temperature (30–55°C) and eluent flow-rate (0.8–2.5 mL/min) demonstrated that optimum resolutions are obtained with the highest flow-rate applicable (remaining on the safe side with a column pressure of $\ll$3500 p.s.i.; 1 p.s.i.=6894.76 Pa) in the temperature range 30–50°C. Twenty-five amino acids, eluting in 31 separate, characteristic derivatives, were determined on all six columns (the main component, asparagine, present in overwhelming excess, together with the minor constituents glutamine, β-alanine, γ-aminobutyric acid, homoserine, and homoarginine). Optimum conditions in the case of both derivatives were obtained on the same type of column (Hypersil, 5 μm), as follows: for the OPA/MPA amino acids with programmed flow-rate [1.3–2.3 ml/min; column, 200(+20 guard)×4 mm], at 50°C, while, for the OPA/NAC amino acids at 2.1 ml/min flow rate, at 30°C [column, 150(+20 guard)×4 mm], with 40 and 37 min run times, including equilibration. Responses of the corresponding amino acids proved to be independent of the column used; reproducibility in the concentration range 6–12 000 pmol, related to the injected amount of amino acids, was <3.4% RSD (average relative standard deviation percentage). The utility of the protocol was demonstrated in the quantitation of the free amino acid content of five apple varieties (Jonagored, Idared, Jonica, Florina, Freedom) on various harvesting dates and after different storage times. Derivatization of the apple pulp was performed with filtered samples, applying any special isolation processes.

Keywords: Derivatization, LC; Apples; Fruits; Food analysis; Amino acids

1. Introduction

Our recent papers [1–3] dealing with the stability and characteristics of the *o*-phthaldialdehyde–3-mer-

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captopropionic acid (OPA/MPA) and *o*-phthaldialdehyde–*N*-acetyl-L-cysteine (OPA/NAC) derivatives of amino acids [1,2], outside the chromatographic system [1] and under high-performance liquid chromatography (HPLC) conditions [2], as well as with the method development of the 27 phenylthiocarbamyl amino acids present in apples [3], served as the basis of this study. The importance of knowledge of the amino acid content of apples has been detailed elsewhere [3]. The apple amino acids have been quantitated by paper chromatography [4], ion-exchange chromatography (IEC) [5–7], gas chromatography (GC) [8], and HPLC [8–13]. HPLC, after precolumn derivatization with *o*-phthaldialdehyde (OPA) and the chiral thiols [*N*-isobutyryl-L-cysteine (IBLC) and/or *N*-isobutyryl-D-cysteine (IBDC)] [8], or with Marfey's reagent [(1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide] [13], made possible the chiral resolution of the L- and D-amino acids in apples [8,13]. Reports dealing with the quantitation of amino acids as OPA/MPA [14–17], OPA/NAC [18–22] and OPA/IBLC(IBDC) [8,23] derivatives present in various matrices [8,18–23] (Table 1) revealed that: (i) the existence of the double

Table 1
HPLC of the OPA/MPA [14–17], OPA/NAC [18–22] and OPA/IBLC(IBDC) [8–23] amino acid derivatives (literature data)[a]

Column		Product	Eluents	$\lambda_{ex}/\lambda_{em}$	RSD (%)	Analyte	Matrix/etc.	Not resolved	No/min	Ref.
cm×mm	μm									
25×4	4	Supersper C_8	A: 12.5 m*M* sodium phosphate (pH 7.2); B: ACN–12.5 m*M* sodium phosphate (pH 7.2) (1:1)	330/445	<3.8	1–10 p*M*	Biological tissues	Asp/Ser	27/50	[14]
25×4 +guard	5	LiChrosorb C_{18}	A: ACN–12 m*M* sodium phosphate (pH 7.2) (4:96); B: ACN–12 m*M* sodium phosphate (pH 7.2)–THF (47.5:47.5:5)	330/450	–	1–800 p*M*	Cell protein metabolism	Asp/Glu, Thr/Gly, Phe/Trp	15/50	[15]
15×4.6	2–3	Spherisorb	A: 12.5 m*M* sodium phosphate (pH 7.0), cont. 7 mL/L THF; B: 12.5 m*M* phosphate (pH 7.0) –ACN–THF (57:40:3)	338/440	<8.7	35 p*M*	Physiological amino acids	Asp/Glu, Arg/GABA, Thr/Trp	30/22	[16]
10×4.6	3	Microsphere	A: 9 m*M* KH_2PO_4 (pH 6.9)+4 mL TEA; B: 9 m*M* KH_2PO_4 (pH 6.9)–Met–ACN (50:35:15)	230/389	<7.2	60 n*M*	Cerebrospinal fluid	–	26/17	[17]
20×6.0	5	Develosil ODS	A: 50 m*M* sodium acetate; B: Met (25°C)	360/405 post col.	<2.28	50 p*M*	Amino acid standards	Five pairs	14 pair/70	[18]
15×4.0	7.5	ISC-07/S1504[b]	A: 0.2 *M* sodium citrate, cont.7% Et (pH 3.2); B: 0.6 *M* sodium citrate (pH 10.0); C: 0.2 *M* NaOH (50°C)	348/450 post col.	<1.3	1 n*M*	Amino acids in biological samples	Thr/Ser/Glu/Pro Gly/Ala, Ile/Leu	17/60	[19]
12×4.0	3	Hypersil ODS	A: 50 m*M* sodium phosphate (pH 7.0); B: 50 m*M* sodium phosphate (pH 7.0)–Met–THF (35:65:1)	344/443	–	–	Amino acids in peptide hydrolyzates	L-/D-thr	Six pair/30	[20]
25×4.0	5	Hypersil ODS	A: 23 m*M* sodium acetate (pH 5.95); B: Met–ACN (474:39, w/w)	F230/445	–	100 p*M*	Amino acids in fruits and vegetables	Three pairs	17 pair +2/85	[8]
12×4.6	5	Spherisorb ODS	Isocratic: Na_2HPO_4–H_3PO_4 (pH 3.0), cont. 0.05 *M* SDS+cont. 3% propanol	UV 336	<2.2	–	Amino acids in pharmaceuticals	–	Two pair/20	[21]
25×4.6	–	Econosphere	A: Met; B: 50 m*M* sodium acetate (pH 5.4)–Met (92:8)	F340/450	–	1–2 p*M*	α-Dialkyl amino acids in geological samples	–	3–5/25 1995	[22]
12.5×4.0 + guard	4	Supersper C_{18}	A,B: 25 m*M* sodium acetate (A, pH 7; B, pH 5.3); C: Met (20°C)	F330/445	<10.2	0.5–200 p*M*	Amino acids in sea water and fossil samples	Four pairs	20 pair +2/95	[23]

[a] IBLC/IBDC, *N*-isobutyryl-L-cysteine/*N*-isobutyryl-D-cysteine; ACN, acetonitrile; Met, methyl alcohol; Et, ethyl alcohol; SDS, sodium dodecyl sulfate.

[b] Cation-exchange column.

[glycine, γ-aminobutyric acid (GABA), β-alanine)] and triple products (ornithine, lysine) that are present in selected cases [2], in non-negligible concentrations, were first evaluated in the frame of a basic research study in our laboratory [1,2] (with GABA the only exception [8]); (ii) in practice, the extremely different concentrations of amino acids to be separated and determined were not taken into account; (iii) several amino acids, including aspartic/glutamic acids, asparagine/serine, could not be separated from their neighbours, even when they were present in commensurable concentrations (Table 1, 'Not resolved').

The aim of this study was to extend our earlier experiences [1–3] in order to determine the possibility for the analysis of 25 amino acids as their OPA derivatives applying photodiode array (PDA) and fluorescence (FL) detection simultaneously, taking into consideration that (i) the multiple derivatives providing amino acids should be evaluated on the basis of all of their separable peaks, including the possible apple constituents; (ii) the time-consuming/tedious isolation process of the apple amino acids should be shortened [8,10,12,33,35,36].

2. Experimental

2.1. Materials

OPA, 3-mercaptopropionic acid (MPA), *N*-acetyl-L-cysteine (NAC) and amino acids were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade methanol, acetonitrile and tetrahydrofuran were purchased from Romil Chemicals (Leics., UK). All other reagents were of the highest purity available. Authentic apple varieties, Jonagored, Idared, Jonica, Florina and Freedom, were obtained from the Research Garden of the University of Horticulture and Food Industry (Szigetcsép, Hungary). Apple samples harvested on three consecutive dates (09.09.98, 16.09.98 and 05.10.98) are indicated by numbers 1–3, i.e. Jonagored1, Jonagored2, and Jonagored3, while the consecutive dates of their storage times (26.01.99, 22.02.99 and 23.03.99) are denoted by the fractional figures 1/1–3/3, i.e. Jonagored1/1–1/3, Jonagored2/1–2/3 and Jonagored3/1–3/3, etc.

2.2. Standard solutions

Standard solutions of free amino acids were prepared in distilled water [16] in two stock solutions: standard solution 1 (ST-1) and standard solution 2 (ST-2). ST-1 contained $2.5\cdot10^{-4}$ M/L each of L-aspartic and L-glutamic acids, L-serine, L-histidine, glycine, DL-homoserine, L-threonine, β-alanine, L-arginine, L-alanine, GABA, L-homoarginine, L-tyrosine, L-valine, L-methionine, L-cysteine, L-tryptophan, L-phenylalanine, L-isoleucine, L-leucine, L-ornithine and L-lysine, with the exception of L-glutamine and L-asparagine (ST-2). ST-2 contained various amounts of L-asparagine ($2.5\cdot10^{-2}$–$2.5\cdot10^{-4}$ M/L) and L-glutamine ($2.5\cdot10^{-3}$–$2.5\cdot10^{-4}$ M/L), and was prepared in all second week: ST-1 and ST-2 were further diluted to obtain [OPA]/[MPA]–([NAC]):$[AA]^{T}\cong 6.89{:}1–120{:}1$.

The stock solution of OPA contained 0.256–0.512 g OPA (weighed with analytical precision) in 25 mL methanol (below: methanolic OPA solution).

2.3. Buffer solution

Borate buffer was mixed in 50:50 volume ratios from 0.2 *M* boric acid (dissolved in 0.2 *M* potassium chloride)–0.2 *M* sodium hydroxide (pH 9.9±0.05).

2.4. Akaline solution for neutralization of apple pulp (below: neutralization solution)

Ten grams sodium hydroxide, 7.4 g potassium chloride and 6.1 g boric acid were dissolved in 100 mL distilled water (pH≅12.4).

2.5. Reagent solutions

OPA/MPA reagent was obtained by mixing, in order of listing, 5.0 mL methanolic OPA, 20.0 mL borate buffer and 50–200 μL MPA solutions: final pH 9.3±0.05. OPA/NAC reagent was prepared from 5 mL methanolic OPA solution and 20.0 mL borate buffer containing 0.1–0.4 g NAC: final pH 9.3±0.05. The mole ratios of OPA to MPA and NAC were, without exception, OPA/MPA(NAC)=1/3.

2.6. Derivatization

2.6.1. Characterization of the reagent solutions

Blank measurements were performed with freshly prepared (reagent age ≥90 min [2]) reagent solutions, stored in a refrigerator at ~4°C, for various times and injected by the robotic autosampler at least two times each day (Waters 717, thermostated at ~4°C).

2.6.2. Method development study with OPA/MPA(NAC)–amino acid solutions

Derivatizations were performed with reagents prepared at least 90 min before use and retained no longer than ≤9 days [2]. The same amounts of reagent solution (120 μL) were mixed with 380 μL ST-1+ST-2 amino acids, and reacted for 7 min before injection.

2.6.3. Reproducibility study of isoindole derivatives

Derivatizations were carried out with reagent solutions prepared at least 90 min before use and retained no longer than ≤9 days. The same amounts of reagent solution (120 μL) were mixed with variously diluted (380 μL) ST-1 and ST-2 amino acids and injected as detailed above.

2.6.4. Preparation of apple pulp prior to derivatization

Peeled apples were homogenized and sieved. Pulps were used immediately, or stored in a refrigerator at −20°C. Prior to derivatization, 2–5 g of pulp was weighed and filtered as follows: a 10 mL volumetric flask, with a funnel (diameter 5 cm), covered with a GF/F glass microfibre paper, were weighed (i.e. flask+funnel with the paper) with 0.01 g precision (the microfibre paper was also weighed separately with analytical precision). The apple pulp (2–5 g) was pipetted into the funnel and weighed again with 0.01 g precision. The amount of apple pulp was obtained from the difference. After filtration, the undissolved residue was washed with 4×1 mL distilled water and the homogenized filtrate was made alkaline (pH 9.3–10.2, determined by a pH meter) with the neutralization solution (0.35–0.40 mL). The volume was then made up to 10.0 mL (apple stock solution). Derivatization was performed with 100 μL apple stock solution according to Section 2.6.3. The funnel with the paper containing the undissolved apple pulp residue was placed in an oven, dried at 50°C, and the dried filter+residue was weighed with analytical precision.

2.7. Chromatography

The system was a Waters HPLC instrument (Waters Pharmaceutical Division, Milford, MA, USA) consisting of Waters 996 PDA and Waters 274 fluorescence detectors, a Waters 600 Controller quaternary pump with thermostattable column area, and a Waters 717 Autosampler, operating with the Millennium software (version 2010, 1992–95, validated by ISO 9002). Test columns T-1 to T-3 were Hypersil ODS bonded phase. T-1, 150 mm×4 mm, 3 μm; T-2, 150 mm×4 mm, 5 μm; T-3, 200 mm×4 mm, 5 μm (Shandon; Supplier: BST, Budapest, Hungary). T-4 was 125 mm×4 mm, 3 μm, Gromsil (Grom Analytic+HPLC; Supplier: LAB-COMP, Budapest, Hungary). T-5 and T-6, 150 mm×4 mm Nucleosil 120, C_{18}, 3 μm and 5 μm columns (Macherey–Nagel; Supplier: BST). Columns T-1 to T-3, T-5 and T-6 were used with 20 mm×4 mm, while T-4 was used with 10 mm×4 mm guard columns (henceforth columns T-1 to T-6).

2.7.1. Detection

Detections were performed simultaneously, applying the PDA (Waters 996) and fluorescence (Fl) (Waters 274) detectors, connecting them in order of listing. Blank tests, concentration dependence and the apple pulp were detected between 190 and 400 nm (PDA), evaluated at 334 nm [OPA/MPA(NAC) amino acids], as well as at the optimum excitation (λ_{ex})/emission (λ_{em}) wavelengths: at $\lambda_{ex}/\lambda_{em}$=337/454 nm.

2.7.2. The eluent system

The eluent system consisted of two components: (A) eluent 0.05 *M* sodium acetate, pH 7.2 [in its final version containing 1% (v/v) tetrahydrofuran (THF)], while eluent (B) was prepared from 0.1 *M* sodium acetate–acetonitrile–methanol (46:44:10) (mixed in volume ratios and titrated with glacial acetic acid or 1 *M* sodium hydroxide to pH 7.2): separate gradient programs were followed (Table 2).

Table 2
Optimum gradient programs for the OPA/MPA- (Prg-1, column T-3, 50°C) and for the OPA/NAC-amino acids (Prg-2, column T/2, 30°C)

OPA/MPA program (Prg-1)					OPA/NAC program (Prg-2)				
Step	Time (min)	Flow (mL)	A (%)	B (%)	Step	Time (min)	Flow mL (mL)	A (%)	B (%)
1	0.00	1.3	99.5	0.5	1	0.00	2.1	99.5	0.5
2	6.00	1.3	99.0	1.0	2	4.00	2.1	99.0	1.0
3	8.00	1.3	97.0	3.0	3	8.00	2.1	90.0	10.0
4	9.00	1.3	93.0	7.0	5	10.0	2.1	75.0	25.0
5	11.00	2.3	85.0	15.0	6	15.0	2.1	65.0	35.0
6	12.00	2.3	81.0	19.0	7	19.0	2.1	55.0	45.0
7	16.00	2.3	72.0	28.0	8	21.0	2.1	0.0	100.0
8	19.00	2.3	72.0	28.0	9	24.0	2.1	0.0	100.0
9	22.00	2.3	65.0	35.0	10	26.0	2.1	99.5	0.5
10	26.00	2.3	0.0	100.0	11	37.0	2.1	99.5	0.5
11	28.00	2.3	0.0	100.0					
12	29.00	2.3	99.5	0.5					
13	40.00	2.3	99.5	0.5					

3. Results and discussion

Preliminary studies revealed [2] that, in order to determine 25 or 24 amino acids as their OPA/MPA or OPA/NAC derivatives (L-homoserine was not available, and we did not want to deal with the enantiomers of the OPA/NAC derivatives), at least 29 or 28 components, members of the solutions ST-1 and ST-2, have to be separated and evaluated.

Based on our earlier experience [2,3,23–34] (which demonstrated that RP C_{18} Hypersil and Nucleosil phases have comparable properties), this work was started in parallel with RP C_{18} Hypersil and Nucleosil columns of 5 and 3 μm particle size.

3.1. Gradient program study, method development

Introductory tests demonstrated that gradient programs would need to be optimized in successive steps.

3.1.1. OPA/MPA derivatives (optimum elution: Prg-1, Table 3, Fig. 1)

First, the separation of asparagine/serine, as well as those of histidine/glycine/homoserine, was studied as a function of the pH of the eluent. In this respect, pH 7.2 proved to be the optimum. To enhance the elution of aspartic and glutamic acids, as well as to increase the resolution between histidine/glycine/homoserine, the THF content of eluent A needed to be varied. On increasing the THF content of eluent A (0–1.5%), 1% proved to be the optimum (details not shown).

The next elution steps assured the separation of arginine, alanine, glycine2, β-alanine2 and GABA2, while the last steps improved the resolution of phenylalanine and isoleucine, and allowed the quantitation of ornithine3 and lysine3.

The optimum separation could be strongly influenced by the temperature of elution and by the eluent flow-rate. With regards to the elution temperature, 50°C, in particular, proved to be advantageous on all six columns tested (Fig. 1, elutions on columns T-1 to T-6), while in order to obtain acceptable separations for all 29 compounds with the best resolution with Hypersil columns of 5 μm particle size (columns T-2 and T-3), a programmed rate has to be followed (Table 2, Prg-1: between 0 and 9 min 1.3 mL/min, from 9 to 40 min 2.3 mL/min). From the best resolution figures (Table 3, values in parentheses) for the OPA/MPA derivatives, column T-2 and Prg-1 were chosen as optimum conditions (henceforth: optimum condition).

3.1.2. OPA/NAC derivatives (optimum elution: Prg-2, Table 4, Fig. 2)

In contrast to the OPA/MPA derivatives, the OPA/NAC derivatives also provided acceptable separation at lower temperatures (Fig. 2, columns T-1 to T-6). The successively developed elution steps were in-

Table 3
Reproducibility of the quantitation of [OPA]/[MPA]/[amino acid]^{T}=8.99·10^{-7}M/27·10^{-7} M/5.67·10^{-8} M, obtained at 50°C elution temperature, with 1% tetrahydrofuran containing eluent A, on column T-3 (Hypersil, 200+20 mm×4 mm, 5 μm), varying the eluent flow-rate

Amino acid	Arbitrary units/1 p*M* amino acid[a]						Average	SD	RSD (%)
	1.3[b] (mL/min)	1.5[b] (mL/min)	1.8[b] (mL/min)	2.1[b] (mL/min)	2.3[b] (mL/min)	1.3⇒2.3[b] (mL/min)			
1 Aspartic acid	4.53	4.41	4.46	4.27	4.65	4.32	4.44	0.140	3.2
2 Glutamic acid	5.12	5.10	5.12	5.27	5.33	5.08	5.17	0.104	2.0
	(1.62)	(1.57)	(1.50)	(1.44)	(1.44)	(1.52)			
3 Asparagine	3.92	3.90	3.89	4.04	4.10	3.90	3.96	0.090	2.3
4 Serine	3.89	3.50	3.66	4.02	3.84	3.73	3.77	0.184	4.9
5 Glutamine	4.65	4.70	4.25	4.90	4.80	4.20	4.58	0.291	6.4
6 Histidine1	0.95	0.94	0.77	1.00	0.95	0.90	0.92	0.081	8.8
7+16 Gly1	3.66	3.55	3.76	3.91	3.75	3.55	3.70	0.140	3.8
+Gly2	(1.15)	(1.18)	(1.07)	(0.96)	(b.r.)	(1.07)			
8 Homoserine	4.42	4.41	4.63	4.90	4.95	4.52	4.64	0.238	5.1
	(1.08)	(1.06)	(1.03)	(1.02)	(0.99)	(1.17)			
9 Threonine	3.26	3.33	3.55	3.70	3.78	3.33	3.49	0.17	6.2
10+20 β-Ala1									
+β-Ala2	2.96	3.03	3.14	3.27	3.13	3.09	3.10	1.103	3.3
11 Arginine	4.02	3.87	3.87	4.07	4.08	4.04	3.99	0.097	2.4
12 Alanine	3.71	3.44	3.36	3.59	3.83	3.79	3.62	0.191	5.3
	(1.71)	(1.31)	(1.43)	(1.31)	(1.16)	(1.38)			
13+21 GABA1									
+GABA2	4.15	3.98	3.93	3.84	3.73	4.18	3.97	0.175	4.4
14 Homoarginine	5.49	5.26	5.37	5.43	5.34	5.49	5.40	0.090	1.7
	(1.74)	(2.39)	(3.11)	(3.62)	(3.86)	(1.90)			
15 Tyrosine	5.10	5.09	5.12	5.16	5.15	5.25	5.15	0.059	1.2
17 Valine	5.02	5.06	5.29	5.32	5.54	5.46	5.28	0.208	3.9
18 Methionine	5.02	5.09	5.20	5.20	5.32	5.27	5.18	0.115	2.2
	(1.25)	(1.24)	(1.24)	(1.42)	(1.43)	(1.26)			
22 Tryptophan	4.42	4.48	4.72	5.00	5.18	5.12	4.82	0.328	6.8
23 Phenylalanine	4.89	4.84)	4.88)	4.96	5.07	4.99	4.94	0.084	1.7
24 Isoleucine	5.90	5.58	5.63	5.51	5.69	5.76	5.68	0.136	2.4
	(b.r.)	(0.85)	(1.01)	(1.08)	(1.10)	(1.05)			
25 Leucine	(n.r.)	5.05	5.24	5.13	5.19	5.13	5.15	0.071	1.4
26+28 Ornith2									
+Ornith3	(n.r.)	1.39	1.59	1.50	1.39	1.43	1.46	0.085	5.9
27+29 Lysine2									
+Lysine3	1.91	1.90	2.03	1.98	2.03	2.02	1.98	0.060	3.0

n.r., no resolution; b.r., bad resolution, i.e. ≪1.
[a] Obtained from at least three separate tests. Resolution in parentheses.
[b] Calculated to 1 mL/min elution rates.

tended to ensure the optimum resolution of aspartic/glutamic acids, histidine1/glycine1, glycine2/valine (strongly influenced by the THF concentration of eluent A), as well as the separation of methionine/β-alanine2, tryptophan/isoleucine, ornithine2/phenylalanine and, finally, very small amounts of ornithine3 and lysine3 (optimized by the temperature and flow-rate of the elutions).

After a detailed investigation between 25 and 40°C, varying simultaneously the elution flow-rate and the THF content of eluent A, the use of column T-2 and Prg-2 was chosen as optimum conditions on the basis of the most advantageous resolution values (Table 4, values in parentheses).

These optimum gradient programs (Table 2) were the basis of the separation of 25 (OPA/MPA)- or 24

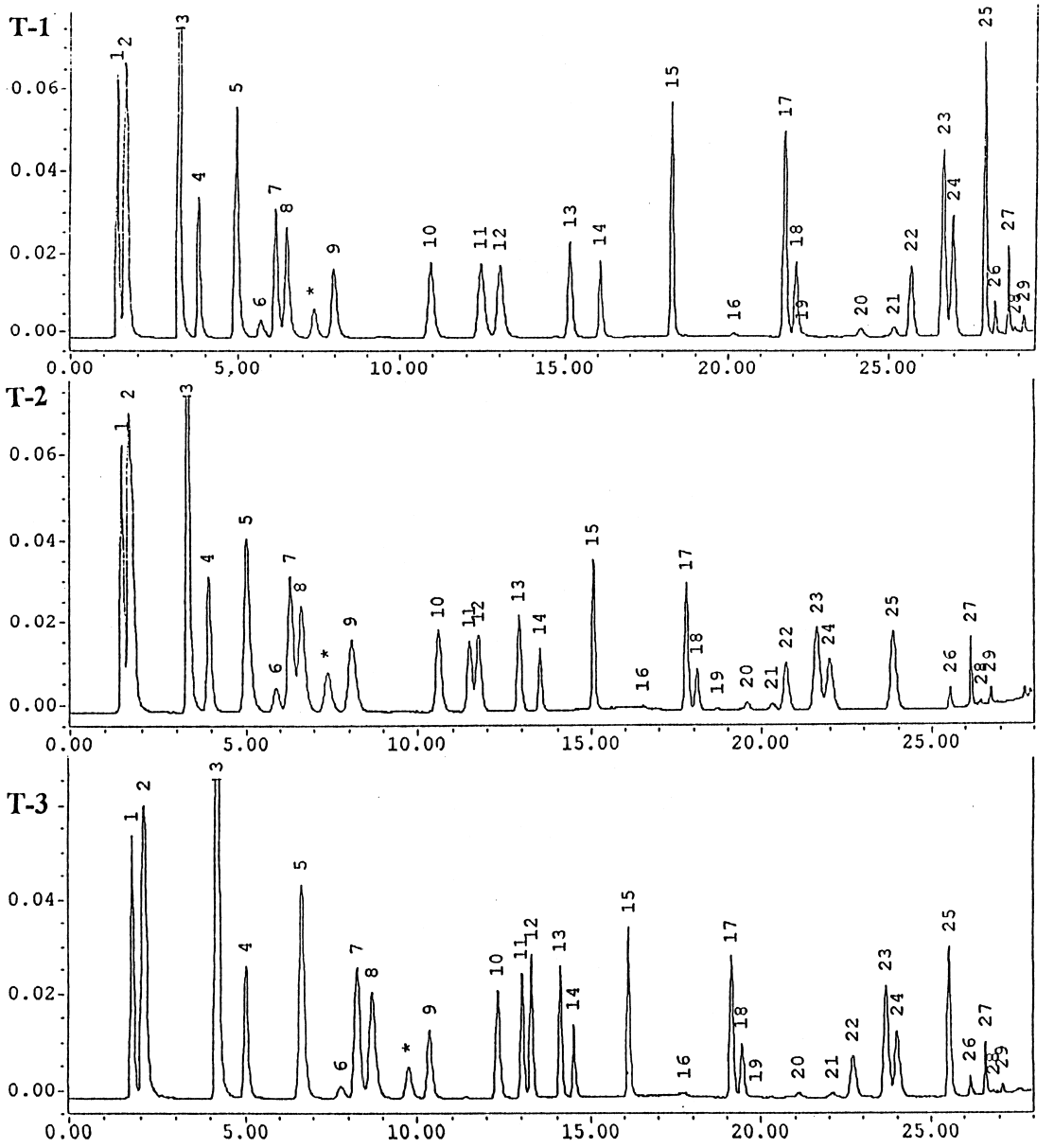

Fig. 1. Elution profile of 25+4 OPA/MPA-amino acids [(a) columns T-1 to T-3; (b) T-4 to T-6] obtained with model solutions. Peaks: (1) aspartic acid, (2) glutamic acid, (3) asparagine, (4) serine, (5) glutamine, (6) histidine, (7) glycine, (8) homoserine, (9) threonine, (10) β-alanine, (11) arginine, (12) alanine, (13) GABA, (14) homoarginine, (15) tyrosine, (16) glycine2, (17) valine, (18) methionine, (19) cyst(e)ine, (20) β-alanine2, (21) GABA2, (22) tryptophan, (23) phenylalanine, (24) i-leucine, (25) n-leucine, (26) ornithine2, (27) lysine2, (28) ornithine3, (29) lysine3. *System peaks; **not amino acid type impurities in apple; ***unknown apple constituents providing amino acid type spectra [2]. Time scale in minutes.

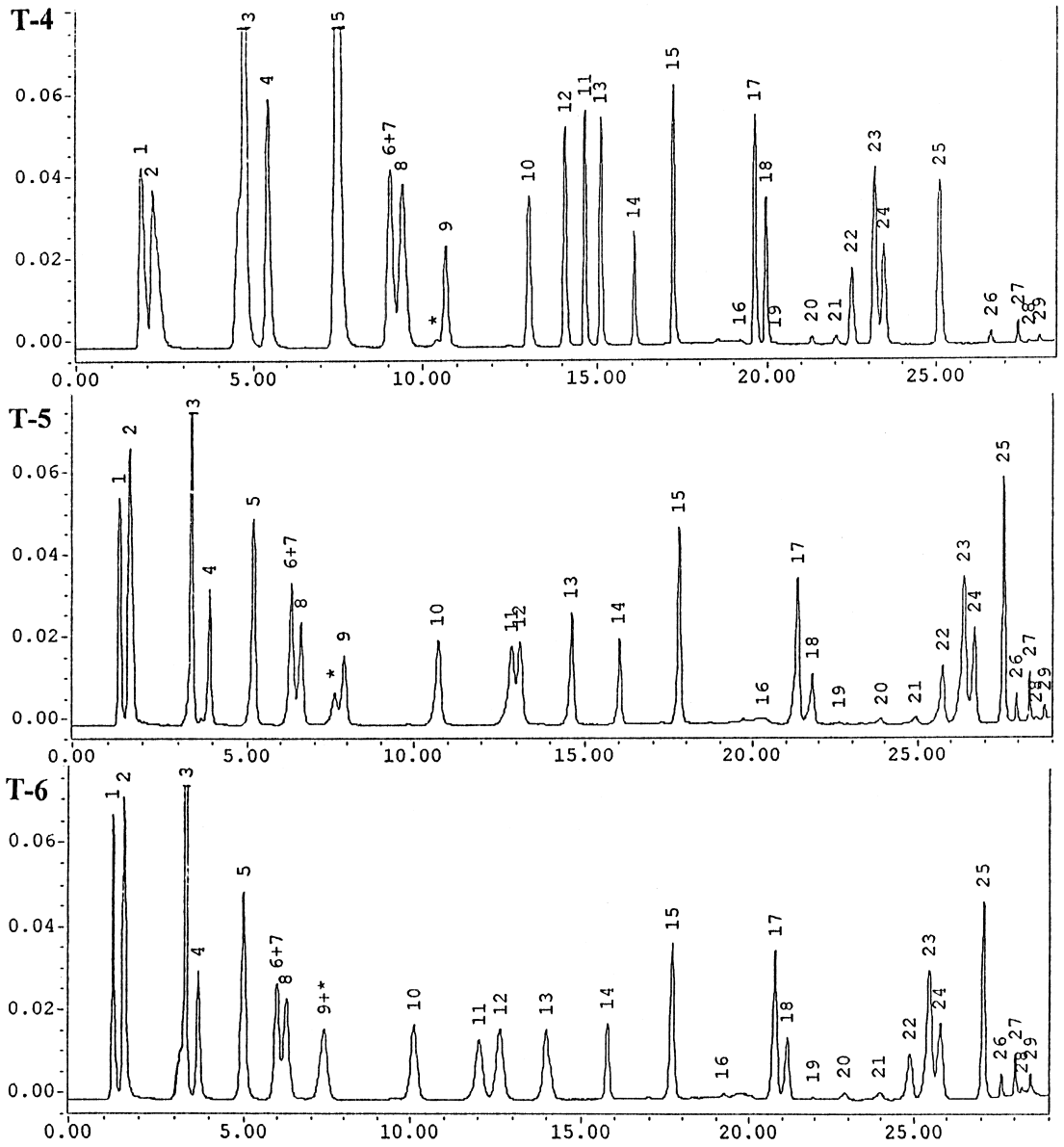

Fig. 1 (*continued*).

(OPA/NAC)-amino acids. The response factors of the amino acids proved to be independent of elution temperature and flow-rate, providing acceptable reproducibilities under all conditions measured (Tables 3 and 4): characterized by the relative standard deviations (RSDs), which were <6.4% for the OPA/MPA and <5.6% for the OPA/NAC derivatives.

3.2. Determination of the free amino acid content of apples

3.2.1. Reproducibility study in model solutions

The reproducibility of selected, different amounts of amino acids was determined as their OPA/MPA derivatives, in spite of the fact that analysis of the

Table 4
Reproducibility of the quantitation of [OPA]/[NAC]/[amino acid]^{T}=8.97·10^{-7} *M*/27·10^{-7} *M*/5.17·10^{-8} *M*, obtained at various elution temperatures, elution flow-rates and tetrahydrofuran containing eluent A, on column T-2 (hypersil, 150+20 mm×4 mm, 5 μm)[a]

	Integration units/p*M* amino acid[b]												Average	SD	RSD (%)
mL/min[c]:	2.1						1.7	1.9	2.1	2.3	2.1				
Elution temp. (°C):	25	30									3.5	40			
THF (%) (v/v): eluent A: Amino acid	1.0	0.8	0.9	1.0	1.1	1.2	1.0								
1 Aspartic acid	3.09	2.97	3.09	3.11	3.05	3.08	3.10	3.13	3.11	3.07	3.1	3.07	3.08	0.043	1.4
2 Glutamic acid	2.08	2.08	2.12	2.18	2.10	2.10	2.10	2.09	2.15	2.13	2.24	2.26	2.13	0.063	3.0
			(2.21)	(1.96)	(1.67)	(1.19)									
3 Asparagine	2.33	2.33	2.39	2.44	2.63	2.41	2.34	2.32	2.35	2.36	2.43	2.43	2.37	0.044	1.9
4 Serine	3.76	3.59	3.53	3.57	3.47	3.59	3.63	3.73	3.72	3.65	3.80	3.72	3.64	0.100	2.8
			(2.12)	(2.23)	(2.16)	(2.35)									
5 Glutamine	2.50	2.50	2.54	2.47	2.48	2.55	2.51	2.45	2.50	2.51	2.61	2.60	2.52	0.048	1.9
6 Histidine1	1.78	n.r.	1.62	1.68	1.82	1.82	1.60	1.69	1.65	1.62	1.65	n.r.	1.69	0.084	5.0
			(b.r)												
7 +16 Gly1+Gly2	4.24	n.r.	4.22	4.26	4.13	4.17	4.25	4.22	4.28	4.37	4.52	n.r.	4.27	0.110	2.6
			(b.r)	(1.43)	(1.55)	(1.77)									
9 Threonine	2.85	2.96	2.96	2.82	2.70	2.73	2.73	2.74	2.74	2.96	2.90	2.77	2.82	0.102	3.6
10 +20 β-Ala1 +β-Ala2	3.55	3.65	3.62	3.70	3.43	3.48	3.48	3.44	3.49	3.64	3.70	3.58	3.56	0.097	2.7
11 Arginine	2.47	2.47	2.47	2.50	2.42	2.50	2.51	2.41	2.42	2.42	2.53	2.50	2.47	0.040	1.6
13 +21 GABA1 +GABA2	3.39	3.52	3.51	3.57	3.40	3.38	3.41	3.36	3.40	3.38	3.53	3.44	3.44	0.073	2.1
			(1.56)	(2.10)	(1.97)	(2.16)									
12 Alanine	3.55	3.60	3.41	3.46	3.44	3.39	3.46	3.56	3.55	3.51	3.63	3.51	3.51	0.075	2.1
14 Homoarginine	2.71	2.71	2.65	2.71	2.65	2.79	2.71	2.62	2.59	2.71	2.76	2.79	2.70	0.065	2.4
15 Tyrosine	2.80	2.85	2.90	2.82	2.82	2.80	2.83	2.85	2.85	2.85	2.95	3.00	2.86	0.062	2.2
17 Valine	3.25	3.30	3.39	3.41	3.25	3.25	3.37	3.38	3.41	3.31	3.47	3.55	3.36	0.094	2.8
18 Methionine	2.38	2.52	2.63	2.55	2.52	2.55	2.43	2.47	2.58	2.45	2.52	2.58	2.53	0.061	2.4
22 Tryptophan	2.49	2.56	2.56	2.64	2.49	2.59	2.56	2.53	2.58	2.56	2.64	n.r.	2.56	0.052	2.0
24 Isoleucine	3.11	3.31	3.35	3.43	3.26	3.35	3.24	3.28	3.30	3.31	3.46	n.r.	3.31	0.094	2.8
26 +28 Ornith2 +Ornith3	1.06	0.96	0.92	1.04	0.96	0.92	1.00	1.00	0.92	0.92	1.06	0.96	0.98	0.054	5.6
23 Phenylalanine	2.61	2.66	2.71	2.78	2.68	2.74	2.76	2.68	2.71	2.71	2.84	2.87	2.73	0.075	2.7
25 Leucine	2.54	2.54	2.56	2.61	2.54	2.57	n.r.	2.55	2.56	2.61	2.70	2.68	2.59	0.057	2.2
27+29 Lysine2 +Lysine3	1.39	1.46	1.43	1.43	1.38	1.39	n.r.	1.47	1.38	1.39	1.38	1.38	1.41	0.036	2.6

[a,b,c] See Table 3.

OPA/NAC derivatives proved to be more advantageous regarding the derivatization and chromatographic conditions: (i) NAC is odourless in comparison with MPA and (ii) it is more convenient to work at 30°C than at 50°C. However, to deal with the double amount, i.e. with the L- and D-enantiomers of amino acids, would need further study. Thus, amino acids were derivatized with the OPA/MPA reagents in different amounts expected to cover the concentration range of amino acids present in apples in free form (Table 5: 95.5–12012 p*M*). Comparing response values obtained with extremely different amino acid concentrations and applying various mole ratios of the reactants (OPA/MPA:amino acid^{T}= 6.89:1–110.4:1, Table 4, last column: RSDs ≤7.9%) produced results almost as good as those measured with a commensurable concentration of amino acids (Tables 3 and 4, RSDs)

3.2.2. Reproducibility study with apple pulp

Our method development — in order to separate the free amino acids in apples as their OPA/MPA

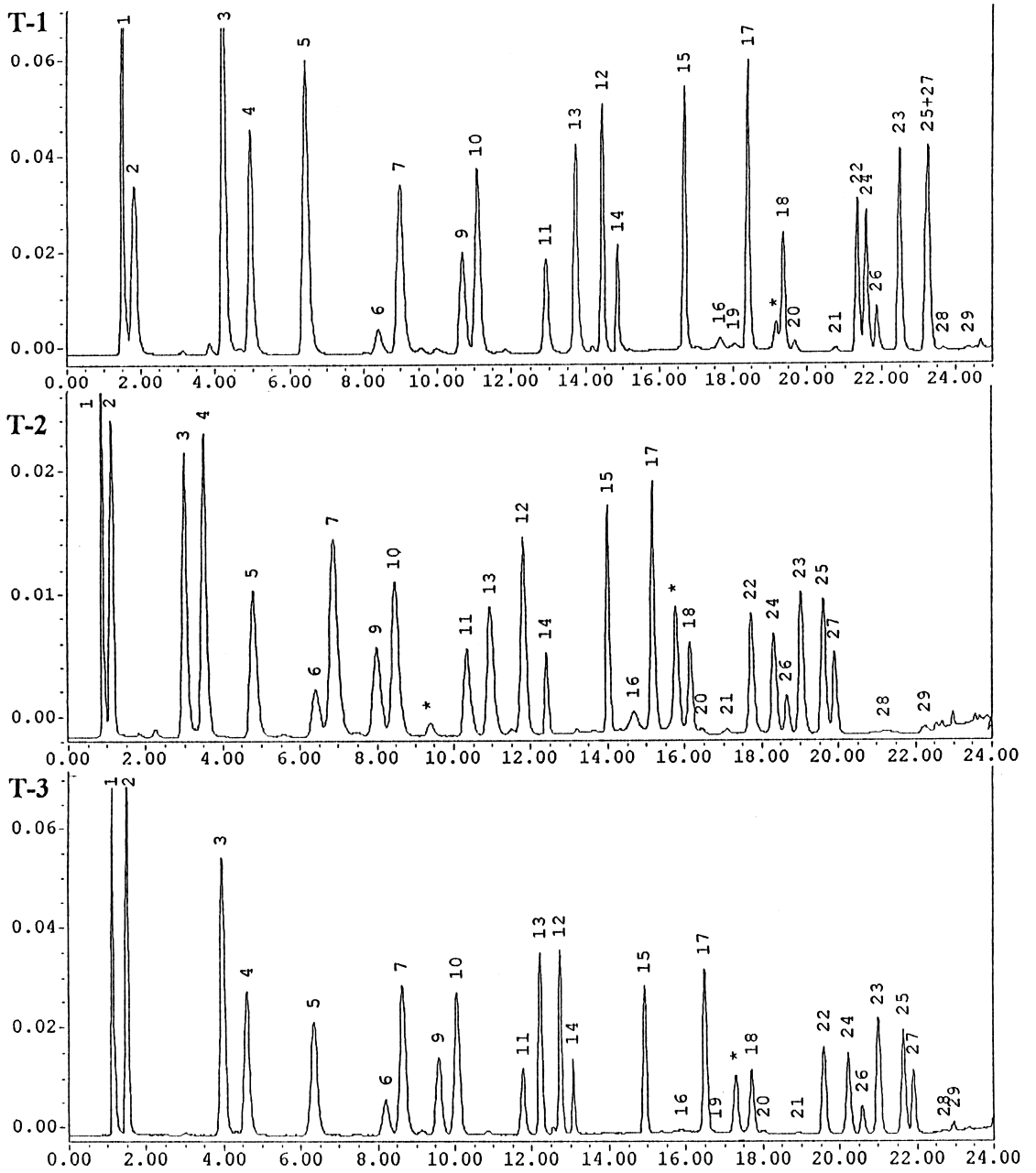

Fig. 2. Elution profile of 24+24 OPA/NAC-amino acids [(a) columns T-1 to T-3; (b) T- 4 to T-6] obtained with model solutions. Peaks as in Fig. 1.

derivatives — has been optimized paying particular attention to the pulp preparation process prior to derivatization. Our 'isolation process' was intended to be as simple and as fast as possible. On the basis of the corresponding literature data [8,10,12,33,36], it became clear that (i) combining the fast OPA derivatization technique with a simple and fast preparation process, and (ii) omitting the time-con-

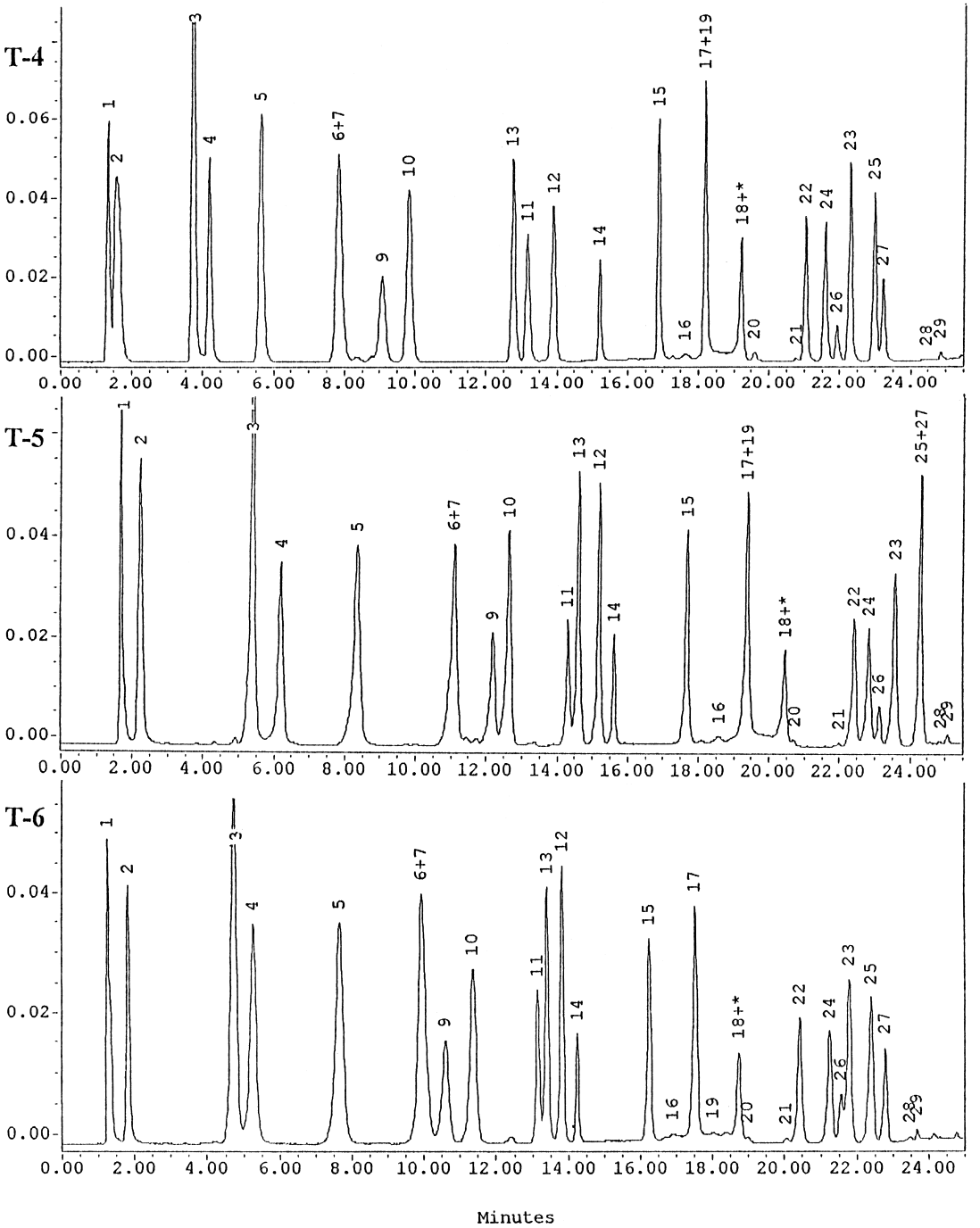

Fig. 2 (*continued*).

Table 5
Reproducibility of the quantitation of different amounts of OPA/MPA-amino acid derivatives in model solutions on the basis of their fluorescence intensities

Amino acid	Arbitrary units/1 p*M* amino acid					Average	RSD (%)
	p*M* injected	[OPA/(MPA)]/[amino acid]^T					
		6.89:1[a]	13.8:1[a]	27.6:1[a]	110.4:1[a]		
1 Aspartic acid	5960	2.94	3.07	3.13	3.09	3.05	2.8
2 Glutamic acid	2630	3.78	3.84	3.79	3.93	3.80	1.8
3 Asparagine	12 012	3.15	3.34	3.42	3.56	3.30	5.2
4 Serine-*1	1724	3.96	4.02	3.83	3.84	3.94	2.4
5 Glutamine	262.1	4.15	4.08	3.96	4.00	4.06	2.1
6 Histidine	286.6	*1.33*	2.04	1.95	2.16	2.05	5.1
7 +16 (Gly1–*2)+Gly2	351.8	2.83	2.86	2.86	2.77	2.85	1.5
8 Homoserine	219.7	4.13	4.11	4.20	3.95	4.15	2.5
9 Threonine	241.8	2.45	2.39	2.30	2.51	2.38	3.8
10 +20 β-Ala1+β-Ala2	370.6	2.12	2.05	1.82	*1.45*	2.00	7.9
11 Arginine	278.6	2.40	2.29	2.12	2.09	2.27	6.4
12 Alanine-*3	408.3	2.35	2.32	2.35	2.33	2.34	0.64
13 +21 GABA1+GABA2	360.3	2.47	2.44	2.36	*2.08*	2.42	2.3
14 Homoarginine	95.5	2.51	2.51	2.49	2.58	2.50	1.6
15 Tyrosine-*4	227.0	2.68	2.76	2.81	2.88	2.75	3.1
16 Valine-*5	234.7	2.59	2.60	2.57	*2.58*	2.59	0.50
18 Methionine	230.4	2.40	2.30	2.09	*1.51*	2.26	7.0
19 Cysteine	237.9	0.050	0.048	0.047	0.055	0.050	7.1
22 Tryptophan	229.5	2.05	1.97	1.87	1.84	1.96	4.9
23 Phenylalanine-*6	266.1	2.57	2.63	2.63	2.48	2.61	2.7
24 Isoleucine	195.9	2.85	2.83	2.83	2.77	2.84	1.2
25 Leucine	317.4	2.73	2.76	2.85	2.93	2.78	3.3
26 +28 Orn2+Orn3	329.3	0.695	0.701	0.666	0.680	0.687	2.3
27 +29 (Lys2–*7)+Lys3	248.9	0.960	0.980	0.959	1.012	0.966	2.6

Obtained from at least three separate tests.
Averages, obtained with [OPA/(MPA)]/[amino acid]^T mole ratios of 6.89:1, 13.8:1, 27.6:1, and 110.4:1.
Data in italics have been omitted from the mean.
[a] [OPA]=$5.73 \cdot 10^{-6}$ *M*.

suming and incomplete recovery isolation procedures, such as cation-exchange [8,33,36], classical ethanolic extraction [10,12], and solid-phase microextraction (SPME) [13], will result in a substantially reduced preparation time and in the complete recovery of amino acids.

We applied a simple filtration technique (Section 2.6.4, Table 6) which demonstrated that (i) by working with different amounts of pulp (2.51–5.09 g/10 mL), (ii) derivatizing various aliquots of the filtered stock solutions (50–200 μL), (iii) the reproducibility percentages in the quantitation of amino acids was comparable to values obtained with model solutions (<7.7%); (iv) with regards to the recovery provided by the cation-exchange cleanup, it has been reported [33] that it varies considerably (Table 6, last column: 37.2–106%).

Evaluating the distribution of identified and determined components as their OPA/MPA derivatives, it can be stated that (Fig. 3, Tables 7 and 8) (i) the overwhelming part of the peaks, on the basis of their retention times and their PDA spectra (detailed analysis of the spectra was performed in the range 205–400 nm [2]), are identical to those for authentic OPA/MPA amino acids. In addition, we found apple components with the characteristic spectra of OPA/MPA amino acids of unknown origin [Fig. 3 (indicated by ***), Tables 7 and 8]. As can be seen the

Table 6
Reproducibility of the quantitation of the free amino acid content of different amounts of apple pulp (Jonagored2) as their OPA/MPA-amino acid derivatives, applying optimum conditions

	Measured μg amino acid/g wet, filtered apple pulp							Ave.[c]	RSD (%)	IE[c,d] 4.00⇒ 1.0 mL	Recovery (%)
Weighed pulp/10 mL:[a]	4.99–0.0611			5.07–0.0750	5.09–0.0506	2.51–0.0118	3.82–0.0330				
μL taken from 10 mL: Amino acid[b]	50	200	100		100					25, 50	
1 Aspartic acid	37.2	36.7	38.2	36.3	35.6	36.4	36.3	36.7	2.3	35.5	96.8
2 Glutamic acid	20.1	18.8	21.0	19.9	21.0	20.0	20.4	20.2	3.7	20.3	101
3 Asparagine	330	307	334	315	318	309	316	318	3.2	292	91.7
4 Serine-*1	10.9	10.4	10.8	10.9	10.9	10.9	10.6	10.8	1.8	10.3	95.3
5 Glutamine	1.08	1.12	1.09	1.10	1.12	1.13	1.14	1.10	2.0	0.77	69.0
6 Histidine	3.00	3.02	3.10	3.20	2.94	3.01	2.97	3.00	2.9	3.20	106
7+16 (Gly1-*2) +Gly2	3.07	2.73	3.22	3.21	2.87	3.07	3.23	3.1	6.3	3.10	102
8 Homoserine	0.185	0.177	0.176	0.189	0.179	0.180	0.185	0.180	2.7	0.100	57.8
9 Threonine	5.69	5.89	6.07	5.81	5.90	5.75	5.95	5.90	2.2	4.90	83.0
10+20 β-Ala1 +β-Ala2	0.663	0.676	0.590	0.650	0.615	0.581	0.598	0.620	6.1	0.600	95.5
11 Arginine	1.54	1.51	1.46	1.49	1.48	1.38	1.37	1.50	4.4	1.30	91.9
12 Alanine-*3	6.12	6.07	6.38	6.29	6.22	6.05	6.27	6.20	2.0	5.50	87.9
13+21 GABA1 +GABA2	2.54	2.65	2.53	2.49	2.55	2.52	2.63	2.60	2.3	2.20	84.3
14 Homoarginine	0.148	0.130	0.145	0.134	0.158	0.150	0.157	0.150	7.3	0.140	93.4
15 Tyrosine-*4	0.944	0.940	1.07	1.00	0.938	0.946	1.00	0.980	5.1	0.700	71.5
16 Valine-*5	1.60	1.52	1.76	1.57	1.58	1.57	1.60	1.60	7.7	1.40	85.8
18 Methionine	0.453	0.450	0.507	0.478	0.505	0.469	0.463	0.480	4.9	0.180	37.2
22 Tryptophan	0.263	0.249	0.23	0.227	0.229	0.246	0.255	0.240	5.8	0.210	87.2
23 Phenylalanine-*6	1.93	1.88	1.96	1.85	1.89	1.82	1.89	1.90	2.5	1.70	92.3
24 Isoleucine	1.41	1.29	1.44	1.35	1.33	1.37	1.39	1.40	3.7	1.30	93.5
25 Leucine	1.46	1.31	1.29	1.34	1.39	1.37	1.42	1.40	4.4	1.00	76.4
26+28 Orn2+Orn3	1.88	1.81	1.88	1.69	1.73	1.82	1.88	1.80	4.2	1.00	56.2
27+29 (Lys2–*7) +Lys3	1.99	1.82	1.78	1.82	1.86	1.83	1.81	1.80	3.7	1.10	57.2

[a] Undissolved, dried residues, obtained according to Section 2.6.4.
[b] *1–*6, impurities of the OPA/MPA reagent [2].
[c] Obtained from at least three separate tests with [OPA]/[(MPA)]/[amino acid]^T mole ratios of $5.72\cdot10^{-6}$ M/~$1.7\cdot10^{-5}$ M:$1.38\cdot10^{-7}$ M=41.5/~120:1, in the cases of injected aliquots of filtrates corresponding to ~9000 pM [amino acid]^T; the total amount of impurities was 113 pM and 203 pM found in 100 μL filtrate and in an equal amount of ion-exchanged sample.
[d] 4.00 mL wet pulp was ion-exchanged [33] and 1.00 mL stock solution was prepared.

amount of these unidentified amino acids is ≪2% of the total of the authentic amino acids.

3.2.3. Changes in the amino acid content of jonagored apples as a function of harvesting and storage time (Table 7)

The total amino acids is limited by the amount of the main constituent, i.e. asparagine. The correlations reported earlier [10,12] for an increasing serine content of apples harvested with increasing date could not be confirmed.

3.2.4. Distribution of the amino acid content of various apple varieties (Table 8)

Selected samples of apple derivatives were taken at the same harvesting time. The free amino acid content proved to be particularly different relating

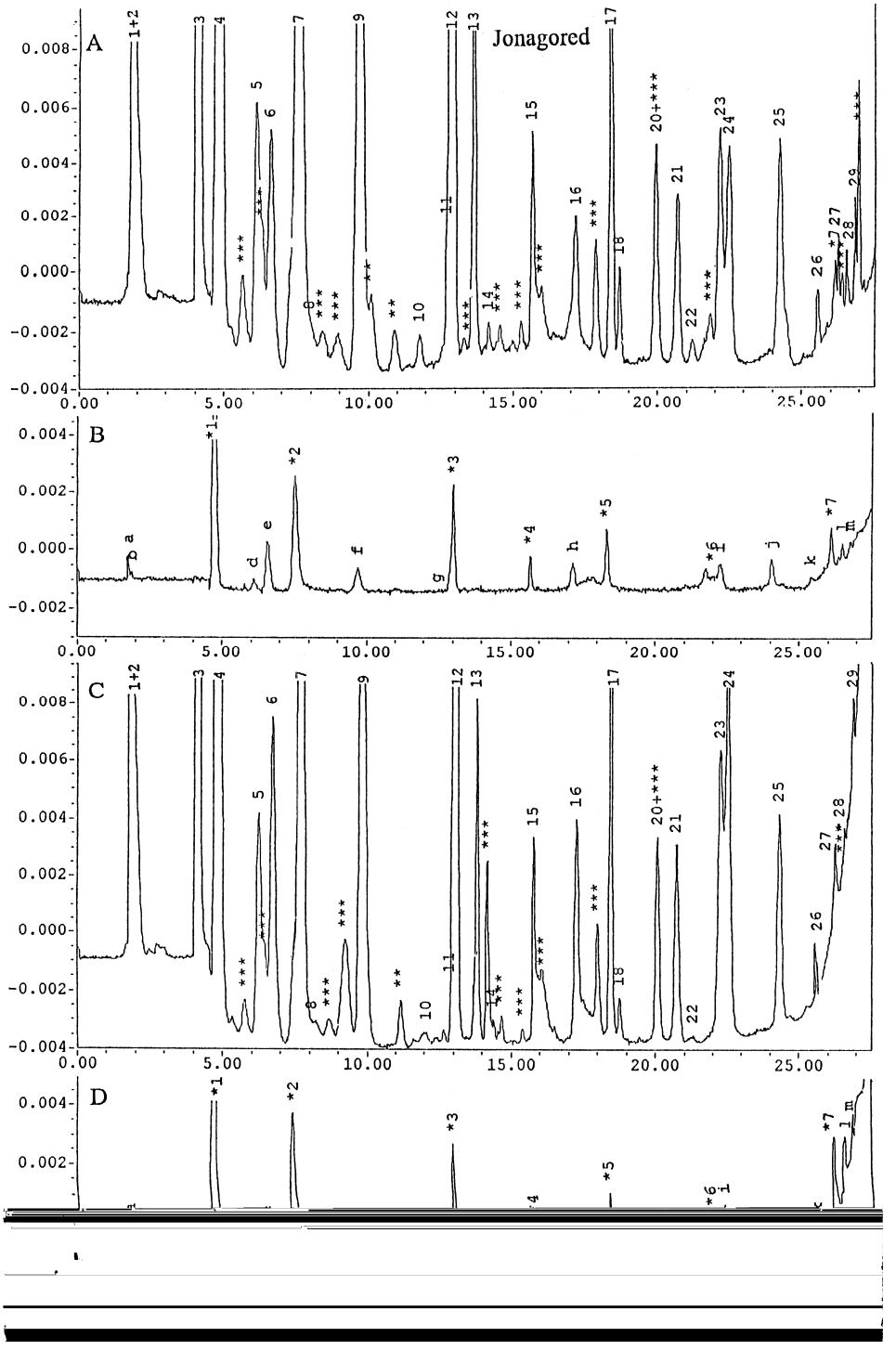

Fig. 3. Elution profile of OPA/MPA-amino acid derivatives of Jonagored apple on column T-3: (A) derivatized according to Section 2.6.4; (B) blank test, belongs to elution A; (C) derivatized in the eluate of a cation-exchanged pulp [33]; (D) blank, obtained from the cation-exchange process, belongs to elution C. Peaks as in Fig. 1.

Table 7
Reproducibility of the quantitation of the free amino acid content of the jonagored variety after various harvesting dates and storage times as their OPA/MPA amino acid derivatives, applying optimum conditions

	Measured μg amino acid/g wet, filtered apple pulp											
Sample:	1	1/1	1/2	1/3	2	2/1	2/2	2/3	3	3/1	3/2	3/3
Weighed pulp/10 mL:	5.22–	5.32–	5.27–	5.12–	4.99–	5.27–	5.17–	3.82–	5.24–	5.11–	5.13–	5.20–
derivatized μL: 100[a]	0.0717	0.0946	0.0859	0.1219	0.0611	0.0805	0.0586	0.0330	0.0460	0.0748	0.0816	0.1168
Amino acid[b]												
1 Aspartic acid	44.1	20.8	13.3	16.9	35.1	26.2	43.3	24.2	72.9	76.1	10.0	10.3
	(3.4)	(1.3)	(3.6)	(1.1)	(2.3)	(3.3)	(0.09)	(1.6)	(0.65)	(3.6)	(6.6)	(1.3)
2 Glutamic acid	50.6	16.8	9.86	13.9	21.5	18.9	26.6	19.0	48.1	44.2	6.44	10.7
	(0.83)	(2.4)	(0.85)	(1.8)	(1.1)	(3.5)	(4.0)	(1.0)	(0.02)	(6.7)	(3.0)	(5.3)
3 Asparagine	108	182	205	263	318	296	536	387	545	844	123	143
	(0.47)	(2.5)	(0.39)	(2.3)	(0.93)	(0.10)	(1.9)	(0.41)	(0.45)	(1.3)	(5.9)	(0.32)
4 Serine-*1	10.8	7.41	3.25	6.15	10.9	5.03	8.53	6.02	9.64	12.4	1.63	2.39
	(4.5)	(1.9)	(1.9)	(3.1)	(0.92)	(2.0)	(5.3)	(0.18)	(1.6)	(1.9)	(1.8)	(1.3)
5 Glutamine	1.95	1.92	1.27	2.14	1.12	1.84	3.45	1.89	1.25	7.68	1.04	0.791
	(1.2)	(0.80)	(1.9)	(1.9)	(1.6)	(2.4)	(0.78)	(3.2)	(0.68)	(0.80)	(5.0)	(0.9)
6 Histidine	1.28	2.29	1.67	2.59	2.94	2.36	5.53	2.89	0.980	2.22	0.501	0.942
	(4.3)	(0.78)	(1.0)	(2.8)	(3.3)	(0.76)	(0.42)	(0.26)	(5.9)	(0.03)	(6.5)	(5.8)
7+16 (Gly1-*2)	1.85	3.40	1.22	2.51	2.95	1.34	2.44	1.95	1.60	3.28	0.617	1.00
+Gly2	(8.8)	(2.4)	(3.4)	(3.8)	(5.3)	(1.2)	(2.4)	(0.29)	(0.68)	(2.0)	(0.73)	(3.0)
8 Homoserine	0.104	0.354	0.364	0.396	0.179	0.304	0.717	0.281	0.117	2.01	0.818	0.259
	(3.0)	(3.8)	(2.5)	(0.48)	(6.9)	(2.0)	(0.78)	(0.25)	(2.9)	(1.5)	(4.8)	(3.2)
9 Threonine	2.79	2.29	1.49	2.37	5.6	2.78	5.4	3.26	7.87	6.19	0.657	0.864
	(3.7)	(1.8)	(0.69)	(2.7)	(5.2)	(0.09)	(2.8)	(1.6)	(3.1)	(0.22)	(6.9)	(0.84)
10+20 β-Ala1	0.335	0.593	0.491	0.642	0.615	1.48	2.91	1.70	2.63	5.61	0.815	0.845
+β-Ala2	(5.7)	(2.2)	(1.5)	(2.5)	(0.95)	(5.1)	(1.3)	(8.0)	(5.9)	(3.2)	(1.3)	(7.4)
11 Arginine	1.01	1.08	1.01	1.29	1.48	0.887	1.80	1.48	2.57	3.96	1.13	0.995
	(2.6)	(2.0)	(6.5)	(5.6)	(5.1)	(2.2)	(3.0)	(3.2)	(6.6)	(1.3)	(6.5)	(3.6)
12 Alanine-*3	4.49	3.94	3.37	5.53	6.22	4.51	8.84	5.77	6.13	18.4	3.38	2.71
	(2.8)	(1.6)	(0.31)	(1.5)	(3.5)	(0.20)	(1.1)	(1.0)	(0.69)	(0.57)	(4.2)	(0.57)
13+21 GABA1	2.48	1.73	1.28	1.78	2.55	2.87	3.59	2.23	6.04	7.80	0.935	1.36
+GABA2	(0.86)	(1.8)	(4.5)	(2.4)	(2.7)	(3.2)	(1.3)	(0.25)	(1.9)	(0.74)	(8.1)	(0.21)
14 Homoarginine	0.0203	0.821	0.269	0.569	0.158	0.680	1.57	0.848	0.627	2.48	0.714	0.441
	(9.4)	(2.9)	(1.3)	(1.4)	(2.3)	(1.7)	(0.76)	(1.0)	(7.1)	(2.2)	(7.6)	(0.41)
15 Tyrosine-*4	0.565	0.821	0.269	0.569	0.938	0.601	0.879	0.545	0.451	0.878	0.901	0.215
	(7.5)	(2.9)	(1.3)	(1.4)	(0.54)	(1.3)	(3.6)	(1.9)	(1.6)	(1.1)	(4.6)	(0.86)
16 Valine-*5	0.875	1.17	0.582	1.12	1.58	1.00	2.02	1.33	1.59	2.13	0.384	0.524
	(4.6)	(0.06)	(0.12)	(2.2)	(5.4)	(1.3)	(1.9)	(0.08)	(0.07)	(0.80)	(3.5)	(2.2)
18 Methionine	0.464	0.356	0.319	0.461	0.505	0.541	0.609	0.664	0.249	0.963	0.129	0.182
	(0.57)	(2.5)	(2.6)	(3.8)	(1.6)	(5.4)	(0.54)	(3.6)	(6.1)	(1.6)	(1.3)	(3.8)
22 Tryptophan	0.113	0.325	0.209	0.327	0.229	0.297	0.529	0.411	0.175	0.300	0.142	0.131
	(5.6)	(0.07)	(5.6)	(6.3)	(2.2)	(3.2)	(1.7)	(1.7)	(1.8)	(4.4)	(9.4)	(9.0)
23 Phenylalanine-*6	0.717	0.938	0.474	1.01	1.89	1.10	1.22	1.36	0.861	1.66	0.187	0.407
	(5.4)	(4.0)	(2.6)	(4.3)	(0.56)	(4.3)	(2.5)	(1.0)	(1.3)	(1.2)	(9.9)	(2.3)
24 Isoleucine	0.639	1.94	1.32	1.47	1.33	1.82	3.34	1.63	2.28	3.79	0.412	0.730
	(0.78)	(1.5)	(0.06)	(1.7)	(3.0)	(1.1)	(0.62)	(0.67)	(1.0)	(0.62)	(1.3)	(0.60)
25 Leucine	0.600	1.03	0.505	0.850	1.39	0.669	1.45	0.873	1.05	1.59	0.257	0.416
	(3.3)	(2.0)	(1.4)	(2.6)	(1.2)	(0.60)	(1.2)	(1.4)	(1.1)	(0.080)	(2.8)	(3.1)
26+28 Orn2+Orn3	1.56	3.05	0.845	2.08	1.73	0.184	1.34	1.34	1.34	2.05	0.458	1.02
	(6.3)	(5.2)	(3.1)	(0.43)	(11)	(5.9)	(3.4)	(7.2)	(0.20)	(7.4)	(9.4)	(4.1)
27+29 (Lys2–*7)	0.977	1.85	1.49	1.83	1.86	1.89	2.91	4.32	2.79	6.60	1.44	3.46
+Lys3	(2.5)	(3.1)	(2.0)	(6.3)	(1.6)	(6.8)	(5.3)	(5.0)	(2.9)	(5.9)	(6.1)	(2.7)
Unknown	3.07	3.09	3.34	3.65	3.17	3.29	4.83	3.18	6.06	7.88	1.48	2.49
amino acid type[c]	(1.0)	(3.8)	(0.7)	(1.6)	(0.25)	(2.3)	(0.40)	(1.5)	(0.13)	(0.50)	(8.7)	(3.5)
Amino acids total	237	257	251	330	421	373	664	472	716	1057	156	184

[a,b] See Table 6.

[c] Unidentified components having the characteristic maximum at 332–334 nm [2], also indicated in Fig. 3 by three asterisks. Harvesting dates in order of listing, Jonagored1, 2 and 3 (09.09.98, 16.09.98 and 05.10.98); taken from storage in order of listing, Jonagored 1/1, 2/1, 3/1 (26.01.99), Jonagored1/2, 2/2, 3/2 (22.02.99), and Jonagored 1/3, 2/3 and 3/3 (23.03.99).

them to the dry matter content (%, w/w) of the apple pulp, in order of dry matter (%)/amino acid^{T} (%): Jonagored, $14.2/2.9\cdot10^{-2}$; Idared, $13.4/4.1\cdot10^{-2}$; Jonica, $13.4/3.9\cdot10^{-2}$; Florina, $8.0/6.1\cdot10^{-2}$; and Freedom, $10.7/6.6\cdot10^{-2}$.

Detailed analysis of the spectra revealed that, also

Table 8
Reproducibility of the quantitation of the free amino acid content of different apple varieties (Jonagored, Idared, Jonica, Florina, Freedom) harvested on 16.09.98 and measured immediately as their OPA/MPA-amino acid derivatives applying optimum conditions[a]

	μg amino acid/1 g wet pulp				
Sample:	Jonagored	Idared	Jonica	Florina	Freedom
Weighed pulp/10 mL:[b]	4.99–	5.13–	5.11–	5.13–	5.18–
derivatized μL: 100	0.0611	0.0911	0.0414	0.0458	0.1210
Amino acid[c]					
1 Aspartic acid	35.3 (2.3)	105 (0.18)	67.3 (2.7)	37.1 (0.80)	39.5 (3.1)
2 Glutamic acid	21.0 (1.1)	71.6 (0.75)	42.5 (1.1)	30.1 (0.30)	23.7 (0.36)
3 Asparagine	318 (0.90)	315 (0.35)	371 (1.7)	355 (2.1)	588 (0.11)
4 Serine-*1	10.9 (0.90)	18.3 (1.1)	9.39 (6.3)	9.30 (2.5)	13.7 (2.5)
5 Glutamine	1.12 (1.6)	0.799(0.30)	3.50 (2.6)	1.62 (1.9)	1.46 (2.6)
6 Histidine	2.94 (3.3)	2.06 (2.3)	1.93 (1.2)	1.95 (2.0)	4.51 (3.0)
7+16 (Gly1-*2)					
+Gly2	2.95 (5.3)	2.15 (2.5)	2.03 (5.1)	1.85 (2.7)	1.98 (7.6)
8 Homoserine	0.179 (6.9)	0.130 (4.0)	0.156 (2.1)	0.108 (2.9)	0.120 (5.2)
9 Threonine	5.90 (5.2)	4.90 (0.25)	4.77 (2.1)	4.14 (0.10)	5.78 (0.20)
10+20 β-Ala1					
+β-Ala2	0.615 (0.9)	1.19 (0.43)	1.52 (5.3)	0.940 (6.2)	1.28 (0.96)
11 Arginine	1.48 (5.1)	1.43 (0.58)	1.54 (3.5)	1.66 (9.9)	1.60 (3.4)
12 Alanine-*3	6.22 (3.5)	8.72 (0.19)	5.15 (2.9)	4.29 (0.20)	3.84 (1.7)
13+21 GABA1					
+GABA2	2.55 (2.7)	4.38 (1.3)	3.66 (0.31)	4.45 (2.2)	3.93 (1.1)
14 Homoarginine	0.158 (2.3)	0.050 (2.9)	0.310 (4.0)	0.188(0.11)	0.247 (2.4)
15 Tyrosine-*4	0.938(0.50)	0.540 (4.8)	0.490 (5.5)	0.484 (4.2)	0.552 (6.3)
16 Valine-*5	1.58 (5.4)	1.24 (1.3)	1.11 (4.1)	2.58 (0.60)	2.46 (1.0)
18 Methionine	0.505 (1.6)	1.71 (1.6)	0.515 (2.7)	0.627 (1.1)	0.862(0.08)
22 Tryptophan	0.229 (2.2)	0.178 (1.4)	0.207 (3.3)	1.27 (1.6)	0.734(0.61)
23 Phenylalanine-*6	1.89 (0.60)	0.611(0.73)	0.810 (1.7)	1.18 (1.3)	1.84 (0.28)
24 Isoleucine	1.33 (3.0)	3.10 (0.92)	0.830(0.31)	13.1 (0.60)	1.55 (4.6)
25 Leucine	1.39 (1.2)	0.66 (1.1)	0.825 (2.9)	0.837 (1.4)	0.910 (4.6)
26+28 Orn2+Orn3	1.73 (4.2)	1.17 (4.3)	1.01 (3.9)	0.703 (6.6)	0.898 (9.6)
27+29 (Lys2–*7)					
+Lys3	1.86 (1.6)	1.13 (6.1)	0.935(0.38)	1.19 (5.8)	1.30 (7.3)
Unknown, amino					
acid type[d]	3.17 (0.25)	4.54 (0.19)	3.69 (1.6)	4.08 (3.9)	4.34 (0.19)
Amino acids total	421	552	526	479	705

[a] Obtained from at least three separate tests with mole ratios of ([OPA]/[MPA])/[amino acid]^{T}=$5.72\cdot10^{-6}$ M/~$1.7\cdot10^{-5}$ M:$1.38\cdot10^{-7}$ M=41.5/~120:1. RSD amino acid in parentheses.
[b,c,d] See Table 7.

in the case of different varieties, the unknown amino acid type components do not exceed 1% of the total.

4. Conclusions

1. Twenty-five OPA/MPA- and 24 OPA/NAC-amino acids were separated on six columns, with the same gradient program, applying various temperatures and eluent flow-rates, within 40 min (OPA/MPA-amino acids) and 37 min (OPA/NAC-amino acids), including equilibration time.
2. Response values of the 25/24 amino acids proved to be independent of the chromatographic conditions used.
3. Reproducibility studies, carried out with extremely different amounts of amino acid derivatives, furnished acceptable relative deviation percentages (RSD), both in model solutions (RSD $\leq$7.1%) and in apple pulp (RSD $\leq$9.0%).
4. Our 'isolation process', i.e. the simple filtration of apple pulp combined by the fast OPA derivatization technique, resulted in a substantially reduced

preparation time and in the complete recovery of amino acids.

5. Special spectrum characteristics of OPA/MPA-amino acids have been recognized and utilized in the identification in apple pulp.

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