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Temperature, eluent flow-rate and column effects on the retention and quantitation properties of phenylthiocarbamyl derivatives of amino acids in reversed-phase high-performance liquid chromatography[1]

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Abstract

The separation and identification possibilities of 27 PTC-amino acids (with particular attention to those present in apples in free forms), are reported on seven RP columns such as, Nucleosil, 3 and 5 μm: 150(+20 guard)×4.0 mm; Gromsil 3 μm; 150(+10 guard)×4.0 mm; Hypersil 5 μm: 130(+20 guard)×4.0 mm, 150(+20 guard)×4.0 mm and 200(+20 guard)×4.0 mm, as well as, Hypersil 3 μm: 150(+20 guard)×4.0 mm: a UV range photodiode array (PDA) detection was employed. Optimization studies carried out in model solutions, as a function of the temperature (30–55°C) and flow-rate (0.8–2.5 ml/min) of eluents proved that optimum resolutions are associated with the highest flow-rate applicable, (remaining on the safe side with a column pressure of <3500 p.s.i., 1 p.s.i.=6894.76 Pa), in the temperature range of 30–50°C. Twenty-seven amino acids, characteristic in apples in free forms, have been separated and determined on all seven columns, performing the same gradient program, (the main component asparagine, present in overwhelming excess, and the minor constituents glutamine, β-alanine, γ-aminobutyric acid, homoserine, homoarginine and 1-aminocyclopropane-1-carboxylic acid). Optimum conditions, at 2.1 ml/min, at 50°C, with 40 min run time, including equilibration, have been obtained with the Hypersil, 150(+20 guard)×4 mm column, performing elutions. Responses of the corresponding amino acids proved to be independent of the column used; reproducibility in the concentration range of 15–1500 pmol was <4.0% R.S.D. (relative standard deviation). Detailed study of the PDA spectra revealed that in addition to the identification/peak purity possibilities further characteristics can be obtained taking advantage of the difference in maximum values and of those of their special ratio values, respectively. The utility of the protocol was shown in the quantitation of the free amino acid content of three apple varieties.

Keywords: Apples; Food analysis; Fruits; Amino acids

1. Introduction

Our first paper dealing with the optimization of the buffer and pH dependence on the retention of

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phenylthiocarbamyl (PTC)-amino acids in reversed-phase (RP) high-performance liquid chromatography (HPLC) was the basis [1] for the quantitation of 19 essential amino acids as their PTC derivatives, applying the Waters Pico Tag Work Station. Further efforts [2–10] resulted in the optimization of hydrolysis conditions of proteins [3–10] and in the quantitation of all essential amino acids from a single protein hydrolysate, including tryptophan [3–5], cyst(e)ine [2,9] and methionine: from one solution by one injection.

The importance of knowledge of amino acid content in apples is based on scientific and practical point of views: (i) the formation, transformation and catalytic activity of amino acids play a key role in the biosynthesis of fruit proteins. (ii) The amount of chosen amino acids are in direct relationship with the ripening of the fruits. (iii) The presence or absence of the special apple amino acids in different kind of pulp samples involves the proof of their authenticity and/or adulteration. (iv) The combination of amino acids with saccharides, (in the frame of a possible Maillard reaction or with enzymatically oxidised phenols resulting in melanine formation), can cause the browning of fruits [11].

The HPLC of the free amino acids in apples, to our knowledge, as PTC derivatives, has not yet been presented. The only report, relating exclusively to the quantitation of the PTC-1-amino-1-cyclopropane carboxylic acid (ACPCA) content of apple, did not deal with the evaluation of other PTC-amino acid derivatives: the apple extract was only utilized for the analysis of its ACPCA content [12]. In order to quantitate amino acids in apple paper chromatography [13], ion-exchange chromatography (IEC) [14–17] and recently HPLC [18–23] and GC [23] have been applied. HPLC, after pre-column derivatization with *o*-phthaldialdehide (OPA) and the chiral thiols (*N*-isobutyryl-L-cysteine and/or *N*-isobutyryl-D-cysteine) [23], or with the Marfey's reagent [(1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide] [22], made possible the chiral resolution of the L- and D-amino acids in apples [22,23].

Recent papers, dealing with the quantitation of free amino acids as PTC derivatives present in various matrices [24–38], as well as offered by the leading column manufacturers [35,36] (Table 1) revealed that (i) in practice, the fast elutions using the Waters, Pico Tag column [25,27,33] provide incomplete resolution also for the separation of 18 amino acids [27,33], even more so for 23 ones [25]. (ii) As to the long-lasting elutions (>50 min), extended elution time did not fulfil expectations either for the classical 20 amino acids [24,35,36], or for more amino acids [24,26,30–32,34,37]. (iii) However, excellent separations have been shown recently [35,36] by the manufacturers, (promising complete separations within unbelievably short elution times; 4.5 min for 17 [35], 8 min for 18 [36] amino acids, without equilibration times), practical realization with these protocols has not yet been published. The HPLC of 27 PTC-amino acids within 88 min seems to be the best separation achieved to date [37].

The aim of this study was to extend our earlier experiences [1–10] in order to find the possibility for the simultaneous analysis of 27 amino acids, including the possible apple constituents, such as, the main component asparagine (being present in overwhelming excess), as well as the minor constituents glutamine, β-alanine, γ-aminobutyric acid (GABA), homoserine, homoarginine and ACPCA, together with all others, within a reasonable elution time, utilizing the advantages of their detailed spectra provided by the UV photodiode array (PDA) detection. Introductory investigations proved that this would be a challenging task, since, the characteristic apple amino acids proved to be present in enormously different concentrations, and several of them manifest very similar retention properties.

2. Experimental

2.1. Materials

Triethylamine (TEA), PITC and amino acids were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade acetonitrile and methanol were purchased from Romil Chemicals (Leics., UK). All other reagents were of highest purity available. Derivatization tubes were supplied with the Waters Pico Tag Work Station (Waters, Milford, MA, USA). Authentic apple varieties, such as Granny Smiths, Red Spur and Jonathan were obtained from the Research Garden of

Table 1
HPLC conditions suggested for the determination of PTC-amino acids: literature data

Practical conditions			Detection/quantitation			Remarks			Ref.
Column (cm×mm, μm)	Product of	Eluents: composition, pH, temperature	Detector	R.S.D. (%)	Analysis	Matrix/ etc.	Amino acids not resolved	Amino acid No./time (min)	
15×4.6, 5	Hypersil, Shandon	A: 0.05 *M* sodium acetate, pH=7.2, B: A–ACN–methanol (46:44:10) (ambient)	UV, PDA APCI-MS	<6.0	25–800 pmol	Hydr. study, tryptophan, cyst(e)ine	Isol/Leu	19/32	[1–10]
25×4.6, 5	Spherisorb[e]	A: 50 m*M* sodium acetate+0.05% TEA+0.25% ACN+ 1 ppm EDTA, pH=6.8, B: ACN–water (70:30), C: water–ACN (3:7), 55°C	UV254	<5.0	–	Amino acids in wines	Ser/Asn	24/71	[24]
15×3.9, –	Pico Tag	A: 0.14 *M* sodium acetate+0.5 ml/l TEA, pH=6.4, B: 60% ACN in water, 38°C	UV 254	–	0.25–25 nmol	Rat and human plasma amino acids	Asp/Glu, Arg/Thr, Asn/ Ser/Gln/Gly, Isol/Leu	23/23	[25]
25×4.6, 5	Supelcosil LC 18	A: 0.14 *M* sodium acetate+0.5 ml/l TEA, pH=6.4, B: 60% ACN in water, 38°C	PDA	–	250 pmol	Fresh pig muscle, cured ham	Arg/Thr/Ala/Pro	21/50	[26]
15×3.9, –	Pico Tag	A: 0.14 *M* sodium acetate+0.5 ml/l TEA, pH=6.4, B: 60% (v/v) ACN in water, 38°C	UV 254	–	–	Amino acids of glycoproteins	Thr/Ala/Pro, Isol/Leu	18/12	[27]
12.5×4.6, 5	Partisphere[a]	A: 50 m*M* sodium acetate+4 ml/l, TEA, pH=6.4, B: A–ACN–methanol (50:40:10), 25°C	UV 254	–	>10 pmol	Amino acids in mixed fish meals	Asp/Glu/Hyo/Arg/Thr, Isol/Leu	17/30	[28]
15×4.6, 3	Econosphere[b]	A: 0.05 *M* ammonium acetate, pH=5.1–ACN (98:2), B: water–ACN (4:6), 41°C	UV 254	<15.6	>1 pmol	Model study; plasma amino acids	Asn/Gly, Isol/Leu	26/35	[29]
30×3.9, 4	Nova-Pak[c]	A: 0.07 *M* sodium acetate (pH=6.5)–ACN (75:25); B: ACN–methanol–water (45:15:40), 46°C	UV 254	<10	–	Biological samples	Ser/Asn/Gly	38/70	[30]
25×4.6, 5	Alltima[d]	A: 0.1 *M* ammonium acetate, pH=6.4, B: ACN–methanol–water (46:10:44), pH=6.4, 43°C	PDA	<3.66	0.02–3 nmol	Protein toxins	Ser/Asn, Arg/Thr/ GABA/Ala	29/65	[31]
25×4.6, 5	Spherisorb[e]	A: 0.1 *M* sodium acetate, 0.68 ml/l TEA, 0.2 mg/ml SPS B: water, C: ACN, pH=5.27, 35°C,	PDA	<7	>63 pmol	Amino acids in musts, in wine	Ser/Asn, GABA/Arg, PITC/Tyr	26/50	[32]
15×3.9, 5	Pico Tag	A: sodium acetate, pH=6.4, B: 60% ACN (not detailed), 38°C	UV 254	<1.94	>1.25 pmol	Amino acids in kelp	Asp/Glu, Isol/Leu	17/18	[33]
30×3.9, 4	Nova-Pak[c]	A: 50 m*M* ammonium acetate, pH=6.67, B: ACN–20 m*M* $Na_2H(PO_4)$ cont. 5% methanol+ 1.5% THF (50:50), C: water–ACN (3:7), 40°C	UV 254	<5.85	>3.9 pmol	Amino acids in soya bean, in egg	Ser/Gln, Thr/Ala	22/50	[34]
10×4.6, 2	TSKgel[f]	A: 50 m*M* ammonium acetate (pH=6.0)–ACN (97:3), B: ACN–water (6:4), (–)	UV 254	–	–	–	Ala/Pro	17/4[g]	[35]
12.5×4, 3+ 1×4, 3 (guard)	GROM-SIL 80 ODS	A: 50 m*M* sodium acetate (pH=6.5), B: 50 m*M* sodium acetate (pH=6.5)–ACN (50:50), 55°C	UV 254	–	–	–	Complete resolution	18/8[g]	[36]
30×3.9, –	Pico Tag	A: 140 m*M* sodium acetate (pH=6.35), cont. 0.05% TEA, B: ACN–water (6:4), 46°C	UV 254	<5.0	39–1250 pmol	Plasma amino acids	Asn/Ser/Gly,	26/88	[37]
25×4.6, 5	Spherisorb[e]	A: 140 m*M* sodium acetate, cont. 0.5 ml/l TEA (pH=6.2), B: ACN–water (6:4), 30°C	UV 254	<3.98	8.1–13.3 pmol	Amino acids in green bean hydr.	Isol/Leu Thr/Ala/Pro	17/37	[38]

Indications: [a] C_{18}; Whatman; [b] C_{18} Alltech; [c] C_{18} column; [d] C_{18} Alltech; [e] C_{18} ODS-2; [f] Super ODS, TosoHaas product. [g] Without equilibration time; – no data available. SPS=Sodium pentanesulfonate; TEA=triethylamine; THF=tetrahydrofuran; APCI-MS=atmospheric pressure chemical ionization mass spectrometry.

the University Horticulture and Food Industry (Szigetcsép, Hungary). Peeled apples were homogenized in a mixer and the sieved pulps were used for cation-exchange clean-up [39] and derivatization.

2.2. Derivatization of amino acids with PITC

Standards of free amino acids, one by one, (such as aspartic acid, glutamic acid, hydroxyproline, serine, asparagine, glycine, glutamine, homoserine, histidine, GABA, threonine, ACPCA, alanine, proline, arginine, homoarginine, valine, tyrosine, methionine, cyst(e)ine, iso- and *n*-leucine, phenylalanine, tryptophan and lysine), as well as in a mixture, (containing ~$1.5\cdot10^{-8}$–$1.5\cdot10^{-6}$ mol of each amino acid, and the corresponding 5–100-fold excess of asparagine, expected in apples), were placed in 50×6 mm I.D. tubes and dried under vacuum.

Apple pulps, 5.0000 g, were filtered on a Whatman GF/F glass microfibre filter, (particle retention from liquid: 0.7 μm), into a 5-ml volumetric flask acidified with 0.2 ml distilled hydrochloric acid and completed by washing the residue with distilled water up to 5 ml. One- and 2-ml aliquots of these solutions were subjected to cation-exchange clean-up and treated according to our earlier experiences [39]. Samples were dried, by adding 20 μl of ethanol–water–TEA (2:2:1) to each tube. Thereafter to each redried sample 20 μl of derivatization reagent [ethanol–TEA–water–PITC (7:1:1:1)] was added and Vortex mixed. Samples were prepared according to the Waters Picotag Work Station manual.

The derivatization standards were dissolved, in order of listing, in 50 μl acetonitrile, 50 μl water and 900 μl 0.05 *M* sodium acetate solution (pH 7.2) to a final volume 1.00 ml. The stock solution of apples was prepared as described above, with the only exception that their solutions were completed by 150 μl 0.05 *M* sodium acetate solution (pH 7.2) to a final volume of 250 μl. Hence 10 μl of standard containing 15–1500 pmol of each amino acid and the corresponding excess of asparagine, as well as 20 μl of the apple stock solution was injected.

2.3. Chromatography

The system was a Waters HPLC instrument consisting of a Waters 996 PDA detector, Waters 600 controller quaternary pump with thermostattable column area, Waters 717 autosampler, operating with Millennium software (version 2010, 1992–95, validated by ISO 9002). T-1–T-4 test columns were 130, 150 and 200 mm×4 mm Hypersil ODS bonded phase, 5 μm, and 150 mm×4 mm Hypersil ODS bonded phase, 3 μm, (Shandon; supplier: BST, Budapest, Hungary); (henceforth T-1, T-2, T-3 and T-4 columns). 150 mm×4 mm Nucleosil 120, C_{18}, 5 μm and 3 μm, (Macherey–Nagel; supplier: BST) (henceforth: T-5 and T-6 columns), as well as, 125 mm×4 mm Gromsil, 3 μm column (Grom Analytic+HPLC; supplier: LAB-COMP, Budapest, Hungary). T-1–T-6 columns were used with 20 mm×4 mm, T-7, with 10 mm×4 mm guard columns.

Elutions were followed at an absorbance setting of 254 nm; spectra were taken between 205–400 nm, with an optical resolution of 4.8 nm.

The eluent systems consisted of two components: (A) eluent was 0.05 *M* sodium acetate of various pH (6.0, 7.2 and 7.8, optimum pH 7.2), while (B) eluent was prepared with the corresponding pH consisting of 0.100 *M* sodium acetate–acetonitrile–methanol (46:44:10) (mixed in volume ratios and titrated with glacial acetic acid or 50% sodium hydroxide to pH 6.0, 7.2 and 7.8). Different gradient programs were followed (given in details with the corresponding sections), the optimum version is shown in Table 2. (Note: Derivatization procedures were performed at ambient temperature, while elutions were carried out between 30 and 55°C).

Table 2
Optimum gradient program

Step	Time (min)	A (%)	B (%)
1	0.00	100	0.0
2	6.00	99.0	1.0
3	14.00	91.0	9.0
4	17.00	65.0	35.0
5	20.00	65.0	35.0
6	24.00	55.0	45.0
7	26.00	0.0	100.0
8	29.00	0.0	100.0
9	31.00	100.0	0.0
10	40.00	100.0	0.0

3. Results and discussion

Preliminary studies concerning the retention behaviour of the 27 PTC-amino acids revealed, that the newly selected ones which were not included in our earlier optimized protocol [1] eluted in the range of the most critical area of the PTC derivatives i.e., (i) asparagine, (being present in ~5–100-fold excess compared to its neighbours), became inserted between serine and glycine, (ii) homoserine coeluted with glutamine, and, (iii) in addition also GABA and ACPCA were inserted into the hardly separable fives of histidine/threonine/alanine/proline/arginine [1,2,27].

Based on our earlier experiences [1,2] [which proved that RP C_{18} Hypersil and Nucleosil phases (5 μm) were of similar properties, and, equally suitable for the separation and quantitation of 19 essential amino acids as their PTC derivatives], this work was started in parallel with RP C_{18} Hypersil and Nucleosil columns of 3 μm particle size: presuming that in this case improved selectivity would be required. In order to separate fewer constituents at least a column of ≥70 000/m plate number was recommended [24].

3.1. Gradient program study

Introductory tests proved that gradient program would need to be optimized in successive steps.

(i) First, the separation of glycine/asparagine, (asparagine being present in overwhelming excess compared to glycine), was studied as a function of the pH of eluents. It became clear that better separation could be obtained when glycine elutes first. In this respect pH=7.2 proved to be the optimum. To increase the resolution between glycine and asparagine the percentage of B eluent needed to be increased slowly from 0 to 1%. Varying the time of this step from 4 to 7 min, 6 min proved to be the best.

(ii) Under the next elution period the separation of eight components was to be optimized from glutamine to alanine, increasing the 1% of eluent B up to 9%, varying this period of time between 6 and 17 min: 14 min proved to be the optimum.

(iii) The third step allowed the fast increase of eluent B providing acceptable separation up to the PTC-leucine.

(iv) The forthcoming isocratic step was needed in order to separate leucine from isoleucine.

(v) Finally, increasing the ratio of B eluent up to 45%, ensured the elution of the last component lysine. The 3-min run with 100% B eluent ensured the removal of the reagent constituents, followed by the 9-min equilibration process.

This optimum gradient program (Table 2) was the basis of the separation of 27 amino acids which intended to be further optimized varying the length and particle size of stationary phases, temperature and flow-rate of eluents, in accordance/competition with each other, taking into account the responses of the derivatives (the faster the elution the smaller the response) and the column's life time (<55°C temperature and <3500 p.s.i. column pressure were preferred; 1 p.s.i.=6894.76 Pa).

3.2. Column, flow-rate and temperature optimization study

In the possession of the optimum gradient program (Table 2) elaborated on T-4 and T-6 columns at 30°C, a unified exhaustive optimization strategy was followed on all seven test columns (T-1–T-7).

First, at 30°C temperature the flow-rates were varied until the best separation could be achieved. This was close to the highest flow-rate, on all seven test columns, that could be used without exceeding the 3500 p.s.i. limit: it means this limit would be limiting for T-1–T-3 and T-5 columns (5 μm) with a flow-rate of 2.1 ml/min, while for T-4, T-6 and T-7 columns (3 μm) with 1.3 ml/min. The next task to complete the separation of the 27 amino acids was to increase the temperature of elutions (35, 40, 45, 50 and 55°C) performed at 1.3 ml/min (3 μm) and 2.1 ml/min (5 μm) flow-rates, respectively. Table 3 shows detailed results obtained with the T-2 column. As shown at the optimum temperature (50°C), the flow-rate was repeatedly varied (1.8, 2.1 and 2.3 ml/min). These successive steps were performed with all seven columns: optimum conditions were listed (Table 4 Fig. 1).

Evaluating data summarised in Tables 3 and 4 it can be stated that:

(i) Acceptable separations could be achieved,

Table 3
Reproducibility of the quantitation of PTC-amino acids obtained at various elution temperatures and elution flow-rates[a] on a Hypersil column (150+20 mm×4 mm)

Amino acid	Arbitrary units/1 nmol amino acid, normalized to 1 ml/min flow-rate													
	30°C				35°C,	40°C,	45°C,	50°C			55°C,	Average	S.D.	R.S.D.
	1.3 ml/min	1.8 ml/min	2.1 ml/min	2.3 ml/min	2.1 ml/min	2.1 ml/min	2.1 ml/min	1.8 ml/min	2.1 ml/min	2.3 ml/min	2.1 ml/min			(%)
Aspartic acid	660	662	661	661	652	649	652	648	650	653	644	654	6.2	0.94
Glutamic acid	685	685	685	685	676	673	673	672	675	679	671	678	6.1	0.90
Hydroxyproline	671	671	676	681	674	678	675	679	687	697	675	678	7.1	1.1
Serine	689	681	687	692	683	684	682	687	692	699	685	687	5.2	0.76
Glycine	689	686	688	693	684	689	680	682	678	667	663	683	8.4	1.2
Asparagine	716	717	718	718	712	715	704	720	720	737	716	717	7.5	1.0
β-Alanine	578	585	586	588	585	582	580	582	580	595	585	584	4.7	1.8
Glutamine	–	–	–	–	–	*624*	671	660	678	662	638	662	15.0	2.3
Homoserine	–	–	–	–	–	*531*	503	504	497	506	497	501	4.0	0.79
GABA	598	615	626	613	*683*	*646*	615	617	615	623	599	616	8.1	1.3
Histidine	–	–	716	736	–	*663*	718	729	743	731	*760*	729	10.3	1.4
Threonine	–	–	–	–	–	*840*	712	708	706	706	698	707	6.8	0.97
ACPCA	–	–	–	–	–	*427*	462	487	472	472	476	474	8.9	1.9
Alanine	–	–	–	–	–	*670*	667	670	671	675	672	671	2.9	0.42
Proline	706	714	707	711	681	–	*807*	689	689	716	668	699	16.6	2.4
Arginine	674	686	687	659	676	–	*518*	672	692	712	689	678	12.3	1.8
Homoarginine	714	754	695	709	736	723	709	730	723	727	689	725	17.1	2.4
Tyrosine	862	844	848	862	848	824	848	803	789	789	779	797	12.6	1.6
Valine	762	740	736	744	730	715	737	728	738	725	705	734	11.8	1.6
Methionine	633	634	630	611	615	596	600	611	602	629	607	615	13.9	2.3
Cyst(e)ine	234	236	238	232	247	240	220	213	232	232	*155*	230	9.2	4.0
Isoleucine	727	736	709	717	703	718	743	729	750	748	725	727	15.5	2.1
Leucine	726	736	721	718	696	718	743	745	693	706	698	718	18.5	2.6
Phenylalanine	746	738	742	727	769	752	754	740	736	719	710	739	16.4	2.2
Tryptophan	–	–	609	614	–	–	–	610	608	606	644	615	13.1	2.1
Ornithine	–	–	1224	1233	–	–	–	1238	1255	1242	1235	1238	10.3	0.84
Lysine	1118	1127	1111	1116	1112	1097	1130	1116	1119	1123	1103	1116	9.6	0.86

– No resolution; italicised data=omitted from the mean.

[a] Calculated to 1 ml/min elution rates.

Table 4
Reproducibility and resolution features in the quantitation of PTC-amino acids obtained on the seven test columns, performing optimum conditions: at various elution temperatures and elution flow-rates[a]

Peak	Amino acid	Arbitary units/1000 pmol amino acid, normalized to 1 ml/min flow-rate, resolutions in parentheses								
		Column (T-1–T-7), flow-rate, (°C)								
		T-1, 2.3 ml/min, (50)	T-2, 2.1 ml/min, (50)	T-3, 2.1 ml/min, (50)	T-4, 1.5 ml/min, (45–50)	T-5, 2.3 ml/min, (30)	T-6 1.3 ml/min, (50)	T-7, 1.2 ml/min, (45)	Average[a]	R.S.D. (%)
1	Aspartic acid	611	650	*561*	625	685	647	*518*	644	4.0
2	Glutamic acid	656 *(2.1)*	675 *(2.4)*	572 *(2.8)*	660 *(3.6)*	710 *(3.5)*	671 *(2.9)*	680 *(0.95)*	661	6.5
3	Hydroxyproline	683	687	704	745	744	672	716	707	4.1
4	Serine	673 *(2.4)*	692 *(2.6)*	665 *(2.9)*	702 *(4.0)*	751 *(4.0)*	679 *(4.0)*	697 *(2.7)*	694	4.1
5	Glycine	661 *(3.0)*	678 *(3.0)*	689 *(3.6)*	730 *(4.5)*	748 *(3.1)*	681 *(4.4)*	728 *(2.8)*	702	4.7
6	Asparagine	703 *(1.0)*	720 *(1.4)*	695 *(1.4)*	737 *(2.3)*	776 *(3.7)*	703 *(1.7)*	726 *(1.4)*	723	3.8
7	β-Alanine	*821 (3.6)*	*580 (3.4)*	676 *(4.4)*	693 *(4.9)*	766 *(3.0)*	665 *(5.5)*	785 *(3.3)*	717	7.6
8	Glutamine	666 *(1.3)*	678 *(1.3)*	655 *(1.3)*	698 *(1.7)*	738	667*(~0.8)*	716 *(~1.1)*	688	4.4
9	Homoserine	497 *(1.7)*	497 *(1.3)*	461 *(1.9)*	*359 (1.9)*	536	491 *(1.7)*	438 *(~1.1)*	487	6.9
10	GABA	580	615	626	698	*738*	597	607	621	6.6
11	Histidine	658 *(0.72)*	743 *(1.4)*	699 *(0.95)*	*778 (1.1)*	653 *(1.7)*	724 *(1.9)*	*569 (2.2)*	696	5.7
12	Threonine	679 *(2.2)*	706 *(1.7)*	672 *(2.8)*	711 *(2.9)*	*924*	712 *(2.2)*	*876 (1.8)*	696	2.7
13	Alanine	648 *(1.5)*	671 *(1.4)*	637 *(1.7)*	674 *(1.9)*	717	652 *(2.3)*	624	661	4.6
14	ACPCA	486 *(2.1)*	472 *(1.7)*	458 (2.0)	524 *(1.6)*	528	459 *(1.2)*	466 *(1.8)*	485	6.2
15	Arginine	681	692	630	689	753	669	679	685	5.3
16	Proline	674 *(1.3)*	689 *(1.3)*	737 *(1.8)*	770 *(2.2)*	763 *(3.7)*	698 *(2.32)*	689 *(9.7)*	717	5.4
17	Homoarginine	689	723	702	729	752	709	716	717	2.8
18	Tyrosine	768	789	710	802	790	803	798	780	4.2
19	Valine	626	738	641	659	*787*	699	712	679	6.5
20	Methionine	573 *(1.9)*	602 *(1.8)*	538 *(2.1)*	621 *(2.1)*	*662 (3.7)*	580 *(3.2)*	596 *(2.0)*	585	4.9
22	Isoleucine	621	750	*607*	735	*805*	696	689	698	7.2
23	Leucine	673 *(1.6)*	693 *(1.5)*	*606 (1.8)*	725 *(2.0)*	*806 (1.1)*	683 *(2.1)*	666 *(1.2)*	688	3.3
24	Phenylalanine	672	736	667	770	765	753	745	730	5.9
25	Tryptophan	588 *(0.98)*	608 (1.1)	637 (1.2)	599 *(0.7)*	699 *(2.6)*	621 (1.9)	587 *(~2.0)*	620	6.3
26	Ornithine	1286 *(1.0)*	1255 *(0.95)*	1304 *(0.98)*	1328 (*0.7)*	1363 *(–)*	1303 *(0.95)*	*1471 (~0.6)*	1307	2.8
27	Lysine	1081	1119	1094	1168	1191	1140	1103	1128	3.6

Footnotes as in Table 3.
Resolutions in parentheses: given by the software, or estimated (~) [40]; resolutions accordingly to changed retention orders: on T-5 column, β-alanine/homoserine=*(~0.8)*; homoserine/glutamine=*(~1.1)*; histidine/ACPCA=*(~1.1)*; ACPCA/alanine=*(~0.9)*; alanine/threonine=*(1.4)*; on T-7 column, threonine/ACPCA=*(1.2)*; ACPCA/alanine=*(0.93)*.
[a] Averages of responses obtained from the seven test columns, without italicised data.

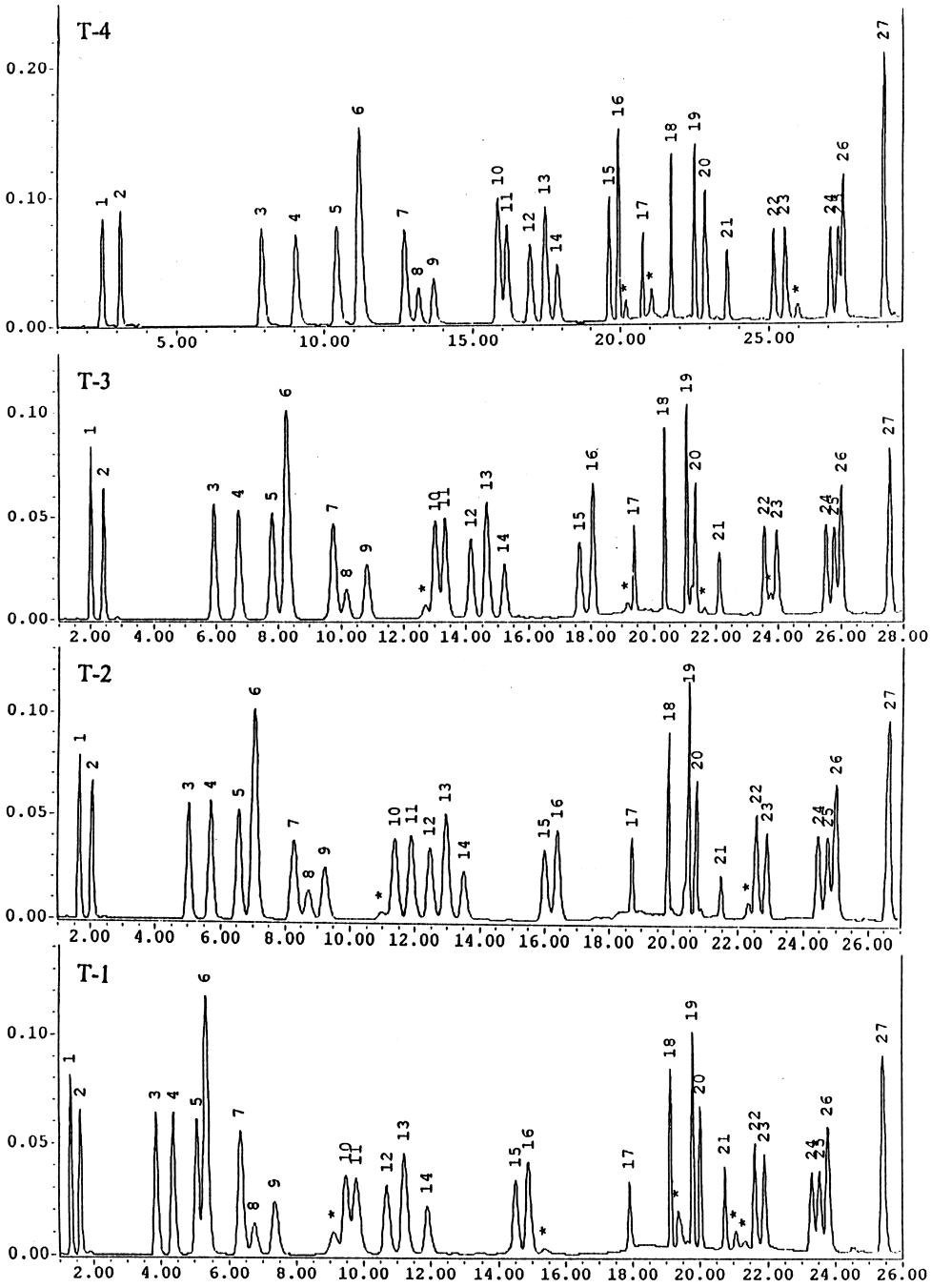

Fig. 1. Elution profile of the 27 PTC-amino acids obtained with model solutions on the (a) T-1–T-4 or (b) T-5–T-7 columns. Peaks: 1=Aspartic-, 2=glutamic acids, 3=hydroxyproline, 4=serine, 5=glycine, 6=asparagine, 7=β-alanine, 8=glutamine, 9=homoserine, 10=GABA, 11=histidine, 12=threonine, 13=alanine, 14=ACPCA, 15=arginine, 16=proline, 17=homoarginine, 18=tyrosine, 19=valine, 20=methionine, 21=cyst(e)ine, 22=isoleucine, 23=*n*-leucine, 24=phenylalanine, 25=tryptophan, 26=ornithine, 27=lysine; *=system peaks, **=impurities of apple; ***=unknown apple constituents providing amino acid-type spectra. Spectra: taken from the apex of 4=serine, 7=β-alanine, ***=unknown apple constituent, providing amino acid-type spectra, 11=histidine, 16=proline, 18=tyrosine, 21=cyst(e)ine, 25=tryptophan, 27=lysine.

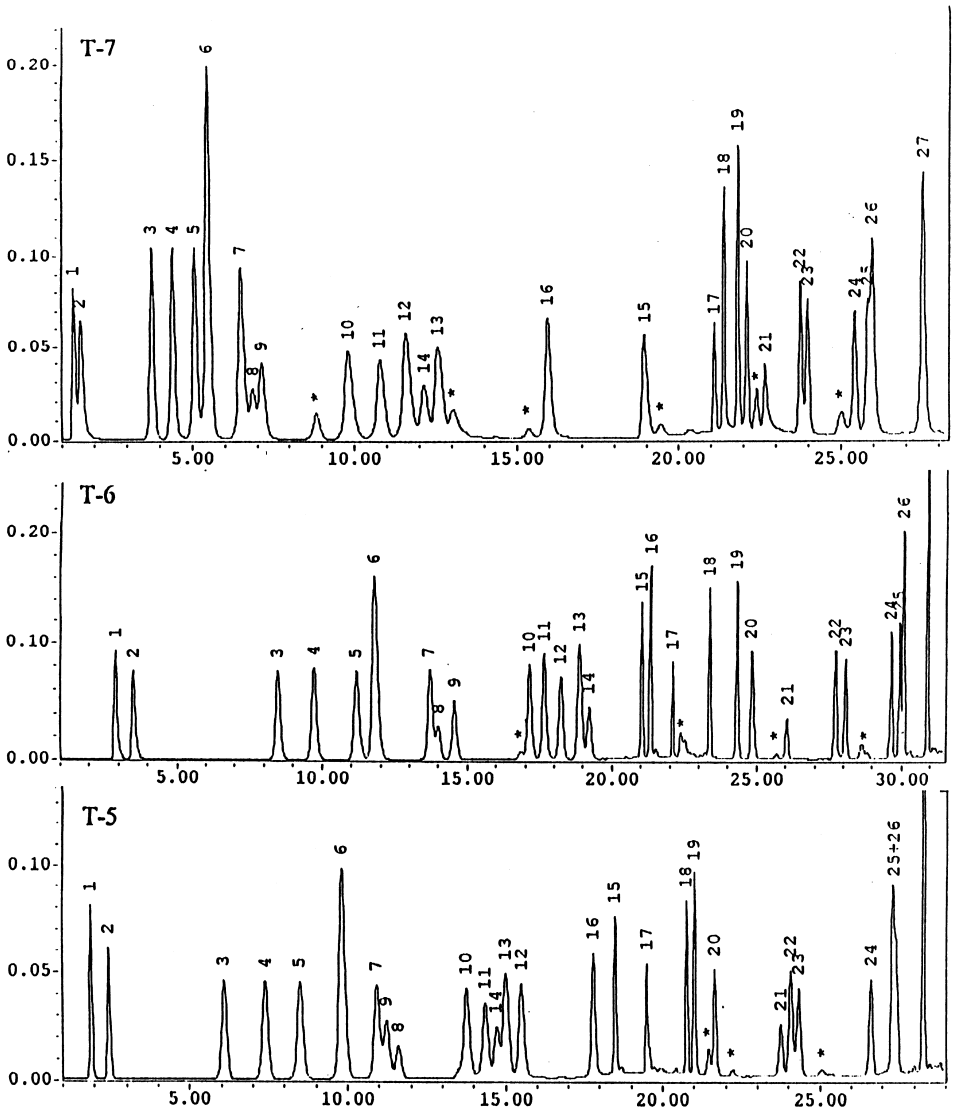

Fig. 1. (*continued*)

performing the optimum gradient program, by all columns tested, in most cases at 45–50°C (with the only exception being column T-5: 30°C), applying the highest flow-rate possible.

(ii) Response factors of amino acids proved to be independent of column length, type of stationary phase, elution temperatures and flow-rates (Table 4). As the most advantageous column, in respect of resolution characteristics (Table 4, values in parentheses), cost and column life time (5 μm), the T-2 column was selected (values in Table 4 Fig. 1).

(iii) Retention orders of selected PTC derivatives were changed by increasing the temperature of elutions, however these changes proved to be also independent of columns (T-1–T-7), as follows:

(1) The retention times of PTC-amines (asparagine, glutamine) by increasing the temperature, decreased relatively faster, comparing them to their

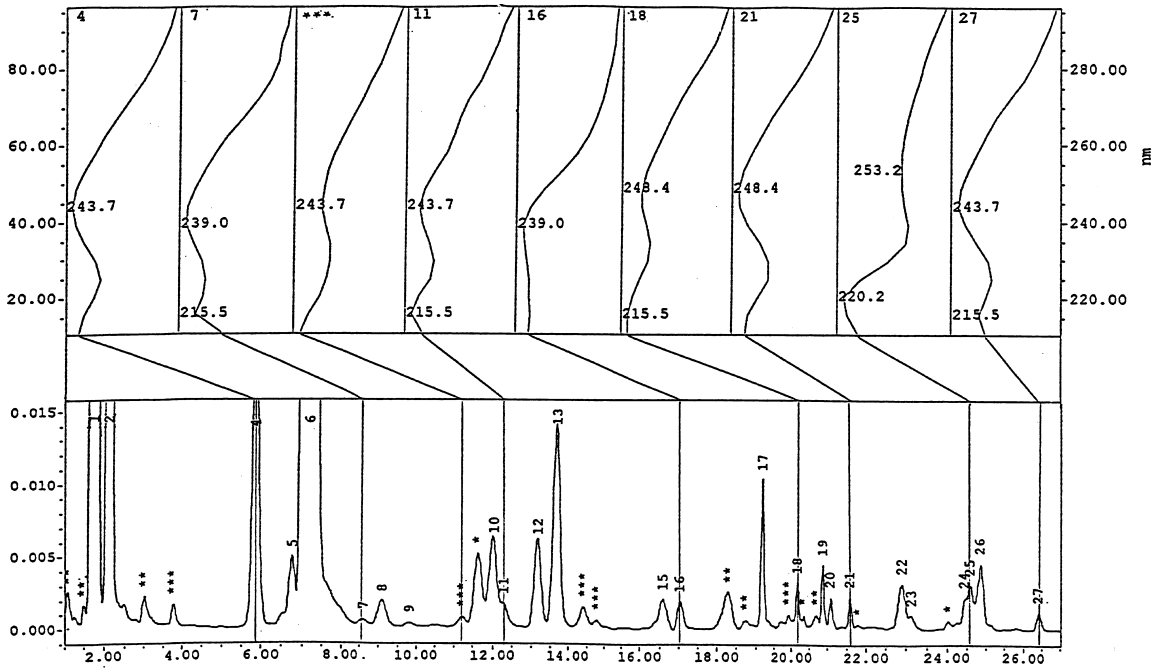

Fig. 2. Elution profile of the 27 PTC-amino acids obtained with Jonathan apple on column T-2. Peaks as in Fig. 1.

neighbours. Thus, this feature of PTC-amines limited the upper temperature level as high as 50°C (compare the resolution of asparagine/glycine on T-1–T-4, T-6, T-7 columns, at ≥45°C to that on T-5, at 30°C).

(2) The decreased retention time of PTC-glutamine resulted in the retention order's change of β-alanine/homoserine/glutamine (30°C) for β-alanine/glutamine/homoserine (50°C).

(3) The retention time of ACPCA was successively increased, it means that the retention order of GABA/histidine/ACPCA/threonine/alanine (30°C) was altered for GABA/histidine/threonine/alanine/ACPCA (50°C).

(4) Similarly the retention orders of proline/arginine and ornithine/tryptophan were also changed by increasing the temperature from 30°C to 50°C.

3.3. Reproducibility study

Comparing response values, obtained on various columns, under different elution conditions (Table 4, R.S.D.s≤7.6%) furnished almost as good results as those obtained on the same column (T-2) varying the amount of amino acids between 15 and 1500 pmol (Table 5, R.S.D.s<4.8%). The exception with its R.S.D. of 9.2% proved to be cyst(e)ine [1].

3.4. Study of the UV PDA spectra taken in the range of 205–400 nm

Detailed analysis of spectra, in the range of 205–400 nm, were investigated in order to complete information to the identification/peak purity data provided by the PDA software. Investigations based on the analysis of the simple, non-derived spectra are summarized in Table 6. Two maxima were evident in the spectra of all 27 PTC derivatives: in the overwhelming part of spectra, at 215.5 and 243.7 nm, due to the absorption of the carboxyl groups (215.5 nm) and to the PTC moiety of the molecule (243.7 nm). The exceptions, associated with their special structures [hydroxyproline, β-alanine, GABA, ACPCA, proline, tyrosine, cyst(e)ine, phenylalanine and tryptophan], furnish their second maximum at 239 nm (hydroxyproline, β-alanine, GABA, ACPCA, proline), at 248 nm (cysteine), or at 253.3 nm (tryptophan) instead of 243.7 nm. For the sake of comparison, we intended to utilize the differences between the extent of these maxima; namely, de-

Table 5
Reproducibility of the quantitation of different amounts of PTC-amino acids obtained on test column T-2, under optimum conditions (50°C, 2.1 ml/min)

Amino acid	Arbitrary units/1000 pmol amino acid, normalized to 1 ml/min flow-rate						Average[a]	R.S.D.
	15 pmol injected	30 pmol injected	60 pmol injected	150 pmol injected	300 pmol injected	1500 pmol injected		(%)
Aspartic acid	634	617	*510*	575	588	630	609	4.3
Glutamic acid	707	672	*618*	*624*	636	675	672	4.3
Hydroxyproline	637	652	645	640	643	687	651	2.8
Serine	688	667	650	642	652	692	665	3.1
Glycine	666	646	628	640	641	678	650	2.9
Asparagine	721	710	709	704	709	720	712	1.0
β-Alanine	*519*	540	573	581	593	580	573	3.5
Glutamine	557	583	589	617	617	*678*	593	4.3
Homoserine	516	524	535	517	518	497	518	2.4
GABA	569	*728*	619	597	591	615	591	3.2
Histidine	617	592	591	646	646	*743*	618	4.4
Threonine	666	*860*	633	641	647	706	659	4.4
Alanine	*731*	659	643	623	631	671	645	3.1
ACPCA	474	471	494	*423*	*427*	472	473	2.8
Arginine	651	667	615	647	650	*692*	646	2.9
Proline	619	660	658	647	653	*689*	647	2.6
Homoarginine	659	662	627	633	653	*723*	646	2.4
Tyrosine	670	688	703	715	727	*789*	700	3.2
Valine	751	746	747	*699*	733	738	743	1.0
Methionine	550	559	561	551	564	602	565	3.4
Cyst(e)ine	*116*	*141*	*157*	279	259	232	256	9.2
Isoleucine	707	704	722	674	640	679	688	4.3
Leucine	720	716	732	711	708	693	722	2.0
Phenylalanine	613	667	686	638	636	654	649	4.0
Tryptophan	717	659	693	656	653	629	668	4.8
Ornithine	1130	1200	1140	1186	1198	1230	1181	3.2
Lysine	983	960	*919*	976	1004	1042	993	3.2

[a] Obtained at least from three separate tests; italicised data=omitted from the mean.

Table 6
Reproducibility of the ratios of maximum values in PDA spectra of selected amino acids obtained on the seven test columns, under optimum conditions

Amino acid	Ratios of maxima at 243.7 nm/215.5 nm[a]											Average[b]	R.S.D.
	T-1	T-2/a	T-2/b	T-2/c	T-2/d	T-2/e	T-3	T-4	T-5	T-6	T-7		(%)
Hydroxyproline	1.006	0.997	1.005	1.005	1.002	1.009	1.015	1.009	1.010	1.000	1.012	1.01	0.53
Serine	1.108	1.111	1.129	1.133	1.105	1.143	1.092	1.114	1.136	1.132	1.136	1.12	1.5
β-Alanine	1.121	1.116	1.117	1.110	1.143	1.187	1.126	1.125	1.127	1.107	1.124	1.13	1.9
GABA	1.092	1.108	1.163	1.128	1.162	1.187	1.101	1.082	1.111	1.096	1.106	1.12	3.1
Histidine	0.934	0.924	0.961	0.955	0.980	0.945	0.930	0.935	*1.127*	0.934	*1.150*	0.94	1.9
ACPCA	0.923	0.920	0.920	0.910	0.939	0.979	0.926	0.930	0.929	0.910	0.927	0.93	2.0
Proline	1.003	1.015	1.014	1.027	1.054	*1.135*	1.009	1.015	1.008	1.016	1.012	1.02	1.4
Tyrosine	0.870	0.885	0.876	0.866	0.869	0.895	0.888	0.881	0.884	0.871	0.882	0.88	1.0
Cyst(e)ine	1.093	1.090	1.099	1.105	*1.169*	1.088	1.097	1.097	1.097	1.069	1.102	1.01	3.3
Phenylalanine	0.989	0.989	0.935	0.998	0.985	*0.849*	0.991	1.002	1.009	0.957	1.006	0.99	2.3
Tryptophan	0.472	0.480	0.498	0.492	0.498	0.498	0.477	0.477	0.490	0.476	0.518	0.49	2.8
Lysine	1.171	1.156	1.172	1.173	1.166	1.157	1.191	1.182	1.188	1.141	1.191	1.17	1.4

Footnotes as in Tables 3–5
T-2/a–T-2/e represent the ratios of maxima values of various amounts of amino acids, in listing order: 1500–30 pmol.
[a] With the exceptions of hydroxyproline, β-alanine, proline, GABA, ACPCA and proline, furnishing their second maximum at 239 nm, with cysteine at 248 nm and with tryptophan at 253 nm, respectively.
[b] Averages of maxima values obtained from the seven test columns, without italicised data.

tailed matching studies revealed that reproducible differences were obtained in the relative extent of maxima values. These differences provided distinguished characteristics in those cases where these maxima values proved to be at the same wavelengths (histidine, tyrosine). All characteristic ratio values have been presented together with those of serine and lysine, representing these two, i.e., serine and lysine all others by providing the common ratio values between 1.12 and 1.17: 1.12 (for serine) and 1.17 (for lysine).

As shown, all these ratio values proved to be independent of conditions applied: columns, eluent flow-rates, temperature, (Table 6, values for T-1–T-7 columns) and amounts of amino acids analyzed (Table 6, values for T-2/a–e columns). The reproducibility of these ratio values (R.S.D. data in Table 6), allowed unambiguous distinction of amino acids

Table 7
Reproducibility of the quantitation of different amounts of apple pulps as PTC-amino acids obtained on test column T-2, under optimum conditions (50°C, 2.1 ml/min)

Amino acid	Apple			
	Granny Smith	Red Spur	Jonathan[a]	
	μg/g wet pulp (R.S.D., %)			Ratio values[b]
Aspartic acid	89.5	160.6	258.9 (0.21)	
Glutamic acid	76.1	48.0	110.2 (0.34)	
Hydroxyproline	0.60	1.40	–	
Serine	32.2	13.6	22.7 (0.24)	1.111
Glycine	4.6	1.7	3.8 (0.50)	
Asparagine	465.5	274.2	542.0 (0.21)	
β-Alanine	1.3	0.42	0.36 (2.0)	1.078
Glutamine	4.2	1.2	2.8 (1.1)	
Homoserine	0.76	–	2.1 (1.9)	
GABA	2.9	2.9	9.3 (0.57)	1.116
Histidine	16.5	9.3	3.7 (2.7)	0.978
Threonine	9.3	7.8	9.4 (0.38)	
Alanine	0.49	0.22	13.3 (0.22)	
ACPCA	–	–	–	
Arginine	5.0	1.2	5.4 (1.7)	
Proline	4.7	2.1	2.0 (0.42)	1.024
Homoarginine	4.4	2.2	12.8 (0.27)	
Tyrosine	1.8	1.2	1.9 (0.91)	
Valine	2.4	1.4	2.1 (0.77)	
Methionine	2.4	1.4	2.1 (1.9)	
Cyst(e)ine	0.42	–	10.0 (2.4)	1.104
Isoleucine	1.5	1.0	4.3 (0.85)	
Leucine	–	0.85	1.1 (1.2)	
Phenylalanine	2.3	3.4	1.9 (1.5)	
Tryptophan	4.8	1.0	8.9 (1.6)	
Ornithine	3.6	2.4	4.5 (0.70)	
Lysine	0.97	1.2	0.99 (0.47)	1.180
Amino acid-type peaks in total[c]	3.2	1.3	5.1	
Amino acids in total	738.0	538.8	1036.6	
%[d]	0.074	0.054	0.104	

[a] Obtained at least from three separate tests.
– Not detectable.
[b] Taken from maxima values, shown in Table 6 and in Fig. 2.
[c] Amino acid-type peaks in total (Fig. 2).
[d] Expressed in the percentages of the wet pulp.

with absorption maxima also at the same wavelengths.

The ratio values, obtained at 243.7/215.5 in most cases, (1.12–1.17), belong to those amino acids, which in their free, underivatized forms do not provide absorbances in the range of 190–400 nm [40]. In ratio values of <1.10 (Table 6) – obtained, in order of listing, for tryptophan (0.49), tyrosine (0.88), phenylalanine (0.98) and cyst(e)ine (1.04) – the corresponding parts of their high molar absorptivities – originating from their free forms [40] – are realized as additive increments (molar absorption of free amino acids: tryptophan at 218 nm: 33 500; tyrosine at 223 nm: 8200; phenylalanine at 236 nm: 63; cystine at 237 nm: 300). These experiences can be attributed to the fact that the intrinsic absorption feature of the compounds are present as additional absorptivities also in their PTC derivatives. Further exceptions of ratio values obtained at 239/215.5 for hydroxyproline (1.01), β-alanine (1.13), GABA (1.12), ACPCA (0.93) and proline (1.02), or for histidine 243.7/215.5=0.94) might be particularly useful in their identification, as these three amino acids have very similar retention indexes (GABA/histidine/ACPCA).

3.5. Determination of amino acids in apples

The separation of 27 PTC amino acids in model solutions, has been optimized with particular attention to the relevant free constituents of apples (Table 7 Fig. 2). Study of their PDA spectra proved that in addition to the software provided identification/peak purity possibilities, those characteristics which have been recognized in model solutions (Table 6, ratios of maxima values) can also be excellently utilized in practice: comparing the ratios of the corresponding maxima values, for all amino acids in apple samples, including also proline, hydroxyproline, homoarginine, cysteine, ornithine, quantitated in three apple varieties (Granny Smith, Red Spur, Jonathan) were determined for the first time (Table 7 and Fig. 2).

4. Conclusions

(1) Twenty-seven PTC-amino acids have been separated on seven columns, with the same gradient program, applying various temperature and eluent flow-rate, within 40 min, including equilibration time.

(2) Response values and spectrum characteristics of the 27 amino acids proved to be independent of the chromatographic conditions used.

(3) Special spectrum characteristics of PTC-amino acids have been recognized and utilized in their identification in apple pulps.

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References

[1] M. Morvai, V. Fábián, I. Molnár-Perl, J. Chromatogr. 600 (1992) 87–92.
[2] I. Molnár-Perl, M. Morvai, Chromatographia 34 (1992) 132–136.
[3] I. Molnár-Perl, M.P. Szakács, M. Morvai, V. Fábián, in: I. Ishiguro, R. Kido, T. Nagatsu, Y. Nagamura, Y. Ohta (Eds.), Advances in Tryptophan Research, Fujita Health University Press, Toyoake, 1992, pp. 195–198.
[4] I. Molnár-Perl, M.P. Szakács, M. Khalifa, J. Chromatogr. 632 (1993) 57–61.
[5] I. Molnár-Perl, M. Khalifa, Chromatographia 36 (1993) 43–46.
[6] E. Tatár, M. Khalifa, Gy. Zárai, I. Molnár-Perl, J. Chromatogr. A 672 (1994) 109–115.
[7] I. Molnár-Perl, J. Chromatogr. A 661 (1994) 43–50.
[8] I. Molnár-Perl, M. Khalifa, LC·GC Int. 7 (1994) 395–398.
[9] K. Schmeer, M. Khalifa, J. Császár, Gy. Farkas, E. Bayer, I. Molnár-Perl, J. Chromatogr. A 691 (1995) 285–299.
[10] M. Khalifa, I. Molnár-Perl, LC·GC Int. 9 (1996) 143–147.
[11] J.R.L. Walker, P.H. Ferrar, Chem. Ind., (1995) 836-839.
[12] D.L. Sanson, C.T. Phan, R.L. Granger, Anal. Biochem. 155 (1986) 322.
[13] K. Linder, Qual. Plant.-Pl. Fds. Hum. Nutr. XXIII (1973) 251–262.
[14] H.J. Bielig, A. Askar, Chem. Microbiol. Technol. Lebensm. 1 (1972) 183–186.
[15] W. Ooghe, A. de Waele, Flüssiges Obst. 11 (1982) 618–628.
[16] H.J. Bielig, H.J. Hofsommer, Flüssiges Obst. 49 (1982) 50–56.
[17] C. Magné, F. Larher, Anal. Biochem. 200 (1992) 115–118.
[18] J.P. Chaytor, J. Sci. Food Agric. 37 (1986) 1019–1026.

[19] D.B. Gomis, A.M.P. Lobo, M.D.G. Alvarez, J.M. Alonso, Chromatographia 29 (1990) 155–160.
[20] A. Dyremark, M. Ericsson, Chomatographia 29 (1990) 51–53.
[21] D.B. Gomis, A.M.P. Lobo, J.M. Alonso, M.D.G. Alvarez, Z. Lebensmm. Unters. Forsch. 194 (1992) 134–138.
[22] D.W. Kunemann, J.K. Braddock, L.L. McChesney, J. Agric. Food Chem. 36 (1988) 6–9.
[23] H. Brückner, T. Westhauser, Chromatographia 39 (1994) 419–426.
[24] M. Callul, J. Fabregas, R.M. Marce, F. Borrul, Chromatographia 31 (1991) 272–275.
[25] V. Fierabracci, P. Masiello, M. Novelli, E. Bergamini, J. Chromatogr. 570 (1991) 285–291.
[26] M. Concepción Aristoy, F. Toldrá, J. Agric. Food Chem. 39 (1991) 1792–1795.
[27] D.E.H. Palladino, R.M. House, K.A. Cohen, J. Chromatogr. 599 (1992) 3–11.
[28] E.Y. Suzuki, R.J. Early, J. Chromatogr. A 657 (1993) 204–207.
[29] M. Hariharan, S. Naga, T. Van Noord, J. Chromatogr. 621 (1993) 15–22.
[30] S.R. Hagen, J. Augustin, E. Grings, P. Tassinari, Food Chem. 46 (1993) 319–323.
[31] J.K. Khan, Y.H. Kuo, N. Kebede, F. Lambein, J. Chromatogr. A 687 (1994) 113–119.
[32] M. Puig-Deu, S. Buxaderas, J. Chromatogr. A 685 (1994) 21–30.
[33] S.F. Shang, H. Wang, Chromatographia 43 (1996) 309–312.
[34] W. Kang-Lyung, H. Que-Chung, K. Hyung-Su, J. Chromatogr. A 740 (1996) 31–40.
[35] Application No. 157, Reversed-Phase Chromatography, TosoHaas.
[36] Application No. 50, Amino Acid Analysis, Grom Analytik+ HPLC, 1994.
[37] A.S. Feste, J. Chromatogr. 574 (1992) 23–34.
[38] M.J. González-Castro, J. López-Hernandez, J. Simal-Lozano, M.J. Oruna-Concha, J. Chromatogr. Sci. 35 (1997) 181–185.
[39] V. Fábián, M. Morvai, M. Pintér-Szakács, I. Molnár-Perl, J. Chromatogr. 553 (1991) 87–92.
[40] J.P. Greenstein, M. Winitz, in: J.P. Greenstein, M. Winitz (Eds.), Chemistry of Amino Acids, Wiley, New York, 1961, Ch. 17, pp. 1688–1695.