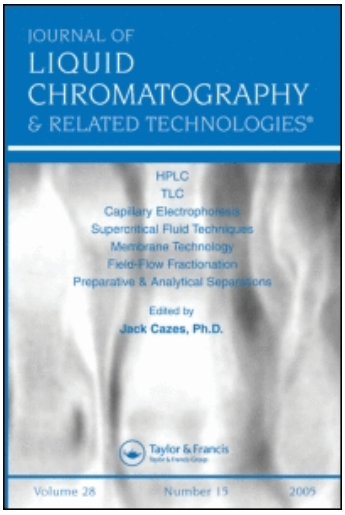

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RAPID ANALYSIS OF FREE AMINO ACIDS IN INFANT FOODS

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ABSTRACT

A new approoch to the analysis of free amino acids from infant foods is described. The method is based on reaction of the free amino acids with phenylisothiocyanate to form stable derivatives which are subsequently separated by liquid chromatography. Sample preparation procedures are described. Separation of all amino acids using this method was completed in 20 min. This method was much faster than the traditional Ion-exchange methods (2-3 h). Variability of the method (expressed as coefficients of variation) for the determination (including preparation of samples, derivatization and liquid chromatography) of all amino acid was less than 10%.

INTRODUCTION

The used of phenylisothiocyanate (PITC, or Edman's reagent) for amino acid analysis was first described by Heinrickson and Meredith (1) and developed

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commercially by Waters (Milford, MA) as the Pico-Tag method (2,3). In the Pico-Tag method, PITC is used for derivatization of amino acids. Reverse-phase gradient elution liquid chromatography is used to separate the phenylthiocarbamyl (PTC) derivatives (normally in less than 20 min), which are then detected by their UV absorbance at a 254 nm. The PITC derivatization method has been reported to be rapid, efficient, and reproducible and to provide results with most amino acids comparable to those obtained by conventional ion-exchange methods (4,5).

The PTC methodology was applied to the analysis of insulin β-chain, human insulin, trypsin and oxytocin (2,6) and has since been applied to several hundred proteins and peptides.

Applications of the PTC derivatization methodology are beginning to appear, such as the analysis of amino acid composition of low levels of growth factors isolated from bovine neural tissue (7), the characterization of angiogenin from human carcinoma cells (8,9). This method has been used to the determination of free amino acids in serum and other phisiological samples such as liver, brain and heart (10).

With some minor modifications in sample preparations and chromatography, the Pico-Tag method has been applied to the determination of amino acids in food matrixes (11,12,13).

EXPERIMENTAL

The Pico-Tag method for sample preparation and analysis was used (Liquid chromatografic analysis of aminoacids in foods using a modification of the Pico-Tag method, revision 1987, Millipore Corp.), with some modifications as described below.

Reagents and Apparatus

(a) Reagents.- Potasium hidroxide, sodium acetate trihydrate, glacial acetic acid, phosphoric acid and ethanol was obtained from Merck (Barcelona, Spain). Disodium hydrogen phosphate and perchloric acid was obtained from Panreac (Barcelona, Spain). All of them were of analytical-reagent grade. Acetonitrile and triethylamine (LC grade) were obtained from Scharlau (Barcelona, Spain).

Phenylisothiocyanate solution (PITC) (Protein sequencing grade) and amino acid standard were purchased from Sigma Chemical Co. (St. Louis, MO).

(b) Equipment.- High purity water generated by a Milli-Q-Water System from Millipore Corp. (Bedford, MA, USA) was used in the preparation of buffers and solutions.

An LC gradient system consisting of a ternary Model SP-8800 pump, a Spectra-physics multiwavelenght detector (Spectra-Chrom 200), a integrator Chromjet (Spectra-physics), a Rheodyne Model 7125 injector with 20 µl loop and a temperature control module was used.

Analyses was performed on a C_{18} Ultrabase (5µm particle size) reversed-phase column (25cm x 0.46cm I.D.) purchased from Scharlau (Barcelona, Spain).

Samples

The study was performed on prepared infant foods which were vacuum packed and are commercially called pots. Four varieties were analysed: vegetable, meat, fish and dessert with six different fruit. These products were supplied for Nestle, S.A. The amino acids content of the samples are shown in Table 1.

Preparation of Samples

Samples of 5 g were dissolved in 50 mL perchloric acid 0.6N, homogeinized in Sorvall. The mixture was then centrifuged at 3500 r.p.m. for 20 min. After that, the supernadant was filtered through a 0.45 mm filter (Millex-GS Millipore) and pH was adjustment with potasium hidroxide (30%) to 7.0 ± 0.2 measured with a pHmeter (CRISON micropH 2001). The filtered was placed the fridge for 5 min. The extracts obtained were frozen for later analysis by HPLC, prior to the measuring of the voiume.

Derivatization of Amino Acids with PITC

Extract (1mL) was evaporated to dryness at 37 °C under liquid nitrogen. The derivatizing solution was prepared just before use, by mixing 70 µL ethanol, 10µL water, 10 µL TEA and 10 µL PITC obtained from Sigma Chemical Co. All amino

TABLE 1
Values obtained from Manufacturer

Amino acid	Vegetable	Meat	Fish	Fruit
Asp	225	100	230	115
Glu	265	500	370	105
Ser	90	90	110	30
Gly	70	90	80	35
His	40	100	60	25
Arg	120	190	130	45
Thr	70	140	100	25
Ala	85	136	110	40
Pro	35	140	20	35
Tyr	50	80	90	20
Val	90	75	30	35
Met	20	80	50	15
Cys	15	25	20	10
Ile	75	100	110	25
Leu	130	125	80	45
Phe	85	140	90	30
Trp	25	40	30	10
Lys	120	250	190	35

acids were derivatizing by adding 20 μL derivatizing solution. The samples were mixed, and the tubes were covered with Parafilm and let to stand for 20 min at room temperature (18 ± 1 °C) for completion of derivatization. The excess reagent was evaporated under liquid nitrogen at 37 °C. The residual which corresponds to derivatized dried samples was redissolved in 200 μL sample diluent. This diluent was prepared by dissolving 710 mg disodium hydrogen phosphate in 1 L of mixed water-acetonitrile (19+1) and by adjusting the pH to 7.40 with phosphoric acid. Finally, 20 μL of sample was then injected into the chromatographic system.

Chromatography

The LC procedures are briefly outline below.

(a) Solvents- Solvent A was prepared by mixing 19 g sodium acetate trihydrate in 1 L water. To this solution 500μL TEA was added. The pH was then adjusted to 6.40 with glacial acetic acid. Finally, 760 mL of this solution was mixing with 60 mL pure acetonitrile.

Solvent B was prepared by adding 600mL acetonitrile to 400mL water followed by mixing and degassing.

Both solvents were degassed in a ultrasonic bath (Sonorex), and filtered using a vacuum pump through 0.45 mash filters (Millipore)

(b) Gradient conditions.-The gradient conditions used are shown in Table 2.

Column temperature was maintained at 38±1 °C. The detection was at 254 nm.

Calculations.

The amounts of free amino acids in the pots were calculated using peak area values. These values yielded concentration values by applying calibration curves obtained from known amino acid concentration.

RESULTS AND DISCUSSION

Four infant formulas (vegetables, meat, fish and fruits) were analyzed using the LC methods described in this report. This method allowed for the separation of essential amino acids.

The amino acid elution profiles of samples of vegetables, meat, fish and fruits are shown in Figures 1, 2, 3 and 4, respectively. In general, all amino acids were satisfactorily resolved and the separation was completed in 20 min.

The gradient shape was chosen to optimize the spacing of the separated peaks in the minimun analysis time.

Normally, hydrolyzed samples are redried with methanol-water-TEA before derivatization (4, 14). Other researches only used methanol for redrying since the use of methanol-water-TEA result in the loss of ammonia (13). But in this work, once the samples were evaporated to dryness, they were resuspended into the derivatization reagent.

Moreover, the commonly used composition of the derivatization reagent (methanol-TEA-water-PITC, 7+1+1+1) (4, 10, 13) was modified (ethanol-TEA-water-PITC, 7+1+1+1) because the resolution of amino acids obtained was better. The resolution of Ser/Gly obtained in this investigation was significantly better than that reported by other authors (10,15).

Lavi et al (16) reported some difficulty in determing lysine, threonine, cysteine and histidine by derivatization with PITC and separation of the derivatized amino

TABLE 2
Gradient Table. The gradient was lineal in all cases.

Time (min)	A(%)	B(%)	Flow (ml/min)
0	100	0	1.00
14.5	54	46	1.00
15.0	0	100	1.00
17.0	100	0	1.50
20.0	100	0	1.50
20.5	100	0	1.00

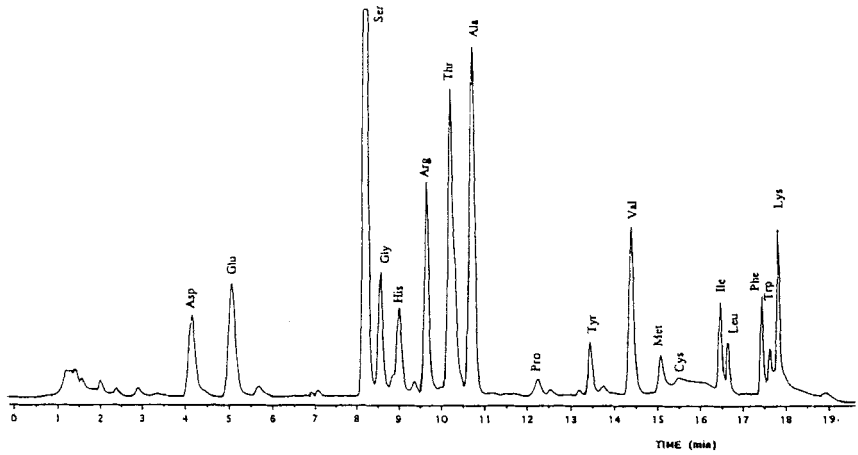

FIGURE 1. Elution profile of vegetable sample showing resolution of PTC-amino acids by liquid chromatography

acids by reverse-phase chromatography. These researches (16) used methanol as deproteinization agents which have been reported (3) to cause considerable losses and reduced yield of many PTC-amino acids. More recently, ultrafiltration has been used for deproteinizing samples prior to analysis by liquid chromatography and selection of appropiate membrane are important in recoveries obtained of 90% or more (10).

Although the resolution of Cys was not ideal, it was adequate for the calculation of this amino acid in most samples.

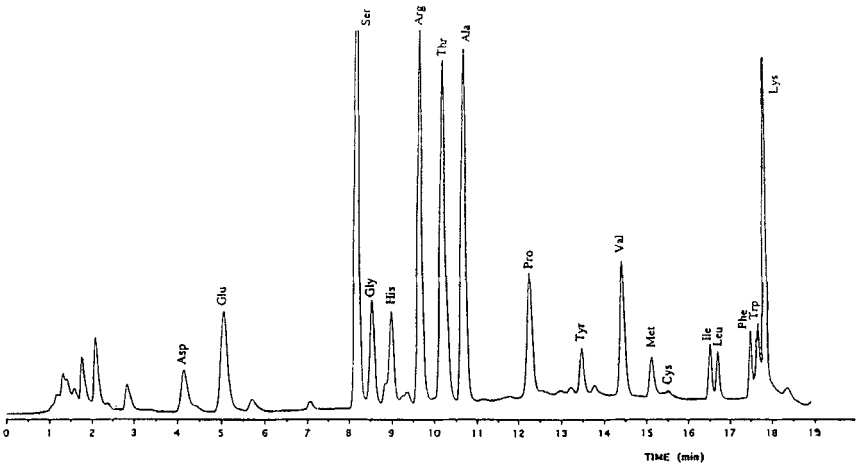

FIGURE 2.-Elution profile of meat sample showing resolution of PTC-amino acids by liquid chromatography

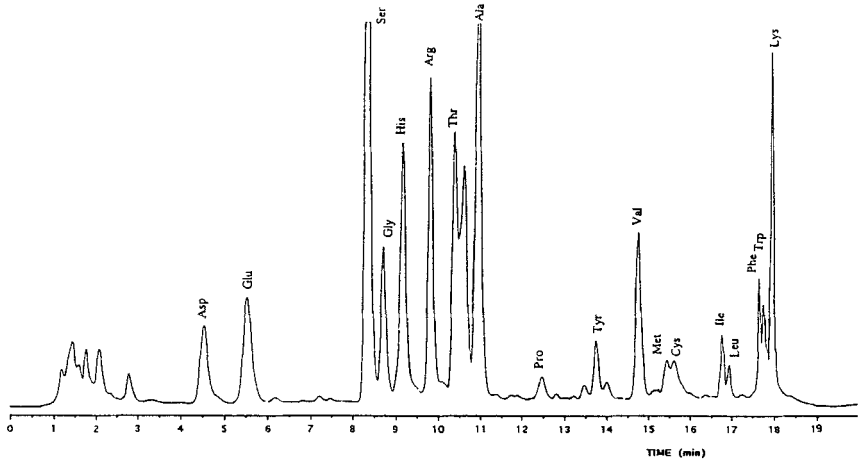

FIGURE 3.- Elution profile of fish sample showing resolution of PTC-amino acids by liquid chromatography

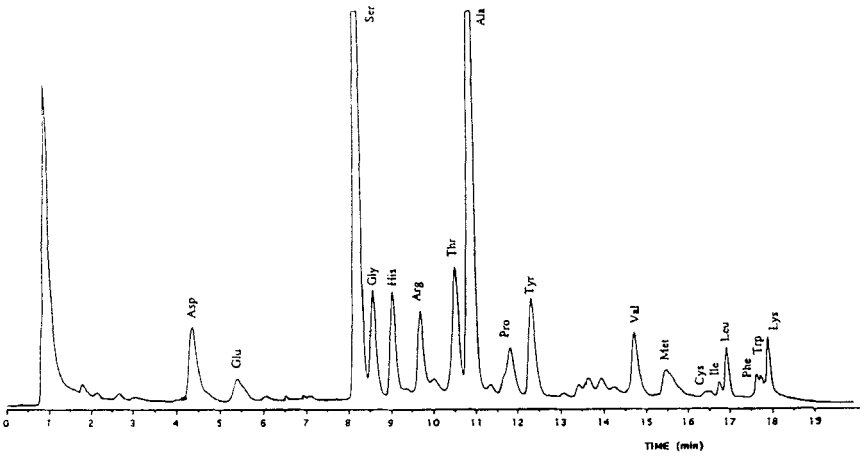

FIGURE 4.- Elution profile of dessert sample showing resolution of PTC-amino acids by liquid chromatography .

Three samples of each infant food were analyzed to estimate the variation of the liquid chromathography method. Moreover, three replicates of the sample extract were analyzed to estimate the variation of the chromatography. The reproducibility data for each sample are shown in table 3, 4, 5 and 6. The variability expressed as coefficient of variation (CV) of the entire analytical procedure, including extraction, derivatization and chromatography, for all amino acids was less than 10%. Variability was highest for cysteine (Cys), tyrosine (Tyr), threonini (Thr) and lysine (Lys) (although never greater than 10% CV).

The coefficient of variation of the entire analytical procedure indicate this method seem to be very precise for amino acid analysis in infant foods. These experimental results can not be compared to those by other authors since research into similar products has not been found in bibliography. Only, Grün et al (17) give lysine values for baby vegetable and beef foods between 173.1-246.4 mg lysine/ 100 g of food and between 24.7-13.7 mg lysine/ 100 g of food for banana samples. The lysine content of baby food samples was determined by Grün et al (17) by using a trinitrobenzenesulfonic acid (TNBS) method, adapted from Hall et al (18). Trinitrophenillysine was measured spectrophotometrically at 415 nm.

TABLE 3

Means and Coefficients of Variation (CV) of Amino Acids in Vegetable Samples determined by LC Methods

Amino acid	Concentration found[a]	CV(%)[b]
Asp	220.39±10.30	4.67
Glu	360.34±5.90	1.91
Ser	91.62±3.65	3.98
Gly	70.92±2.46	4.17
His	80.27±3.59	4.47
Arg	79.46±2.07	2.60
Thr	62.31±3.29	5.28
Ala	76.91±3.32	4.32
Pro	19.12±1.12	5.86
Tyr	63.03±3.49	5.53
Val	66.88±3.40	5.08
Met	38.64±2.04	5.28
Cys	11.38±0.78	6.75
Ile	115.02±2.40	2.09
Leu	83.34±1.93	2.32
Phe	144.17±2.90	2.01
Trp	39.39±1.77	4.49
Lys	81.57±7.85	9.62

[a]Mean ± S.D. (n=6)

[b]Coefficient of variation

TABLE 4

Means and Coefficients of Variation (CV) of Amino Acids in Meat Samples determined by LC Methods

Amino acid	Concentration found[a]	CV(%)[b]
Asp	137.77±7.16	5.19
Glu	351.54±12.75	3.63
Ser	56.65±2.92	5.15
Gly	67.35±3.28	4.87
His	85.36±2.21	2.59
Arg	140.12±5.62	4.01
Thr	65.45±3.43	5.24
Ala	96.40±4.25	4.41
Pro	138.61±5.68	4.09
Tyr	53.19±3.16	5.95
Val	46.34±2.11	4.55
Met	45.27±2.36	5.21
Cys	7.22±0.35	4.85
Ile	64.41±2.02	3.14
Leu	65.44±3.04	4.65
Phe	92.77±2.55	2.75
Trp	60.18±2.91	4.83
Lys	317.07±16.12	5.09

[a]Mean ● S.D. (n=6)

[b]Coefficient of variation

TABLE 5

Means and Coefficients of Variation (CV) of Amino Acids in Fish Samples determined by LC Methods

Amino acid	Cocentration found[a]	CV(%)[b]
Asp	234.60±9.88	4.21
Glu	271.78±15.73	5.79
Ser	110.69±3.72	3.36
Gly	80.33±3.84	4.79
His	126.96±3.95	3.11
Arg	131.13±1.61	1.23
Thr	57.50±3.75	6.52
Ala	122.58±7.32	5.97
Pro	22.11±1.21	5.47
Tyr	65.44±2.60	3.97
Val	52.72±2.27	4.30
Met	40.89±2.26	5.52
Cys	5.21±0.13	2.49
Ile	84.14±2.23	2.65
Leu	43.05±1.41	3.27
Phe	160.37±4.05	2.52
Trp	78.92±3.96	5.02
Lys	214.94±8.69	4.04

[a]Mean ± S.D. (n=6)
[b]Coefficient of variation

TABLE 6

Means and Coefficients of Variation (CV) of Amino Acids in dessert with six different Fruit Samples determined by LC Methods

Amino acid	Concentration found[a]	CV(%)[b]
Asp	103.13±3.87	3.75
Glu	45.62±1.27	2.79
Ser	29.35±1.34	4.56
Gly	28.14±1.22	4.33
His	54.96±2.06	3.75
Arg	19.08±0.90	4.72
Thr	10.25±0.36	3.51
Ala	68.19±3.15	4.62
Pro	32.06±1.16	3.62
Tyr	11.15±0.86	7.71
Val	13.79±0.47	3.41
Met	17.37±0.73	4.20
Cys	5.07±0.21	4.14
Ile	12.24±0.24	1.96
Leu	39.91±1.97	4.94
Phe	19.60±0.87	4.44
Trp	10.05±0.19	1.89
Lys	24.74±1.34	5.44

[a]Mean ± S.D. (n=6)
[b]Coefficient of variation

Amino acids values obtained for us agree well with the values supplied from manufacturer (Table 1).

Other authors have reported PTC-amino acid method agree well with those from the ion-exchange analysis for different foods (4,13).

CONCLUSIONS

This liquid chromatographic method reported in the present study can be used for accurate, rapid (20 min) and reproducible determination of most nutritionally important amino acids in this foods including tryptophan.. Moreover, this method can be used for rapid determination of available lysine.

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