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Analytical Methods Section

Precolumn phenylisothiocyanate derivatization and liquid chromatography of free amino acids in biological samples

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A method for the analysis of free amino acids using precolumn phenylisothiocyanate derivatization and liquid chromatography was tested on a range of sample matrices: bovine plasma, dry cat food, dry infant milk formula and potatoes. The chromatographic resolution of the phenylthiocarbamyl derivatives of free amino acids was satisfactory, with few unidentified compounds present. Standard deviations for triplicate analyses were generally less than 10% of their means. 5-Sulfosalicylic acid (SSA) and a small pore membrane ultrafilter were tested for the deproteinization of bovine plasma. Recoveries of standards were highest when 1% and 4% SSA were used for deproteinization (98% average recovery), and lower (91%) when the ultrafilter was used. The 4% SSA treatment gave higher average free amino acid contents and protein removal than the other deproteinization techniques. These higher values were not due to protein hydrolysis by SSA. Interaction of free amino acids with plasma protein remaining after deproteinization may account for the lower free amino acid contents measured after 1% SSA and ultrafilter treatment. Deproteinization of plasma with 4% (w/v) (final concentration after mixing with the sample) SSA is recommended.

INTRODUCTION

Free amino acids have traditionally been analyzed by continuous postcolumn reaction with ninhydrin after ion-exchange chromatography (Moore *et al.*, 1958). The disadvantages of this method include long analysis times (3 h) and relatively low sensitivities for UV detection (200–500 pmol). In recent years, the analysis of amino acids by reversed-phase liquid chromatography (LC) after derivatization with phenylisothiocyanate (PITC) (to yield phenylthiocarbamyl (PTC) amino acids) has gained popularity because UV detection limits are lower (2–5 pmol) and analysis times are shorter (less than 90 min for free amino acids, including column re-equilibration) than traditional techniques (Cohen & Strydom, 1988). This procedure, marketed by Waters (Milford, MA) as the Picotag method, has been used extensively for hydrolyzate amino acid analysis (Hagen *et al.*, 1989). To date, however, there have been only limited reports on using the Picotag method for free amino acid analysis (Lavi & Holcenberg, 1986; Cohen & Strydom, 1988; Sarwar & Botting, 1990).

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A key step in sample preparation for free amino acid analysis is deproteinization. Several methods have been used, including: chemical precipitation with organic solvents or acids, ion-exchangers, dialysis and ultracentrifugation (Deyl, 1986). 5-Sulfosalicylic acid (SSA) precipitation is a common method used for deproteinization of biological fluids (Cohen & Strydom, 1988). The recovery of underivatized free amino acids after SSA precipitation has approximated 100% (DeWolfe *et al.*, 1967, Mondino *et al.*, 1972). However, the conversion of free amino acids to their PTC derivatives in the presence of SSA may be inhibited (Cohen & Strydom, 1988), although no data were presented. Recently, ultrafiltration of samples with small pore membranes has been used as a means of deproteinization (Cohen & Strydom, 1988). The yield of pure PTC amino acids after ultrafiltration with small pore membranes has approximated 100% (Cohen & Strydom, 1988); however, the yield of PTC amino acids in a sample matrix deproteinized with this technique has not been documented.

One objective of this paper was to assess the versatility of the Picotag method of free amino acid analysis. This was accomplished by analyzing PTC amino acids from a wide variety of sample matrices. A second objective was to determine the yield (recovery) of PTC amino

acids in a sample matrix after SSA precipitation, and ultrafiltration with small pore membranes. This was accomplished with a standard addition and recovery study.

EXPERIMENTAL

The Picotag method of free amino acid analysis was used as previously reported (Cohen & Strydom, 1988), with some modifications as described below.

Reagents and apparatus

Reagents

Reagent grade concentrated hydrochloric acid, ethanol, phosphoric acid (85%), sodium phosphate dibasic, and LC grade acetonitrile, glacial acetic acid, methanol, and sodium acetate trihydrate were obtained from J. T. Baker (Phillipsburg, NJ). Pierce A/N and B amino acid standards, PITC, and triethylamine (TEA) were obtained from Pierce Chemical Co. (Rockford, IL). Methionine sulfone (MET-O_2) and SSA were obtained from Sigma Chemical Co. (St Louis, MO).

Apparatus

High purity water was supplied by a Milli-Q purification system (Millipore, Bedford, MA). Derivatization (6 mm × 50 mm) tubes were Pyrex brand (Corning Glass Co., Corning, NY). Vacuum vials and resealable closures were obtained from Waters (Milford, MA). HV-type membrane filters (0·45 μm pore size, Millipore) were used for aqueous solvent and sample filtration. Millipore Ultrafree-Pf (10000 NMWL polysulfone membrane) ultrafilters were used for the deproteinization of some samples. Samples were derivatized and dried in a Waters Picotag vacuum station.

Sample handling prior to derivatization

All glassware used in sample preparation and analysis was soaked overnight in 6 N HCl (25°C), rinsed with Milli-Q water, and dried overnight at 100°C.

Two general types of samples were analyzed: solid samples (cat food, infant milk formula, and potatoes) and bovine plasma. These two sample types were handled differently, as described below.

Dry cat food (Iams Co. Dayton, OH, lot no. 100190A3BU, 250 g) was homogenized in a Waring Blender (New Hartford, CT) for 1 min at high speed. Subsamples of homogenized cat food (2·5 g) or dry infant milk formula (Similac with iron, lot no. 36453RBSI, Ross Labs, Columbus, OH, 2·5 g) were mixed with 150 ml of 80% (v/v) ethanol and ground for 2 min with a Polytron (Kinematica, Luzern, Switzerland), shaken for 15 min on a reciprocal shaker, and centrifuged for 10 min at 1250 *g*. The supernatant was brought to dryness on a rotary evaporator (Rinco, Greenville, IL) at 35–40°C, and the resulting residue was redissolved in 25 ml of 0·1 N HCl. Potatoes (cv. Russett Burbank, 250 g, mixed 2 + 1 with Milli-Q water) were ground for 1 min at high speed in a Waring Blender. Subsamples of ground potato, including added water (10 g), were extracted with 50 ml of 80% (v/v) ethanol on a reciprocal shaker for 15 min and centrifuged for 10 min at 1250 *g*. Ethanol extraction and centrifugation were repeated twice on the residue. The pooled supernatant was brought to dryness and resuspended as stated above for the cat food and infant milk formula samples. The concentrated extracts of these three foods were mixed 1 : 1 (v/v) with 0·4 mM MET-O_2 in 0·1 N HCl, filtered, and stored at −20°C.

Blood was collected from a Holstein cow using a heparinized 50 ml syringe and stored on ice for transport (less than 30 min) to the laboratory. The heparinized blood was centrifuged at 3500 *g* for 15 min. Aliquots of plasma (1 ml of the supernatant) were mixed with 50 μl of either 0·1 N HCl (unspiked control subsamples) or a 1 : 1 (v/v) mixture of Pierce A/N and B amino acid standards (spiked subsamples). Some of the control and spiked subsamples were deproteinized by SSA treatment by mixing 200 μl of either 6·25% (w/v) or 25% (w/v) SSA with the subsamples. These subsamples were held at 25°C for 30 min, then centrifuged at 25 000 *g* for 30 min at 5°C. The remainder of the subsamples were mixed with 200 μl of Milli-Q water and deproteinized by passage through Millipore Ultrafree-Pf units with 30–60 psi air pressure. All of the deproteinized plasma subsamples were mixed 1 : 1 (v/v) with 0·4 mM MET-O_2 in 0·1 N HCl, filtered, and stored at −20°C.

Derivatization with PITC

Derivatization tubes containing 25 μl of filtrate from the subsamples were placed in vacuum vials which were then sealed with Teflon-lined closures. The tube contents were dried under vacuum (to less than 75 mtorr pressure) in the Picotag vacuum station, then 25 μl of a redry solution was added (LC grade methanol + 1 N LC grade sodium acetate trihydrate + TEA, 2 + 2 + 1, v/v/v). The tube contents were dried again to less than 75 mtorr pressure, then 25 μl of a derivatization solution was added (LC grade methanol + Milli-Q water + TEA + PITC, 7 + 1 + 1 +1, v/v/v/v). The tubes were held at 25°C and atmospheric pressure for 10 min, then dried again to less than 75 mtorr pressure.

After derivatization, the subsamples were either stored dry at −20°C (where they were stable for at least three weeks) or 100 μl of diluent was added (95% 5 mM sodium phosphate dibasic, titrated to pH 7·5 with 10% phosphoric acid, and 5% (v/v) acetonitrile). The subsamples were then transferred to limited volume (300 μl) inserts contained in 4 ml autoinjector vials. The vials were sealed with Teflon-lined closures and analyzed within 24 h.

Standard preparation

The working standard consisted of Pierce A/N and Pierce B amino acid standards, Milli-Q water, and 0·4 mM

MET-O_2 in 0·1 N HCl, mixed 1 + 1 + 4·25 + 6·25 (v/v/v/v). Aliquots of this working standard (25 μl) were derivatized in the same manner as the subsamples.

Chromatography, column cleaning, and quantification

Chromatography

Waters equipment was used: Two M6000A solvent delivery systems controlled by Maxima 820 software (ver. 3.1) via a System Interface Module (SIM); a WISP M712 autoinjector equipped with a 200 μl sample loop; a model M440 fixed wavelength spectrometer (254 nm, 0·05 AUFS). Two Waters columns were tested which gave comparable results: an application specific Picotag amino acid analysis column (3·9 mm × 300 mm, stainless steel) and a Nova-Pak C_{18} column (3·9 mm × 300 mm, stainless steel). The analyses for this paper were made with the Picotag column, which was preceded by a 0·2 μm in-line filter (Waters No. 84560), and held at 46°C by a Waters Temperature Control Module.

Two 10 μl aliquots of the derivatized working standard (containing 500 pmol per injection of amino acid, except for 250 pmol per injection of asparagine, cystine and taurine) were injected at the beginning of each day, and the second was used for calibration. Aliquots (10 μl) of the derivatized subsamples (containing 500 pmol per injection of the internal standard MET-O_2) were injected. The solvent system consisted of two eluents: (1) 975 ml of 0·07 N LC grade sodium acetate trihydrate, titrated to pH 6·50 with LC grade glacial acetic acid, mixed with 25 ml of LC grade acetonitrile; and (2) 450 ml of LC grade acetonitrile, 150 ml of LC grade methanol, and 400 Milli-Q water measured separately and then mixed. These eluents were compared to Waters Picotag free amino acid eluents 1 and 2 (Waters No. 10960 and 10965, respectively) which gave comparable results. The solvent program was as follows (total flow rate of 1 ml min^{-1}): 100% eluent 1 to 13·5 min; 13·5 min, step to 97% eluent 1; 13·5–24 min, concave curve (Waters No. 8) to 94% eluent 1; 24–30 min, convex curve (Waters No. 5) to 91% eluent 1; 30–50 min, linear change to 66% eluent 1; 50–62 min, hold at 66% eluent 1; 62–62·5 min, linear change to 100% eluent 2; 62·5–66·5 min, hold at 100% eluent 2; 66·5–67 min, linear change to 100% eluent 1; 67–87 min, hold at 100% eluent 1. The total run time between injections including column re-equilibration was 87 min.

Column cleaning

Since no guard column is utilized the column must be flushed every 250–300 injections in order to remove highly retained lipids and peptides. Peptides can be removed by flushing with 10 column volumes of 0·1% (v/v) trifluoroacetic acid (TFA) followed by a 20 column volume gradient from 0·1% (v/v) TFA to 40% of the TFA solution/60% acetonitrile. Lipids can be removed by flushing with 10 column volumes of tetrahydrofuran. With proper care and cleaning each column will have a useful life of 1000–1500 injections.

Quantification

Chromatographic data were collected and processed with Maxima 820 software (ver. 3.1) via a SIM link between the spectrometer and an IBM (Armonk, NY) PC/XT computer.

RESULTS AND DISCUSSION

The chromatographic resolution of the PTC derivatives of free amino acids in the working standard, and samples of bovine plasma, cat food, infant milk formula and potatoes, was generally very good (see Figs 1 and 2 as representative chromatograms). The resolution of the working standard was very similar to previous results (Cohen & Strydom, 1988). The working standard did not contain glutamine (GLN). Thus, GLN was not quantitated and values are not reported. However, GLN can be measured if a fresh stock standard solution is prepared and added to the working standard (use within one week). GLN elutes just after glycine and is identified on the plasma sample chromatogram (Fig. 2).

Free amino acid levels (based on quantitation of the PTC derivatives) in cat food, infant milk formula and potatoes are shown in Table 1. Even though the working standard contained 38 amino acids, only 22 that are commonly studied were quantitated in the samples.

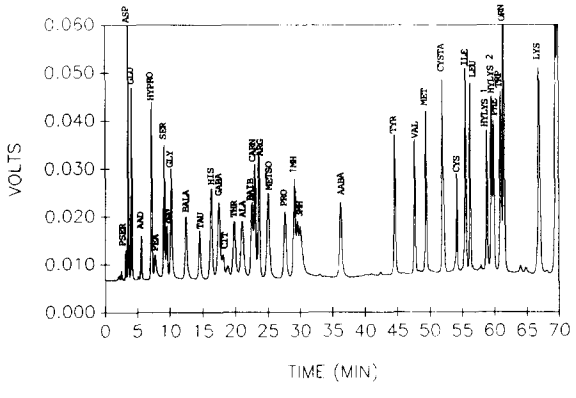

Fig. 1. Elution profile of the 38 amino acid standard showing resolution of each PTC amino acid by liquid chromatography.

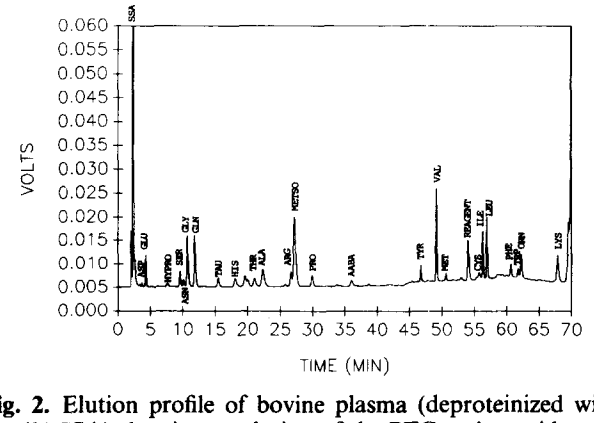

Fig. 2. Elution profile of bovine plasma (deproteinized with 4% SSA) showing resolution of the PTC amino acids.

Table 1. Free amino acid content (μmol g^{-1} dry weight, means and standard deviations, based on PTC derivatives) for triplicate analyses of dry cat food, dry infants milk formula and potatoes

Amino acid	Cat food Mean (SD)	Infant formula Mean (SD)	Potatoes Mean (SD)
ASP	1·5 (0·10)	0·1 (0·02)	22·8 (0·52)
GLU	5·9 (0·61)	1·0 (0·04)	36·6 (0·29)
HYPRO	0·4 (0·06)	ND	1·0 (0·05)
SER	2·5 (0·08)	ND	12·4 (0·14)
ASN	2·6 (0·22)	0·4 (0·05)	198·2 (2·99)
GLY	4·7 (0·53)	0·4 (0·05)	1·4 (0·10)
TAU	13·1 (1·58)	3·5 (0·54)	0·5 (0·05)
HIS	0·4 (0·01)	ND	4·3 (0·02)
THR	1·5 (0·05)	ND	10·0 (0·48)
ALA	7·4 (0·38)	ND	17·6 (0·52)
ARG	1·3 (0·07)	ND	11·4 (0·38)
PRO	3·3 (0·18)	ND	12·4 (0·24)
TYR	0·9 (0·17)	ND	14·7 (0·10)
VAL	2·1 (0·29)	0·1 (0·01)	37·1 (0·38)
MET	28·0 (0·94)	0·1 (0·01)	5·7 (0·29)
CYS	0·4 (0·07)	ND	2·4 (0·14)
ILE	1·0 (0·06)	0·1 (0·01)	10·0 (0·10)
LEU	2·5 (0·25)	0·1 (0·01)	8·6 (0·57)
PHE	1·0 (0·06)	ND	15·7 (0·33)
TRP	0·2 (0·01)	ND	2·4 (0·14)
ORN	0·1 (0·01)	ND	0·5 (0·05)
LYS	1·0 (0·09)	ND	4·8 (0·05)

Standard deviations were generally less than 10% of the mean values. Cat food and infant formula were chosen for analysis, not only because they are very different matrices, but also because they are formulated to contain significant levels of taurine (TAU). TAU analysis has grown in importance since this amine has been found to be essential for cats and possibly primates, including humans (Pion *et al.*, 1987), and because of its probable role in nerve function (Gaull, 1990). Many foods (e.g. potatoes) are susceptible to enzymatic browning, which may be related to free tyrosine content. Thus, potatoes were analyzed as another test of the applicability of the Picotag procedure for free amino acid determination.

The free amino acid content of bovine plasma and the recovery of standards added to the plasma (based on quantitation of the PTC derivatives) was determined for three deproteinization techniques (Table 2). The average recovery of standards was very high when either 1% or 4% SSA was used for deproteinization (98% for 1% and 4% SSA). These SSA concentrations were chosen since they are near the lower and upper range of SSA concentrations generally used for deproteinization (Deyl, 1986). When ultrafilters were used for deproteinization PTC standard recoveries averaged about 91%, about 7% less than when SSA was used. In addition, many recoveries were less than 90%, with two below 80% (Table 2). These results are in contrast with previous indications that SSA deproteinization may cause significant losses of PTC amino acids, and that ultrafilters are the apparatus of choice for protein removal (Cohen & Strydom, 1988). However, it should be noted that sample pH and the membrane used can affect recoveries of free amino acids when ultrafilters are used for deproteinization (Sarwar & Botting, 1990). Average free amino acid content after 1% SSA and ultrafilter treatment was comparable (80 nmol ml^{-1} plasma; Table 2). However, after 4% SSA treatment,

Table 2. Free amino acid content (nmol ml^{-1}, means and standard deviations) and recovery of added standards (based on PTC derivatives) for triplicate analyses of bovine plasma deproteinized with three different techniques

Amino acid	1% SSA			4% SSA			Ultrafiltered		
	Mean	SD	Recov.	Mean	SD	Recov.	Mean	SD	Recov.
ASP	9	(2)	94	11	(3)	89	9	(3)	92
GLU	88	(1)	101	134	(10)	93	86	(3)	90
HYPRO	24	(1)	102	27	(0)	116	26	(2)	113
SER	89	(1)	99	102	(2)	101	87	(3)	90
ASN	62	(2)	101	68	(5)	99	646	(3)	89
GLY	336	(5)	101	363	(4)	99	332	(3)	95
TAU	46	(2)	94	44	(2)	94	44	(5)	84
HIS	36	(1)	98	38	(1)	107	36	(3)	94
THR	79	(3)	101	94	(6)	102	82	(2)	81
ALA	161	(3)	100	178	(4)	95	162	(4)	94
ARG	96	(4)	89	100	(7)	94	95	(5)	83
PRO	61	(3)	94	74	(6)	93	59	(4)	87
TYR	44	(1)	96	47	(3)	94	45	(6)	93
VAL	197	(6)	109	231	(7)	105	209	(2)	93
MET	13	(1)	98	14	(2)	107	14	(1)	74
CYS	31	(3)	99	30	(3)	99	32	(2)	102
ILE	80	(1)	103	97	(2)	96	83	(4)	79
LEU	113	(1)	104	145	(3)	89	110	(2)	91
PHE	38	(2)	96	40	(1)	98	37	(4)	94
TRP	35	(0)	91	29	(2)	91	36	(3)	95
ORN	44	(1)	96	48	(2)	98	43	(1)	89
LYS	73	(1)	95	84	(2)	102	74	(2)	94
AVG	80	(2)	98	91	(4)	98	80	(3)	91

Table 3. Total (6 N HCl hydrolyzate) amino acid content (nmol ml^{-1}, means and standard deviations; based on PTC derivatives) for triplicate analyses of bovine plasma deproteinized with three different techniques

Amino acid	1% SSA Mean (SD)	4% SSA Mean (SD)	Ultrafiltered Mean (SD)
ASP	6050 (103)	121 (4)	6380 (66)
GLU	9480 (44)	640 (17)	12050 (210)
SER	6410 (61)	284 (7)	6210 (79)
GLY	4920 (44)	733 (23)	6110 (40)
HIS	1300 (11)	22 (1)	617 (8)
ARG	3280 (36)	301 (4)	3520 (42)
THR	5000 (23)	317 (4)	5950 (124)
ALA	5580 (57)	498 (15)	7300 (101)
PRO	4940 (61)	475 (61)	7300 (97)
TYR	2520 (15)	173 (9)	1790 (17)
VAL	6280 (88)	466 (4)	7020 (110)
ILE	2720 (22)	246 (4)	3920 (71)
LEU	7040 (46)	485 (5)	8840 (110)
PHE	2750 (21)	258 (2)	3470 (23)
LYS	1860 (94)	164 (4)	1520 (118)

the average was about 11% higher (91 nmol ml^{-1} plasma; Table 2).

An additional experiment was carried out to determine whether deproteinization with 4% SSA hydrolyzed some of the protein in the samples. Aliquots (1 ml) of 5% (w/v) BSA in 0·85% (w/v) NaCl were mixed with 200 μl of 1% and 4% (w/v) SSA and 50 μl of 0·1 N HCl for 30 and 60 min. Deproteinization, derivatization, and chromatography were completed as described above. No PTC amino acids were detected in any of these aliquots, which suggests that the higher free amino acid levels found after deproteinization with 4% SSA were not due to hydrolysis of sample protein.

Measurement of total protein after the deproteinization treatments (Bradford, 1976) indicated 99·6% removal by the 4% SSA, versus 93·7% and 93·2% removal by the 1% SSA and ultrafilter treatments, respectively. Hydrolyzate amino acid analysis (Hagen *et al.*, 1989) of deproteinized plasma confirmed that less protein was removed by the 1% SSA and ultrafilter treatments (Table 3). Thus, the lower free amino acid values found after the 1% SSA and ultrafilter treatments may have been due to binding of these compounds to proteins remaining in the plasma.

In conclusion, the Picotag method used was satisfactory for the analysis of the PTC derivatives of free amino acids in a wide range of sample matrices. A faster and more limited PTC free amino acid analysis is possible (Sarwar & Botting, 1990); however, for complex matrices the longer column and analysis time used in this paper are recommended. For the deproteinization of plasma, 4% (w/v) (final concentration of acid after mixing with the sample) SSA was found to give the most satisfactory recovery and quantitation of free amino acids.

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