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Review

Evaluation of liquid chromatographic analysis of nutritionally important amino acids in food and physiological samples

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ABSTRACT

Significant advances have been made in standardizing methods for amino acid analysis of foods. The methods included standardized hydrolysis of the food proteins followed by separation and quantitation of the released amino acids by ion-exchange chromatography (IEC). IEC is still the main method in use. Its use is, however, being replaced by the faster high-performance liquid chromatographic (HPLC) methods of derivatized amino acids. The HPLC separation of precolumn phenylisothiocyanate (PITC) derivatives has been adapted for rapid analysis of all amino acids in protein hydrolysates (12 min) and nutritionally important amino acids in deproteinized physiological samples (20 min). The inter-laboratory variability of the PITC derivatization method has not been determined although the intra-laboratory variation of the HPLC method was found to be similar to that of IEC. When similar hydrolytic conditions were used in preparing protein hydrolysates, amino acid results obtained with the PITC derivatization method were generally in close agreement with those obtained by IEC. There is, however, room for improvement in the HPLC analysis of amino acids in physiological samples.

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LIST OF ABBREVIATIONS

AABA	α-aminobutyric acid
ACN	acetonitrile
AOAC	Association of Official Analytical Chemists
1CH3HIS	1-Methylhistidine
3CH3HIS	3-Methylhistidine
C.V.	Coefficient of variation
CYSCOOH	Cysteic acid
Dabsyl	Dimethylaminoazobenzenesulphonyl chloride
Dansyl	Dimethylaminonaphthalenesulphonyl chloride
DNPAA	1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide
FAO–WHO	Food and Agriculture Organization–World Health Organization of the United Nations
FDNB	1-Fluoro-2,4-dinitrobenzene
FMOC-Cl	9-Fluoroenylmethyl chloroformate
HPLC	High-performance liquid chromatography
IEC	Ion-exchange chromatography
METONE	Methionine sulfone
MPA	3-Mercaptopropionic acid
OPA	*o*-Phthalaldehyde
PA	Picric acid
PAA	Plasma free amino acids
PCA	Perchloric acid
PER	Protein efficiency ratio
PICA	Piperdine-4-carboxylic acid
PITC	Phenylisothiocyanate
PTA	Phosphotungstic acid
PTC	Phenylthiocarbamyl
PTH	Phenylthiohydantoin
SSA	5-sulphosalicylic acid
TCA	Trichloroacetic acid
TFA	Trifloroacetic acid

In figures, a three-letter abbreviation was used for common amino acids.

1. INTRODUCTION

Protein in foods contains some twenty common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, proline, serine, tyrosine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine). In addition, some food proteins contain hydroxyproline and hydroxylysine. All twenty amino acids are needed for protein synthesis although about nine cannot be synthesized or adequately synthesized in the human body, and are referred to as indispensable or essential amino acids [1]. These essential amino acids which include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine must be supplied in the diet. Dietary requirements for protein and amino acids are based on needs for both total amino nitrogen and essential amino acids to synthesize body protein and other important nitrogen-containing compounds such as peptide hormones and neurotransmitters [1].

There is ongoing interest in the development of reliable and rapid methods of assessing protein quality of foods for regulatory purposes. To be accurate and applicable to a wide range of foods, such methods must measure all the basic parameters that determine the quality of protein, such as quantities of essential amino acids, digestibility of protein and bioavailability of amino acids [2]. Similarly, the nutritional significance and potential utility of changes in plasma free amino acids (PAA) in the evaluation of protein quality, the assessment of protein nutritional status and the evaluation of requirements for essential amino acids have been appreciated for a number of decades [3,4]. Therefore, the availability of reliable methods for amino acid analysis of foods, feces and plasma becomes important for the precise prediction of protein quality of foods and protein nutritional status of humans.

Methods for evaluating protein quality of foods were recently reviewed by a Joint Food and Agricultural Organization–World Health Organization (FAO–WHO) Expert Consultation on Protein Quality Evaluation [5]. It was recognized that significant advancements have been made in standardizing amino acid methodology, human amino acid requirements and determination of digestibility of protein and amino acids in a vari-

ety of foods. Based on these advancements, the Consultation agreed that the protein digestibility–corrected amino acid score method was the most suitable approach for routine assessment of protein quality for humans, and recommended the adoption of this method as an official method at the international level [5].

To calculate a protein digestibility–corrected amino acid score, a test food must be analyzed for proximate and amino acid compositions, and a protein digestibility value must be obtained from a data base or be determined by the rat balance method, the most suitable practical method for predicting protein digestibility in humans [5]. The Consultation noted that methods for the determination of amino acids in foods have been standardized resulting in acceptable inter-laboratory variation expressed as coefficients of variation (C.V.) of about 10%. These methods involved standardized acid or alkaline hydrolysis of the protein followed by separation and quantitation of the released amino acids by ion-exchange chromatography (IEC) using cation-exchange resins and postcolumn derivatization (by a commercial amino acid analyzer or HPLC system) or by precolumn derivatization followed by reversed phase high-performance liquid chromatography (HPLC). In the case of precolumn derivatization HPLC methods, only the phenylisothiocyanate (PITC) method was found satisfactory in giving amino acid results equivalent to IEC. The need for comparative study of other precolumn derivatization reagents and collaborative testing of HPLC methods was noted by the Consultation.

Deyl *et al.* [6] published a comprehensive review concerning preparation and deproteinization of biological samples (body fluids and tissues) for subsequent amino acid analysis by liquid chromatography. For routine clinical screening of a large number of samples, the use of relatively inexpensive and simple planar chromatographic methods was recommended [6]. Both underivatized and derivatized amino acids were separated by the planar methods. A large number of substances besides paper have been used in these methods [6]. Detailed information about the chromatography of underivatized and derivatized (dansyl) amino acids on silica gel was reported by Deyl *et al.* [6]. For more precise and quantitative analysis, the use of modern equipment for cation-exchange chromatography for separating underivatized amino acids or sophisticated HPLC systems capable of separating amino acids as derivatives was suggested. The focus of the excellent review Deyl *et al.* [6] was to discuss applicability of the liquid chromatographic methodology in studying hereditary disorders of metabolism. The purpose of this review was to evaluate liquid chromatographic methods for rapid quantitative analysis of nutritionally important amino acids in foods and physiological samples.

2. REVIEW OF PRINCIPAL LIQUID CHROMATOGRAPHIC METHODS OF ANALYSIS OF AMINO ACIDS

Traditionally, amino acid analyses of protein hydrolysates and physiological fluids have been performed according to the classical procedures of Spackman *et al.* [7] and Hamilton [8]. The free amino acids are separated by cation-exchange chromatography and detected photometrically after postcolumn reaction with ninhydrin. This approach is reliable and the resolution of the amino acids is reasonable but the analysis time is rather long (1 h for hydrolysates and up to 4 h for physiological amino acids) with limited detectability (*ca.* 150 pmol). Although the IEC is still the main method in use, the faster HPLC methods are tending to supersede classical IEC. In the last few years, HPLC methods of derivatized amino acids have replaced IEC for the analysis of protein hydrolysates as they offer reduced analysis time and improved limit of detection of about 1 pmol [9,10]. Similar wide acceptance of the HPLC methods for the analysis of amino acids in physiological fluids has not occurred mainly because of the increased demands of sample preparation including deproteinization and chromatographic separations [9]. HPLC may be used to separate amino acids on ion-exchange columns with postcolumn derivatization with ninhydrin or *o*-phthalaldehyde (OPA) [11] or by pre-

column derivatization followed by separation on reversed-phase octyl- or octadecylsilica. Due to the use of simpler instrumentation in precolumn derivatization and the lower cost of such systems compared with postcolumn derivatization, the precolumn derivatization is generally preferred [10,12]. Recently, commercial systems based on precolumn derivatization methodology have been marketed. Through the use of small particle size (3–5 μm) resins, small-bore columns, high-pressure pumps and injection valves, it is possible to separate complex mixtures of derivatized amino acids in as little as 10 min [10]. Coupled with high-sensitivity detectors, these materials allow for picomole or lower detection limits.

Typical reagents for precolumn derivatization are PITC [13], OPA [14], 9-fluorenylmethyl chloroformate (FMOC-Cl) [15], 1-fluoro-2,4-dinitrobenzene (FDNB) [16], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (DNPAA) [17] and dansyl chloride [18]. Some of the features of these methods for amino acid analysis of protein hydrolysates were recently summarized by FAO [5] and are shown in Table 1. Only the first two reagents listed in Table 1 (*i.e.* PITC and OPA) have been widely used for precolumn derivatization of protein hydrolysates. The PITC method has been shown to give amino acid results with foods equivalent to classical IEC. Comparative studies with other derivatization reagents are needed before their use to replace the IEC can be recommended [5].

Until recently, the most widely reported reagent for precolumn derivatization of protein hydrolysates and physiological fluids was OPA, which, in the presence of a thiol reagent, reacts with primary amines to form highly fluorescent isoindoles well suited for HPLC separation [10]. This derivatization procedure has become popular because the reagent itself does not fluoresce and consequently produces no interfering peaks. Moreover, its solubility and stability in aqueous solution coupled with rapid derivatization make it amenable to automated derivatization and analysis. Amino acids from a protein hydrolysate can be separated in 15 min [10], and separation times as short as 45 min have been reported for amino components in biological samples [19]. Despite the convenience of automated derivatization, improved limit of detection and rapid anal-

TABLE 1

SUMMARY OF METHODS SUITABLE FOR PRECOLUMN DERIVATIZATION AND HPLC DETERMINATION OF AMINO ACIDS IN PROTEIN HYDROLYSATES

Reproduced from ref. 5 with permission. Abbreviations: PITC = phenylisothiocyanate; OPA = *o*-phthalaldehyde; FMOC = 9-fluorenylmethyl chloroformate; FDNPAA = 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; FDNB = 1-fluoro-2,4-dinitrobenzene; dansyl = 5-dimethylamino-1-naphthalenesulfonyl chloride.

	PITC	OPA	FMOC	FDNB	FDNPAA	Dansyl
Derivatization time (min)	20	0.5	5	30	50	30
Removal of reagent by drying	Yes	No	No	Yes	Yes	No
Solvent extraction	No	No	Yes	No	No	No
Determines secondary amine	Yes	No	Yes	Yes	Yes	Yes
Quantitative yield	Yes	Yes	Yes	Yes	Yes	Yes
Stable derivative	Yes	No	Yes	Yes	Yes	No
Interfering side-products	No	No	No	Yes	Yes	Yes
Detection	254 nm	Fluor	Fluor	365 nm	340 nm	Fluor
Sensitivity	pmol	fmol	fmol	pmol	pmol	pmol
Interference by contaminants in eluent	Yes	No	No	No	No	Yes
Chromatogram run time (min)	15	18	30	70	110	30

ysis time, OPA-based amino acid analysis has not achieved the wide level of use initially predicted for this method [10]. Lack of direct reactivity with secondary amino acids has been the main limitation. Proline and hydroxyproline can be rendered reactive by the addition of chloramine T, a highly reactive oxidizing agent, prior to the addition of OPA. However, the poor reproducibility of this method has hindered its widespread acceptance. Even without the oxidation step, OPA analysis has also been reported to be less reproducible than desired for glycine and lysine [10]. Moreover, cystine (unlike other amino acids containing primary amines) yields a derivative with minimal fluorescence [20]. This problem could, however, be overcome by pretreating cystine/cysteine with iodoacetic acid before OPA derivatization.

More recently, an automated precolumn derivatization of amino acids in cheese hydrolysate was reported using OPA–3-mercaptopropionic acid (MPA) for the primary amino acids, followed by FMOC for the secondary amino acids [21]. The addition of piperidine-4-carboxylic acid (PICA) as a hydrolysis-resistant internal standard enabled the quantitation of secondary amino acids. Since lysine formed two derivatives with OPA–MPA, it was quantitated using the sum of both peaks. Quantitative determination of cystine/cysteine, methionine and tryptophan was not achieved due to partial loss of these amino acids during acid hydrolysis. The determination of other amino acids in duplicate hydrolysates was quite reproducible with variations of less than 4% (0.6–3.2%). Similar variability for the IEC method was 0.2–2.2%. A comparison with the classical IEC method gave an excellent correlation for all the determined amino acids [21].

The use of reversed-phase HPLC with precolumn derivatization for the analysis of amino acids in protein hydrolysates and blood plasma (serum) is becoming established as a cheaper alternative to commercial amino acid analysers. The precolumn derivatization methods for the HPLC determination of free amino acids in biological samples [22] and standard mixtures [23] have been compared. The four methods (OPA, automated; FMOC-Cl; PITC; dansyl-chloride) permitted the measurement of 21–26 major free amino acids in 13–40 min in physiological samples (plasma, muscle, liver, kidney and leucocytes) [22]. The results obtained by the HPLC methods compared favourably with those obtained by IEC. For routine analyses of primary free amino acids (except cystine) in biological samples, the use of the OPA method was preferred (Table 2). For the determination of secondary amino acids, the use of the FMOC-Cl

TABLE 2

HPLC ANALYSES OF FREE AMINO ACIDS: COMPARISON OF FOUR DERIVATIZATION METHODS

Reproduced from Fürst *et al.* [22] with permission. Abbreviations: OPA = *o*-phthalaldehyde; FMOC-Cl = 9-fluorenylmethyl chloroformate; PITC = phenylisothiocyanate; Dansyl chloride = 1-dimethylaminonaphthalene-5-sulphonyl chloride.

Parameter	OPA (automated method)	FMOC-Cl	PITC	Dansyl chloride
Limit of detection (pmol) (signal-to-noise ratio = 25)	0.8	1.0	5.0	1.5
Error of the method (C.V., %) (based on duplicate determinations)	1.0–4.7	1.1–5.9	3.6–7.0	1.7–4.5
Reproducibility (C.V., %)	0.4–2.2	1.9–4.6	2.6–5.5	1.5–4.1
Stable adducts	No	Yes	Yes	Yes
Detection of secondary amines/cystine	No/No	Yes/No	Yes/Yes	Yes/Yes
Laborious	–	+ + +	+ +	+
Problematic amino acids	Asp, Trp	His, Trp	Orn, Trp, His, $(Cys)_2$	His, Asn

TABLE 3

COMPARISON OF PRECOLUMN DERIVATIZATION HPLC TECHNIQUES IN TERMS OF THE SPECIFIED ANALYTICAL PARAMETERS

Reproduced from McClung and Frankenberger, Jr. [23] with permission. Abbrevations: dabsyl = dimethylaminoazobenzenesulphonyl chloride; dansyl = dimethylaminonaphthalenesulfonyl chloride; OPA = *o*-phthalaldehyde; PTC = phenylthiocarbamyl; PTH = phenylthiohydantoin.

Limit of detection	OPA > dabsyl = dansyl > PTH = PTC
Precision	dansyl > PTH = PTC > dabsyl > OPA
Stability	PTC = PTH > dabsyl = dansyl > OPA
Resolution	PTC > PTH > OPA > dabsyl = dansyl
Time of analysis	PTH = dabsyl < dansyl < OPA < PTC

method or a combination of the OPA and FMOC-Cl techniques was recommended. For the determination of free cystine or cystine-containing short-chain peptides, the use of the dansyl chloride method was suggested. The PITC method was found useful in cases where sufficient sample was available.

Five precolumn derivatization [OPA; phenylthiocarbamyl (PTC); phenylthiohydantoin (PTH); dansyl chloride and dabsyl chloride] HPLC techniques were compared in terms of limit of detection, precision, stability, resolution and time of analysis (Table 3). The formation of fluorescent derivatives using OPA was found most desirable in terms of limit of quantitation. The main disadvantage of this method lies in the fact that OPA does not react with the secondary amino acids, proline and hydroxyproline. The OPA method may also not be best suited for precolumn derivatization due to the instability of the derivatives formed [23–25]. The use of PTC derivatives for reversed-phase HPLC analysis of precolumn-derivatized amino acids was the preferred method of the derivatization techniques used [23]. The PTC amino acid derivatives formed were quite stable as peak areas of derivatized standards remained constant for two days. PTC amino acids have been reported to be stable for months if stored dry in a freezer [26]. The use of a system which uses stable derivatives such as PTC amino acid derivatives was found suitable in the quantitation of labelled amino acids by reversed-phase HPLC [24]. More recently, the use of electrochemical detection of PTC derivatives was described to virtually eliminate interferences observed during ultraviolet or fluorescence detection in the separation of amino acids in blood or urine [27].

3. HYDROLYSIS OF PROTEINS IN FOOD AND FECAL SAMPLES

Amino acids in proteins must be released by hydrolysis prior to assay. Therefore, hydrolysis of proteins in food and fecal samples is a very important step in amino acid analysis. No matter which technology is applied to the analyses, the hydrolysis procedure is the major limiting parameter for both the precision and accuracy with which the amino acid composition of proteins can be determined [28]. Methods for hydrolysis of proteins were recently reviewed [5].

Hydrolysis with 6 *M* HCl, either in evacuated tubes at 110 ± 0.5°C or refluxed under a stream of nitrogen for 22–24 h, is the most commonly used method [5]. More recently, the usefulness of vapour phase 6 *M* HCl hydrolysis of proteins and peptides for a cleaner hydrolysis and faster sample drying after hydrolysis has been reported [10]. Influence of various hydrolysis parameters such as acid-to-nitrogen ratio, the use of sealed tubes *versus* open reflux, and hydrolysis times have been reviewed [28–31]. A shorter acid hydrolysis time of 4 h at high temperature (145°C) was found to be satisfactory [28]. The results obtained with this method were in close agreement with those obtained after 24 h at 110°C for all amino acids except for valine and isoleucine (8–9% higher) and threonine and serine (9–13% lower). In using the shorter-time hydrolysis, the use of appropriate correction factors for the four amino acids was recommended [28].

The 6 *M* HCl hydrolysis results in large destruction of cyst(e)ine and tryptophan, and in partial loss of methionine especially in carbohydrate-rich foods, necessitating separate hydrolys-

es for accurate determination of these amino acids [5]. A pre-oxidation with performic acid followed by 6 *M* HCl hydrolysis is widely used for accurate determination of cyst(e)ine and methionine as cysteic acid and methionine sulfone, respectively. Similarly, an alkaline (NaOH, BaOH, LiOH) hydrolysis is commonly used for accurate determination of tryptophan [5].

The three hydrolysis procedures (6 *M* HCl, performic acid plus 6 *M* HCl hydrolysis, and 4.2 *M* NaOH) were standardized by Satterlee *et al.* [32] in a collaborative study, and were subsequently adopted as a part of the official first action of the calculated Protein Efficiency Ratio (PER) by the Association of Official Analytical Chemists (AOAC) [33]. The performic acid oxidation and hydrolysis determination of sulfur amino acids and the 4.2 *M* NaOH hydrolysis determination of tryptophan in food and feed ingredients were further standardized and compared in collaborative studies [34,35]. The mean recoveries (average of seven to nine laboratories) of cystine, methionine and tryprophan in the two studies were 95–97, 98–101 and 85%, respectively. The performic acid plus 6 *M* HCl hydrolysis method [34,35] and the alkaline hydrolysis procedure [35] were adopted official first-action AOAC methods for the determination of sulfur amino acids and tryptophan, respectively [33].

A single 6 *M* HCl hydrolysis at 110°C for 24 h can give adequate information for most amino acids for nutritional purposes [36]. Three hydrolyses of different time duration (usually 24, 48 and 72 h) are, however, recommended for more accurate determination of isoleucine and valine (calculated to infinite time) and of threonine and serine (calculated to zero time) [29]. Anderson *et al.* [30] and Mason *et al.* [37] conducted a series of experiments on hydrolytic conditions for oxidised proteins, and recommended optimum conditions for the oxidised hydrolysate suitable for the determination of all amino acids except for tryptophan, tyrosine and histidine. Hydrolysis with organic sulphonic acids has been used to determine tryptophan and all other amino acids in pure proteins but the method was not successful in measuring tryptophan in foods and feeds where the presence of carbohydrates caused losses of tryptophan [38,39]. Very short hydrolysis time (45 min) at high temperature (160°C) was used to hydrolyse all amino acids including cystine and tryptophan in pure proteins [40]. Although further research on such methods is desirable, use of organic sulphonic acid hydrolysis for the amino acid analysis of foods is not recommended for routine use at the present time [5].

4. DEPROTEINIZATION OF PHYSIOLOGICAL SAMPLES

The presence of proteins present in plasma or tissues exerts considerable influence on the determination of free amino acids, necessitating deproteinization of the biological samples prior to free amino acid analysis. The detailed preparation and deproteinization procedures for handling various physiological fluids have been reviewed by Deyl *et al.* [6]. Deproteinization of plasma or serum should be carried out within 30 min after sample collection [6]. Deproteinized supernatants should be analyzed as soon as possible or stored at low temperatures (−20 to −40°C). The deproteinized samples should be stored at −68°C or lower if there is likely to be a long delay before analysis [6,29].

Methods for deproteinization of plasma or serum include precipitation with acids or organic solvents, ultrafiltration and ultracentrifugation [10]. The last method is cumbersome and, consequently, rarely used. Ultrafiltration is a new technique that has been used for deproteinizing a wide variety of samples prior to analysis by liquid chromatography. However, this method has had only limited success in amino acid analysis [6]. Sample pretreatment (including acidification with HCl) and selection of appropriate membrane are important in obtaining recoveries of 90% or higher [10]. Moreover, ultrafiltration was found unsuitable for lipemic plasma samples due to the time required to filter sufficient sample, and occasionally no filtrate could be obtained, presumably due to blockage of membrane pores [9].

5-Sulphosalicylic acid (SSA) is the most common precipitant used for plasma samples, where-

as perchloric, trichloroacetic (TCA) and picric acids are also used for tissue samples [29]. SSA appears to be a desirable deproteinization reagent for separation of amino acids by IEC [6]. The filtrate obtained after SSA deproteinization has a pH of 1.0–2.0, which is ideal for subsequent IEC without the removal of SSA from the filtrate. Although widely different SSA-to-plasma ratios have been used, deproteinization with 100 mg of solid acid per ml of plasma was recommended for obtaining precise results. It was found necessary to wash the precipitate at least twice with sodium acetate buffer (pH 2.2) for obtaining accurate results. If the amount of SSA added to the column exceeds 200 mg, the resolution in the region of threonine and serine can be adversely affected [29]. Picric acid is sometimes preferred though others have found little difference between the use of picric acid, SSA and TCA. The use of acid precipitation reagents leads to over-estimation of free tryptophan due to the release of protein-bound tryptophan from the plasma albumin.

The effects of various deproteinization agents on the levels of free amino acids in human plasma have been investigated [19]. Six precipitation reagents [30% SSA; 1 *M* perchloric acid; 20% trifluoroacetic acid (TFA); acetonitrile; ethanol; and acetone] were studied. Free amino acids in deproteinized plasma were determined by reversed-phase chromatography of automatic precolumn derivatives with OPA–mercaptoethanol. Among the various deproteinization reagents, the use of SSA and perchloric acid gave similar results with high recoveries for most amino acids (Table 4). The variation in recovery was large

TABLE 4

EFFECTS OF VARIOUS DEPROTEINIZING AGENTS ON CONCENTRATIONS OF FREE AMINO ACIDS IN PLASMA OF HEALTHY SUBJECTS

Data are from Qureshi and Qureshi [19] with permission. Abbreviations: SSA = 5-sulphosalicylic acid; TFA = trifluoroacetic acid.

Amino acid	Concentration (mean ±, S.D., n = 8) (μmol/l)					
	SSA	Perchloric acid	TFA	Acetonitrile	Ethanol	Acetone
Arginine	118 ± 27	101 ± 21	105 ± 26	89 ± 21	93 ± 25	82 ± 20
Histidine	93 ± 11	92 ± 10	87 ± 11	74 ± 8	79 ± 12	73 ± 11
Isoleucine	72 ± 16	74 ± 9	68 ± 12	72 ± 11	70 ± 9	71 ± 12
Leucine	116 ± 29	163 ± 25	165 ± 25	142 ± 21	152 ± 27	137 ± 26
Lysine	223 ± 38	212 ± 33	202 ± 38	199 ± 33	201 ± 36	187 ± 24
Methionine	29 ± 5	29 ± 4	28 ± 4	27 ± 5	25 ± 4	27 ± 5
Phenylalanine	66 ± 13	64 ± 10	65 ± 11	66 ± 12	63 ± 10	62 ± 11
Tyrosine	69 ± 8	59 ± 9	51 ± 10	63 ± 11	62 ± 11	51 ± 11
Threonine	121 ± 19	123 ± 17	114 ± 21	118 ± 18	116 ± 21	111 ± 19
Valine	268 ± 41	260 ± 36	217 ± 27	232 ± 31	257 ± 31	230 ± 33
Alanine	396 ± 37	400 ± 42	406 ± 32	366 ± 36	382 ± 39	372 ± 31
Asparagine	68 ± 7	68 ± 6	63 ± 8	56 ± 9	62 ± 7	49 ± 9
Aspartic acid	13 ± 4	12 ± 3	10 ± 4	18 ± 6	17 ± 4	20 ± 5
Glutamic acid	33 ± 5	32 ± 4	33 ± 4	47 ± 6	40 ± 4	49 ± 5
Glutamine	498 ± 40	482 ± 37	483 ± 49	453 ± 48	440 ± 43	436 ± 46
Glycine	232 ± 26	236 ± 31	215 ± 27	219 ± 32	230 ± 32	214 ± 28
3-Methylhistidine	8 ± 4	8 ± 4	6 ± 3	6 ± 4	5 ± 3	5 ± 3
Ornithine	54 ± 7	48 ± 6	47 ± 6	51 ± 5	53 ± 6	50 ± 6
Serine	92 ± 12	92 ± 11	87 ± 12	83 ± 9	83 ± 10	80 ± 12
Taurine	60 ± 5	58 ± 3	55 ± 5	48 ± 5	48 ± 5	37 ± 4

with organic solvents. Only threonine, phenylalanine, isoleucine and ornithine were stable in all precipitating agents. The degradation of asparagine and glutamine was lower in perchloric acid and SSA than in organic solvents. In this study, however, several nutritionally important amino acids such as cysteine/cystine, proline and tryptophan were not determined.

In several other publications on HPLC procedures, there was a clear preference for organic solvents as deproteinizing reagent instead of SSA which is widely used prior to analysis with the automatic analyzer [41]. Organic solvents such as methanol, acetone and acetonitrile are commonly used as protein precipitants in biological fluids. At neutral pH, all of the organic solvents work well, but under the acidic conditions, methanol and to some degree acetone are inefficient [9]. Acetonitrile works well under the acidic conditions forming a firm pellet of protein precipitate and leaving a clear supernatant. However, with a large excess of acetonitrile, PITC reacts to form a derivative with a retention time similar to that of citrulline. This does not occur if acetonitrile is evaporated prior to derivatization.

The desirability of using organic solvents as deproteinizing reagents instead of SSA in HPLC studies may represent a more recent awareness of the disadvantages associated with the use of SSA. A major problem of this method of deproteinization is the interference of SSA with aspartic acid and glutamic acid [42,43]. Deproteinization with SSA and other acids has been reported to result in sharply reduced yield of many PTC amino acids [10,43]. Aristoy and Toldra [43] recently compared various deproteinization techniques including chemical precipitants (TCA, perchloric acid, SSA, phosphotungstic acid, picric acid, and acetonitrile) and ultrafiltration through 10 000 and 1000 molecular mass cut-off membranes for free amino acid analysis in fresh pork muscle and dry-cured ham. PTC amino acids were analyzed by reversed-phase HPLC. The use of SSA, phosphotungstic acid, and ultrafiltration through a 1000 molecular mass cut-off membrane resulted in important losses of some amino acids from a standard amino acid solution (Table 5). The use of the other deproteinization methods, however, gave amino acid recoveries of more than 90%, except poor recovery of tryptophan with the use of PCA. Similar effects of the deproteinization techniques were also noted for both meat and dry-cured ham samples. However, an interfering peak coeluting with arginine was observed. SSA gave poor recoveries of aspartic acid, glutamic acid, histidine, arginine, valine, isoleucine, leucine, and valine and abnormally high recoveries of proline and glycine. Phosphotungstic acid gave especially low recoveries of lysine, arginine, ornithine, aspartic acid, asparagine, glutamine and tryptophan. Similarly, there were important losses of ornithine, lysine, histidine and arginine (basic amino acids) when the 1000 molecular mass cut-off membrane filter was used.

Several publications do not report details of sample preparation and deproteinization. Therefore, caution should be exercised in interpreting the results of such studies, particularly in light of the comprehensive preparation procedures recommended by Deyl *et al.* [6] for handling various physiological fluids.

In normal situations, the low level (about 1%) of urinary protein does not interfere with amino acid analysis. In a pathological situation, deproteinization of urine samples with solid SSA (50-100 mg of solid per ml of urine) is recommended for subsequent analysis by IEC [6].

5. RAPID AND COMPLETE AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES BY LIQUID CHROMATOGRAPHY OF PRECOLUMN PHENYLISOTHIOCYANATE DERIVATIVES

The use of PITC or Edman's reagent for amino acid analysis was first described in detail by Heinrickson and Meredith [26] and developed commercially by Waters (Milford, MA, USA) as the Pico-Tag method [44]. This method was recently reviewed [10].

PITC reacts with free amino acids to yield PTC amino acids, which can then be separated on a special reversed-phase HPLC column. The PTC amino acids have a strong ultraviolet absorption. Therefore, picomole quantities of amino acids

TABLE 5

RECOVERY OF STANDARD AMINO ACIDS AFTER DIFFERENT DEPROTEINIZATION PRETREATMENTS

Reproduced from Aristoy and Toldra [43] with permission. Abbreviations: ACN = acetonitrile; PCA = perchloric acid; TCA = trichloroacetic acid; PA = picric acid; SSA = 5-sulphosalicylic acid; PTA = phosphotungstic acid.

Amino acid	Recovery (%)							
	Chemical precipitants						Ultrafiltration	
	ACN	PCA	TCA	PA	SSA	PTA	10 000	1000
Arginine	99	91	92	100	83	35	103	70
Histidine	100	97	92	100	90	93	100	55
Isoleucine	99	98	98	100	89	96	102	99
Leucine	100	99	97	96	84	102	104	98
Lysine	99	100	97	102	88	10	99	52
Methionine	101	104	106	105	107	91	99	100
Phenylalanine	99	99	99	106	93	98	100	101
Tyrosine	102	93	98	101	96	103	103	105
Threonine	98	97	96	100	98	95	98	95
Tryptophan	–	73	93	101	97	84	98	103
Valine	98	97	98	100	89	98	99	99
Alanine	101	104	104	102	96	100	101	99
Asparagine	97	101	102	102	101	71	99	95
Aspartic acid	98	99	97	98	70	53	97	97
Glutamic acid	99	99	99	98	73	78	98	99
Glutamine	97	99	96	101	92	81	98	94
Glycine	100	104	104	103	113	96	101	106
Ornithine	97	95	94	–	96	39	101	34
Proline	100	104	108	104	150	104	99	102
Serine	101	98	94	99	95	92	101	99
Taurine	101	–	–	–	–	–	99	102

can be detected at a single wavelength (254 nm). Since PITC forms the same chromophore with primary and secondary amines, no additional procedures are necessary for analyzing secondary amino acids.

The derivatization method is not suitable for automation, but most of the manual work before automatic feeding into an HPLC column (*i.e.* derivatization, removal of excess reagent, etc.) for a batch of hydrolysed samples can be done in about 60 min. The PTC amino acid derivatives are separated by reversed-phase gradient elution liquid chromatography (typically in less than 20 min), and then detected by their UV absorbance.

The stability of PTC amino acids is controlled by both temperature and pH [10]. At ambient temperature, maximum stabibility in the pH range 5.0–7.5 is at pH 7.5. In solution, at optimum pH, the loss of derivatives is in the range 0–10% in 10 h (only isoleucine, valine and cystine loose more than 5%). This modest loss can be controlled by refrigeration at 4°C, degradation thus being limited to less than 5% in 48 h. Storage of dry derivatives at −20°C and under reduced pressure has been successful for as long as one month.

Since the PTC amino acid derivatives have been shown to be stable for at least 32 h at ambient temperature [42], a large number of samples (about 60) can be analyzed per 24-h work day.

The PITC derivatization method has been used extensively for the analysis of amino acids in hydrolysates of pure proteins and peptides because of its sensitivity, reproducibility and speed, espe-

cially when compared to traditional ion-exchange amino acid analysis techniques. With some minor modifications in sample preparation and chromatography, this method has been used in the determination of amino acids in foods and feces [13,45–48].

The use of a modified PITC derivatization method [48] enabled the accurate and rapid determination (12 min) of methionine (as methionine sulfone), cystine/cysteine (as cysteic acid) and all other amino acids except tryptophan in hydrolysates of foods and feces. The PTC derivatives of all amino acids in the hydrolysate were well resolved, and the separation was complete in 12 min (Fig. 1). Similarly, the separation of cysteic acid and methionine sulfone (the more stable forms of the sulfur amino acids) in the performic acid plus 6 *M* HCl hydrolysate was good (Fig. 2). The separation of the cysteic acid–aspartic acid–glutamic acid triplet can be further improved by lowering the pH of eluent A (composition of eluent A is given in the legend to Fig. 1) from 6.4 to 5.9 (chromatogram not shown). This method also permitted determination of ammonia (needed for the calculation of amino acid nitrogen). Tryptophan in an alkaline hydrolysate of foods and feces was determined by a simple liquid chromatographic method requiring no derivatization in about 8 min (Fig. 3).

Four samples of a milk-based infant formula were individually hydrolyzed, derivatized, and analyzed to estimate the variation of the entire liquid chromatographic method [48]. Moreover, four replicates of the same derivatized hydrolysate were analyzed to estimate the variation of the chromatography. The variations expressed as C.V. of the entire method (including hydrolysis, derivatization and chromatography) and of chromatography for determining all amino acids were not more than 4 and 2%, respectively. The reproducibility data for the HPLC method obtained in this investigation compared favourably with those obtained for the ion-exchange procedures.

Hagen *et al.* [49] described a precolumn PITC

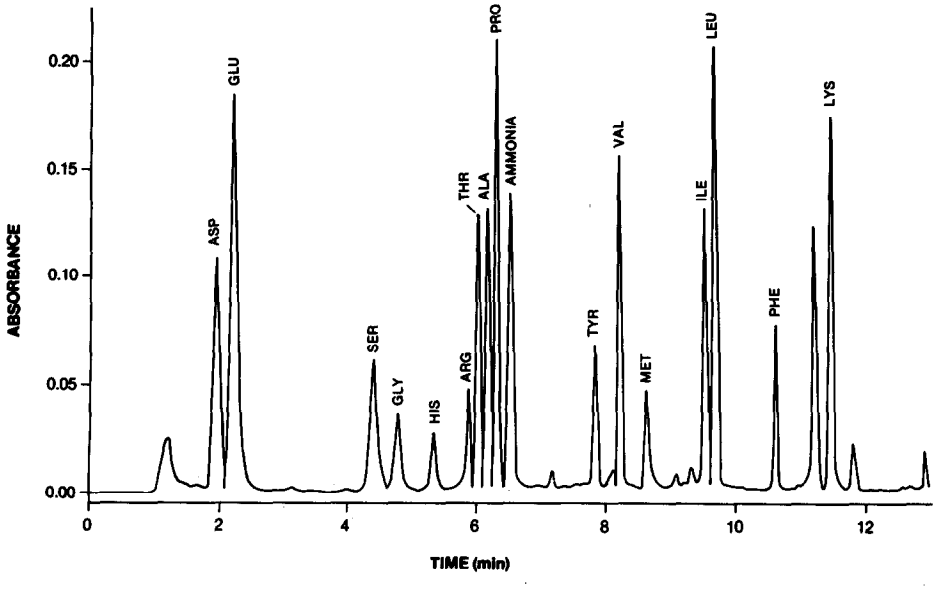

Fig. 1. Chromatogram of all PTC amino acids, except tryptophan, in 6 *M* HCl hydrolysate of milk-based infant formula (from ref. 48 with permission). Eluent A was 0.14 *M* sodium acetate, 0.05% triethylamine, pH 6.40, containing 6% acetonitrile. Eluent B was 60% acetonitrile in water. Gradient: elution was started at 1 ml/min and held at 100% eluent A for 0.5 min. At this time, a convex curve (curve 5) was used to increase eluent B to 46% over the next 9.5 min. Eluent B was then increased linearly to 100% over 0.5 min and maintained for 1.5 min. The column was then equilibrated with eluent A at 1.5 ml/min for 7.5 min. Total analysis time was 20 min. The column was a 150 mm × 3.9 mm I.D. Pico-Tag column which was maintained at 38°C.

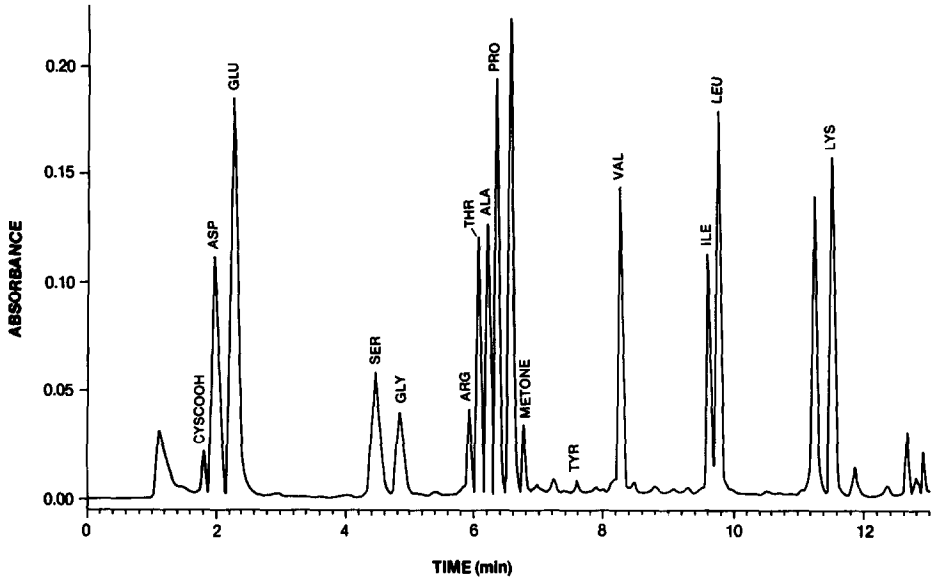

Fig. 2. Chromatogram of PTC cysteic acid (CYSCOOH) and methionine sulfone (METONE) in performic acid plus 6 *M* HCl hydrolysate of milk-based infant formula (from ref. 48 with permission). The chromatographic conditions were the same as noted in the legend to Fig. 1.

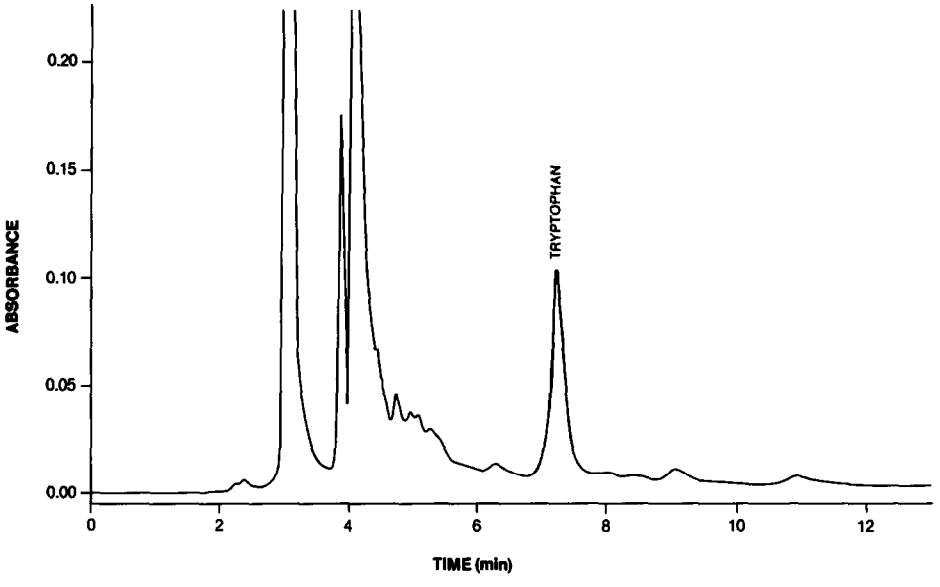

Fig. 3. Chromatogram of tryptophan in 4.2 *M* NaOH hydrolysate of soy-based infant formula (from ref. 48 with permission). Eluent A was 0.01 *M* sodium acetate, pH 5.8, containing 10% acetonitrile. Eluent B was 60% acetonitrile in water. Tryptophan was eluted isocratically at 1 ml/min with 100% eluent A for 10 min. The column was then washed for 2 min with eluent B and subsequently equilibrated with eluent A at 1.5 ml/min for 10 min. Total analysis time was 22 min. The column was a Waters μBondapak C_{18} column (100 mm × 3.9 mm I.D.) which was maintained at 25°C.

method for the determination of amino acids in protein hydrolysates from a wide variety of recipe-prepared foods such as a mixed beef dish, salads, sauces, casseroles, deserts, etc. Duplicate samples of each food were analyzed. The C.V. between duplicate analyses were less than 5% for most amino acids and never greater than 15%. The C.V. between duplicate injections were always less than 5%. Recovery of amino acids from the Pierce H standard added to a canned beef recipe, that was subjected to 6 *M* HCl hydrolysis, averaged 106% (excluding cystine that was not recovered). When the Pierce H standard was hydrolysed with HCl and analyzed, recovery averaged 96% (excluding cystine which was not recovered). Recovery of methionine sulfone, cysteic acid, methionine and cystine added to samples that were oxidized, hydrolyzed with HCl and analyzed averaged 105, 105, 96 and 108%, respectively. While all the recipes were analyzed after freeze-drying (to ensure adequate homogenization and preservation, two recipes (chocolate pudding and sweet and sour pork) were also analyzed before freeze-drying. There were no significant differences in amino acid content between the wet and dry samples. The amino acid contents, as determined by the PITC method, agreed well with published values when normalized for differences in protein content.

As mentioned earlier, analysis times for protein hydrolysates with the PITC method are very short: typically only a 12-min run time required with an 8-min equilibration, which may be increased to 13 min if required [45]. The IEC system, however, requires run times of 75 min (*i.e.* more than six times longer than the PITC analysis time), followed by a regeneration and equilibrium period of about 13 min. The chromatography obtained with the PITC method is excellent, with clear advantages over that produced from IEC. Most notable features, in addition to shorter analysis times, are excellent baseline stability, very good resolution and sharper peaks with very consistent retention times, which allow automatic peak integration and calculation. In the original PITC technique, the use of the Sep-Pak system for sample clean-up was recommended. More recently, however, it was demonstrated that wrong results can be obtained after using this system [45].

The PITC derivatization method has been used to determine the amino acid composition of a variety of foods with both speed and accuracy. Results agree well with the literature and with those obtained by conventional IEC.

6. RAPID ANALYSIS OF NUTRITIONALLY IMPORTANT FREE AMINO ACIDS IN BLOOD PLASMA (SERUM) AND OTHER PHYSIOLOGICAL FLUIDS BY LIQUID CHROMATOGRAPHY OF PRECOLUMN PHENYLISOTHIOCYANATE DERIVATIVES

The development of rapid, accurate and reproducible liquid chromatographic methods using precolumn PITC derivatization for determining amino acids in protein hydrolysates provided the impetus for its application to physiological samples such as plasma (serum) liver, heart and brain. Amino acid analysis of plasma (serum) and organs is of ongoing interest to research centres conducting nutritional studies.

A 15-cm-long amino acid analysis column is normally used for determining amino acids in protein hydrolysates. A longer (25–30 cm) and more expensive column is, however, required for better resolution of all amino acids in physiological fluids. For example, the separation of 23 PTC amino acids from physiological samples was achieved in 80 min by using a 25-cm-long column [50]. Similarly, 40 PTC amino compounds were separated in about 66 min by using a 30-cm-long column [10]. Several of the amino compounds separated using the longer columns have limited nutritional significance. Therefore, a rapid method for determining nutritionally important amino acids in physiological samples was developed [51–53].

Early and Ball [51] reported separation of plasma amino acids using the faster-eluting amino acid analysis column (15 cm) used for protein hydrolysates. They demonstrated good separation of 17 PTC amino acids, but were unable to achieve adequate separation of some of the common protein amino acids such as glutamic acid,

glycine, glutamine, serine and tryptophan (an essential amino acid).

Sarwar and Botting [52] modified the PITC method for protein hydrolysates for its application in rapid analysis of nutritionally important free amino acids in serum and tissues such as liver, brain and heart. By using a 15-cm-long amino acid analysis column, separation of 27 PTC amino acids in physiological samples was achieved in 20 min by the modified method. The standard containing 27 amino acids revealed good separation of each amino acid (Fig. 4). Similarly, satisfactory resolution of all amino acids in human serum (Fig. 5), rat liver (Fig. 6), rat brain (Fig. 7), and rat heart (Fig. 8) was achieved. Total time for analysis and equilibration was 30 min. The modified method was much faster than the traditional ion-exchange methods (2–3 h) or the other liquid chromatographic methods using PITC derivatization (66–80 min) for determining nutritionally important free amino acids in plasma and tissues.

To estimate the variation of the liquid chromatographic method, four samples of rat serum and seven samples of pig liver were individually analyzed (Table 6). The variation, expressed as C.V. of the entire method, including deproteinization, derivatization and chromatography, for all amino acids was less than 5% in both the samples. To obtain information on the efficiency of the method, a sample of rat serum or pig liver was analyzed with or without added standard. The average values for the recovery of amino acids in serum and liver samples were 100 and 97%, respectively.

Fierabracci *et al.* [53] reported another rapid

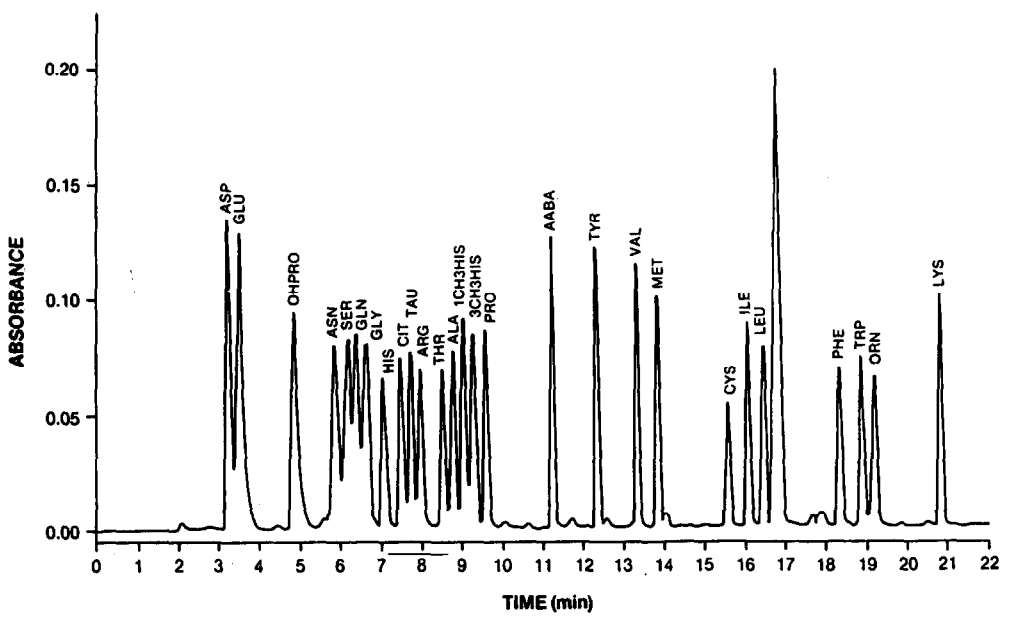

Fig. 4. Elution profile of a 27-amino acid standard showing resolution of each PTC amino acid by liquid chromatography (from ref. 52 with permission). The standard amino acid mixture consisted of 150 nmol/ml of each amino acid except for cystine, 75 nmol/ml. The compositions of eluents A and B were the same as noted in the legend to Fig. 1. Gradient: elution was commenced at 0.5 ml/min with 100% eluent A. A convex curve (curve 5) was used to increase eluent B to 10% over 6 min. The flow-rate was increased linearly to 1 ml/min from 6 to 7 min. Then curve 4 was used to increase eluent B to 25% over the next 8 min. The eluent composition and flow-rate were held at these levels for the next 2.5 min. Between 17.5 and 20.9 min, both flow-rate and eluent B were increased linearly to 1.6 ml/min and 40%, respectively. Eluent B was then increased linearly to 100% over the next 0.5 min and held at the level for 2.4 min. The column was then returned to 100% eluent A and equilibrated for 6.2 min. A Pico-Tag amino acid analysis column (150 mm × 3.9 mm I.D.) was used and maintained at 47.5°C. OHPRO = Hydroxyproline; AABA = α-aminobutyric acid; 1CH3HIS and 3CH3HIS = 1- and 3-methylhistidine.

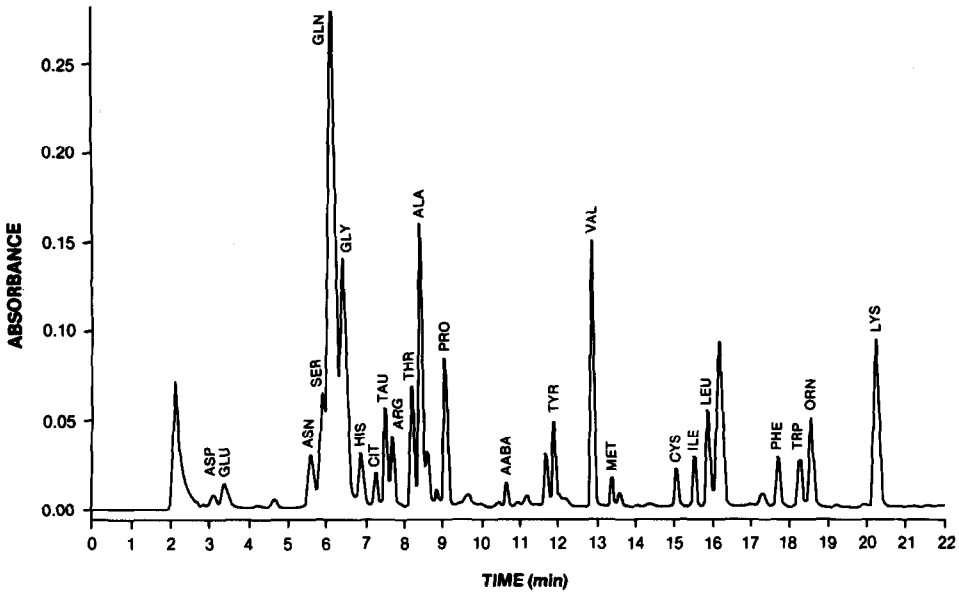

Fig. 5. Elution profile of human serum showing resolution of PTC amino acids by liquid chromatography (from ref. 52 with permission). The chromatographic conditions were the same as noted in the legend to Fig. 4.

method for amino acid analysis of biological samples, by reversed-phase HPLC after precolumn derivatization with PITC. Preparation of samples included deproteinization with 3% SSA and careful removal under high vacuum of residual PITC. A 15-cm-long column was used, immersed in a water-bath at 38°C. In rat or human plasma, separation of 23 individual amino acids

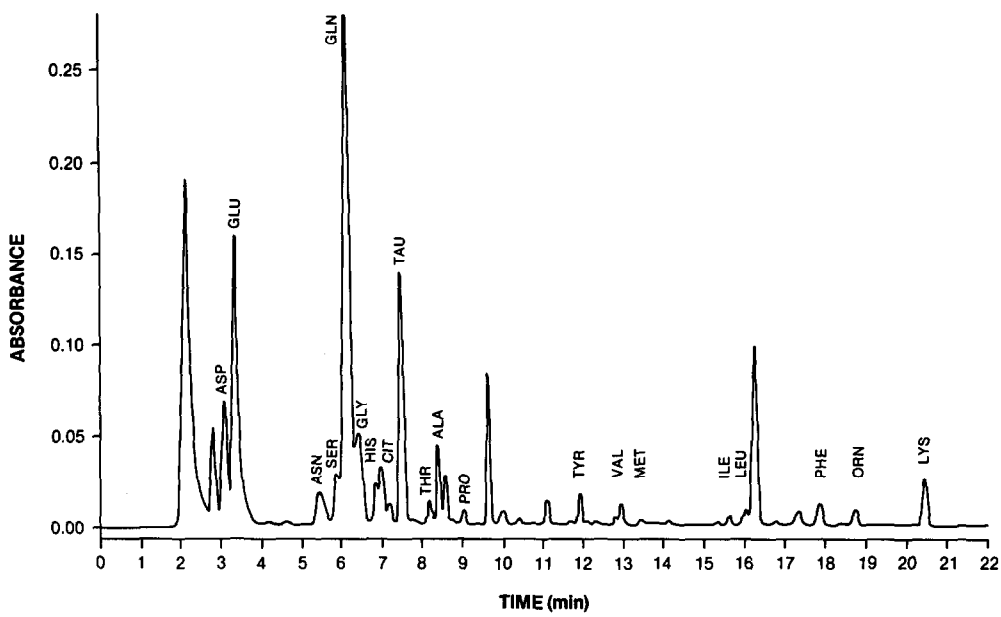

Fig. 6. Elution profile of rat liver showing resolution of PTC amino acids by liquid chromatography (from ref. 52 with permission). The chromatographic conditions were the same as noted in the legend to Fig. 4.

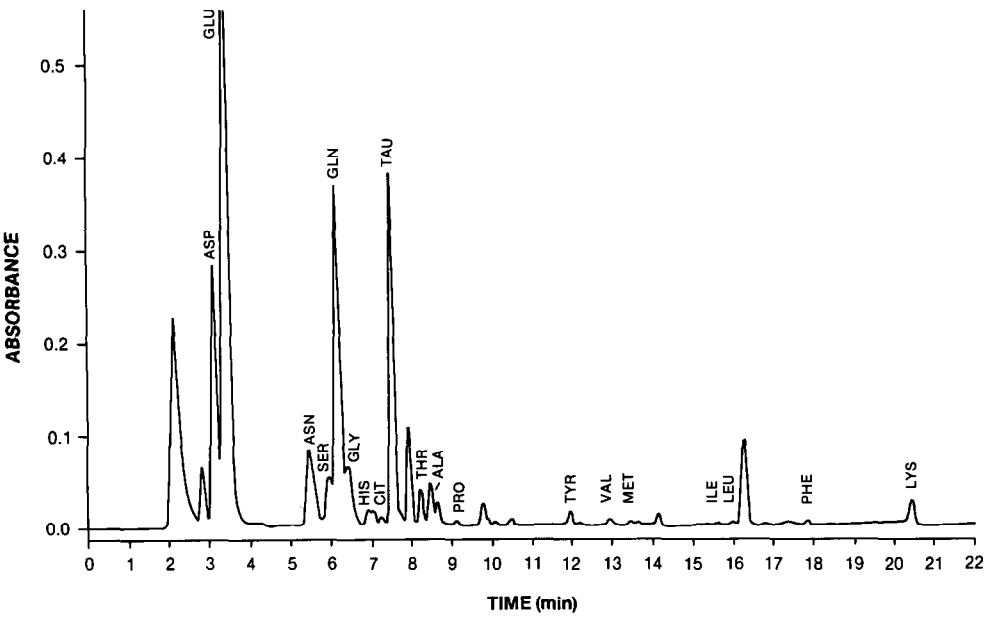

Fig. 7. Elution profile of rat brain showing resolution of PTC amino acids by liquid chromatography (from ref. 52 with permission). The chromatographic conditions were the same as noted in the legend to Fig. 4.

plus the unresolved pair of tryptophan and ornithine, was achieved within 13 min. Including the time for column washing and reequilibration, samples could be chromatographed at 23-min intervals. The C.V. for retention times and peak areas for each amino acid were on the average less than 1 and 4%, respectively. The linearity for each standard amino acid was remarkable over

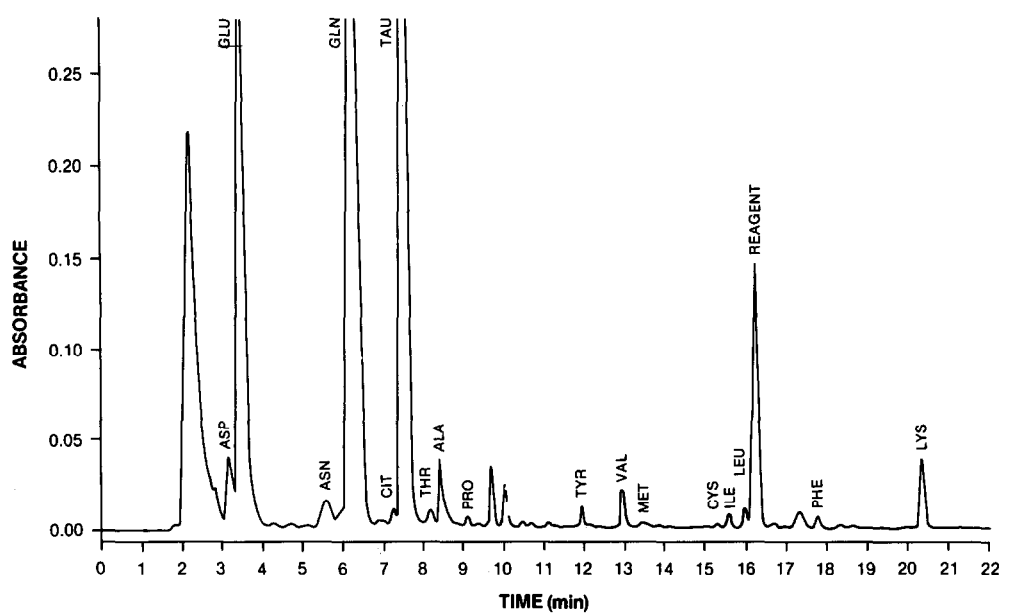

Fig. 8. Elution profile of rat heart showing resolution of PTC amino acids by liquid chromatography (from ref. 52 with permission). The chromatographic conditions were the same as noted in the legend to Fig. 4.

TABLE 6

CONCENTRATIONS OF FREE AMINO ACIDS IN RAT SERUM AND PIG LIVER AS DETERMINED BY LIQUID CHROMATOGRAPHY OF PRECOLUMN PHENYLISOTHIOCYANATE DERIVATIVES

Abstracted from Sarwar and Botting [52].

Amino acid	Rat serum (n = 4)		Pig liver (n = 7)	
	Concentration (mean ± S.D.) (μmol/100 ml)	C.V. (%)	Concentration (mean ± S.D.) (nmol/mg freeze-dried tissue)	C.V. (%)
Arginine	11.9 ± 0.10	0.8	5.3 ± 0.13	2.4
Citrulline	11.9 ± 0.10	0.8	1.2 ± 0.02	1.7
Histidine	11.4 ± 0.14	1.2	28.4 ± 1.02	3.6
Isoleucine	8.7 ± 0.15	1.7	12.7 ± 0.31	2.4
Leucine	8.0 ± 0.08	1.0	31.2 ± 0.88	2.8
Lysine	24.8 ± 0.26	1.0	5.0 ± 0.20	4.0
Methionine	7.6 ± 0.20	2.6	9.1 ± 0.30	3.3
Cystine	–	–	0.9 ± 0.02	2.2
Phenylalanine	5.2 ± 0.15	2.9	12.3 ± 0.30	2.4
Tyrosine	8.0 ± 0.05	0.6	11.4 ± 0.50	4.4
Threonine	30.3 ± 0.22	0.7	15.9 ± 0.75	4.7
Tryptophan	9.8 ± 0.14	1.4	3.0 ± 0.09	3.0
Valine	11.2 ± 0.05	0.4	19.9 ± 0.55	2.8
Alanine	88.3 ± 0.88	1.6	76.4 ± 1.46	1.9
Asparagine	5.4 ± 0.14	2.6	21.5 ± 0.46	2.1
Aspartic acid	3.0 ± 0.08	2.6	11.3 ± 0.21	1.8
Glutamic acid	18.2 ± 0.06	0.3	55.6 ± 1.08	1.9
Glutamine	68.6 ± 1.54	2.2	29.0 ± 1.06	3.6
Glycine	34.1 ± 0.42	1.2	79.9 ± 3.57	4.5
Hydroxyproline	5.6 ± 0.13	2.3	1.4 ± 0.04	2.8
Ornithine	13.8 ± 0.40	2.9	20.8 ± 0.80	3.8
Proline	24.5 ± 0.60	2.4	18.8 ± 0.30	1.6
Serine	38.6 ± 0.45	1.2	52.5 ± 2.28	4.3
Taurine	27.2 ± 0.24	0.8	16.7 ± 0.80	4.8

the concentration range 30–50 nmol/ml. The mean recovery of amino acid standards added to plasma prior to derivatization was 97 ± 0.8% except for aspartate (82%) and glutamate (81%). The lower recovery of aspartate and glutamate could be explained by the use of SSA as the deproteinization agent. This is the most rapid method with satisfactory precision, sensitivity and reproducibility for most free amino acids in biological fluids and tissue homogenates [53]. The drawback of this method is that it involves a lengthy drying step to remove the excess derivatization reagents under vacuum (90–120 min). Moreover, tryptophan and ornithine are not properly resolved by this method. Accurate determination of tryptophan, an essential amino acid, is critical in nutritional studies.

7. COMPARISON OF AMINO ACID RESULTS BY REVERSED-PHASE HPLC AND ION-EXCHANGE METHODS

Several workers have compared amino acid results obtained by ion-exchange and reversed-phase HPLC of precolumn PTC derivatives [9,44,45,48,54]. These comparisons included both

protein hydrolysate and deproteinized plasma samples. When comparable hydrolytic conditions were used, results obtained with the HPLC method were generally in good agreement with those obtained by the ion-exchange method.

Sarwar *et al.* [48] compared amino acid profiles of protein hydrolysates of an infant formula and a sample of rat feces (Table 7) as determined by HPLC and ion-exchange methods. The differences for most amino acids due to the analytical procedures were less than 10%. Similar observations about agreement of the two methods for determining amino acids in protein hydrolysates of food/feed samples have been reported by others [44,45,49]. Feste [54] and Davey and Ersser [9] compared plasma acid profiles determined by reversed-phase chromatography of PTC amino acid derivatives and by IEC. The mean concentrations and ranges (for most amino acids) determined by each method were similar. Values for most amino acids, as determined by the two methods, had correlation coefficients of greater than 0.8. Several nutritionally important amino acids such as cystine, threonine, tryptophan, glutamic acid, proline and serine were, however, not studied by Feste [54].

TABLE 7

COMPARISON OF AMINO ACID PROFILES (g/16 g NITROGEN) DETERMINED BY TWO ANALYTICAL PROCEDURES

Reproduced from Sarwar *et al.* [48] with permission.

Amino acid	Milk-based formula		Rat feces	
	Ion exchange	HPLC[a]	Ion exchange	HPLC[a]
Arginine	3.18	3.35	3.86	4.02
Histidine	2.16	2.32	1.60	1.81
Isoleucine	5.84	5.62	4.38	4.43
Leucine	10.32	10.46	5.17	5.20
Lysine	7.49	7.40	4.67	4.40
Methionine	1.79	1.74	2.18	2.03
Cystine	1.52	1.48	2.19	2.10
Phenylalanine	4.00	4.13	2.48	2.62
Tyrosine	3.87	4.35	2.74	2.62
Threonine	5.76	5.56	4.20	4.63
Valine	6.24	6.14	4.85	4.58
Alanine	4.19	4.35	4.68	4.86
Aspartic acid	9.34	9.22	9.86	9.57
Glutamic acid	19.20	19.70	13.36	12.42
Glycine	1.95	1.92	3.75	3.74
Proline	8.03	8.32	3.30	3.58
Serine	5.50	5.54	6.38	5.86
Ammonia	2.10	2.28	3.00	2.96

[a]The amino acids were analyzed as PTC derivatives. The chromatographic conditions are described in the legend to Fig. 1.

The results of collaborative studies concerning the intra- and inter-laboratory variations in amino acid analysis of protein hydrolysates by IEC were recently summarized [5]. Intra- and inter-laboratory variations (expressed as C.V.) for most amino acids in foods determined by IEC of 6 *M* HCl hydrolysates were reported to be less than 3 and 10%, respectively [36,55]. The inter-laboratory variations for tryptophan (up to 24%), cystine (up to 18%) and methionine (up to 16%) were, however, large. The relatively high inter-laboratory variations for tryptophan, cystine and methionine may be due to the failure to follow the same detailed conditions for preparation of hydrolysate (4.2 *M* NaOH for tryptophan and performic acid plus 6*M* HCl for sulfur amino acids) by the participating laboratories. Inter-laboratory variation due to chromatographic or analytical measurements is considerably lower than that produced by hydrolysate preparation in different laboratories [28]. Improvements in inter-laboratory variation for tryptophan (C.V. = 4–16%), cystine (C.V. = 7–17%) and methionine (C.V. = 5–12%) in food and feed ingredients were noted by meticulous attention to details of prechromatographic handling of samples, including preparation of hydrolysate [35].

The precision of the IEC in determining the amino acid content of blood plasma from a preruminant and a ruminant calf was studied in a collaborative study at four laboratories [56]. Samples of plasma were deproteinized using SSA by the organizing laboratory and by the participating laboratories using their own methods. The within- and between-laboratory variations for most amino acids in deproteinized plasma samples were considerably larger than those reported previously for protein hydrolysates. The wider range of amino acid concentrations and the prob-

lem of peak resolution caused by the presence of many more amino acids and related compounds in plasma than in protein hydrolysates were suggested as contributory causes for the poor resolution of amino acids in the plasma samples. Moreover, the preparatory steps prior to analysis were found to have a major influence on the precision of the analysis. Further research on the deproteinization of physiological fluids was recommended.

Although the inter-laboratory variability of the HPLC methods has not been determined, the intra-laboratory variation of the PITC derivatization method was found to be similar to that of ion-exchange. Several investigators have estimated the within-laboratory variation of the reversed-phase chromatography of PTC amino acids in protein hydrolysates and physiological samples. Variation expressed as C.V. for the determination of all amino acids in protein hydrolysates and physiological samples such as rat serum and pig liver was less than 5% [48,52]. Similarly, a within-laboratory variation of 2.1–5.5% for amino acid analysis of a meat and bone meal hydrolysate was reported by Bidlingmeyer *et al.* [13]. The intra-assay variability of the HPLC method (C.V. of less than 8% for all amino acids except 20% for methionine, 16% for citrulline and 14% for hydroxyproline) compared favourably with that of the ion-exchange method in analyzing free amino acids in plasma (Table 8). The inter-assay (or within-laboratory) variability of the method was less than 10% for all amino acids except methionine, glutamic acid, hydroxyproline and ornithine. In the case of the latter amino acids, the variability ranged from 11 to 14% (Table 8).

Feste [54] determined the within-run and between-run precisions of concentrations of human plasma and urinary PTC amino acid derivatives. The within-run range of precision for plasma amino acids was 1.6 to 17.0%, and for urinary amino acids was 4.0 to 115.0% (Table 9). The errors for urinary amino acids was especially high for ornithine (115%), methionine (91%) and proline (56%). These may be due to the low amounts of these amino acids (3.0–4.6 μmol/l).

TABLE 8

COEFFICIENTS OF VARIATION FOR PLASMA AMINO ACIDS DETERMINED BY HPLC AND ION-EXCHANGE METHODS

Reproduced from Davey and Ersser [9] with permission.

Amino acid	Coefficient of variation (%)		
	Intra-assay		Inter-assay
	HPLC (n = 11)	Ion-exchange (n = 18)	HPLC (n = 18)
Arginine	3.2	7.4	5.8
Histidine	4.8	1.9	N.D.
Isoleucine	2.1	0.4	9.9
Leucine	0.5	0.4	7.0
Lysine	1.4	1.5	5.6
Methionine	20.5	4.5	12.2
Cystine	N.D.	N.D.	N.D.
Phenylalanine	1.5	1.4	6.0
Tyrosine	1.5	0.8	6.7
Threonine	1.7	2.5	5.3
Tryptophan	6.5	2.6	9.1
Valine	5.4	1.2	4.7
Alanine	1.2	3.8	5.4
Asparagine	3.1	8.1	4.9
Aspartic acid	N.D.	N.D.	N.D.
Citrulline	15.7	18.7	N.D.
Glutamic acid	1.1	1.2	10.9
Glutamine	3.5	8.0	5.4
Hydroxyproline	14.3	N.D.	14.5
Ornithine	3.9	0.9	11.6
Proline	5.5	7.9	5.9
Serine	2.2	1.2	4.0
Taurine	5.5	5.3	7.8

The errors for other amino acids, except these three, were 4.8–18.6% (Table 9). The between-run (or within-laboratory) precision ranges for plasma amino acids were 2.0–15.3% (Table 9). Of the 21 urinary amino acids reported, 7 had between-run errors ranging from 5.7 to 10.0%. Isoleucine, methionine, ornithine, threonine and proline had between-run errors that ranged from 31.6 to 129%. All of these amino acids were, however, present in concentrations lower than 11.0 μmol/l. The overall conclusion is that a laboratory skilled in the HPLC method obtains ami-

TABLE 9

WITHIN- AND BETWEEN-RUN PRECISION OF CONCENTRATIONS OF HUMAN PLASMA AND URINARY PTC AMINO ACID DERIVATIVES

Reproduced from Feste [54] with permission.

Amino acid	Coefficient of variation (%)			
	Plasma		Urine	
	Within-run (n = 10)	Between-run (n = 20)	Within-run (n = 10)	Between-run (n = 20)
Arginine	3.4	2.0	–	–
Histidine	2.6	3.1	5.1	6.4
Isoleucine	5.2	5.7	10.0	31.6
Leucine	2.2	3.4	7.3	14.2
Lysine	1.6	5.6	5.9	13.6
Methionine	4.1	10.1	91.0	61.4
Cystine	8.7	14.5	–	–
Phenylalanine	3.4	3.4	10.1	18.0
Tyrosine	3.2	4.6	7.9	7.6
Threonine	2.7	11.4	8.1	103.0
Tryptophan	1.9	3.5	18.6	14.9
Valine	–	–	14.6	12.5
Alanine	3.2	3.6	8.5	8.4
Asparagine	2.4	2.3	12.0	16.9
Aspartic acid	17.0	15.3	–	–
Glutamic acid	4.6	8.2	–	–
Glutamine	1.7	2.4	4.7	16.3
Glycine	2.7	2.6	5.1	11.5
3-Methylhistidine	–	–	4.0	5.9
Ornithine	3.1	3.3	115.0	66.1
Proline	1.9	2.0	56.3	129.0
Serine	2.7	3.2	6.3	10.8
Taurine	–	–	4.8	8.6

no acid results and precision comparable to those of the ion-exchange method.

8. CONCLUSIONS

(A) The availability of reliable methods for amino acid analysis of foods, feces and plasma is important in accurate determination of bioavailability of amino acids and quality of protein in foods, and in assessment of protein nutritional status of humans.

(B) Methods for amino acid analysis of foods were recently reviewed by a Joint FAO–WHO Expert Consultation on Protein Quality Evaluation, and were found to have been standardized to an acceptable level as measured by inter-laboratory variation of about 10%.

(C) The methods included standardized hydrolysis (4.2 M NaOH for tryptophan; performic acid plus 6 M HCl for sulfur amino acids; 6 M HCl for other amino acids) of the protein followed by separation and quantitation of the released amino acids by IEC, as adopted by AOAC.

(D) The IEC is still the main method in use. Its use is, however, being replaced by the faster and

more sensitive HPLC methods of derivatized amino acids in protein hydrolysates.

(E) Similar wide acceptance of the HPLC methods for the amino acid analysis of physiological fluids has not occurred mainly due to lack of standardized conditions for sample preparation including deproteinization and chromatographic separations.

(F) The use of different deproteinization agents (acids, organic solvents, ultrafiltration and ultracentrifugation) has been reported to have significant effects on the accuracy and precision of the analysis of free amino acids in plasma or serum.

(G) SSA appeared to be an ideal deproteinization reagent in free amino acid analysis of plasma by IEC, while organic solvents including acetonitrile were found more desirable in the analysis by HPLC.

(H) Deproteinization of physiological fluids is still considered a major problem in amino acid analysis. Therefore, its standardization deserves urgent attention.

(I) HPLC may be used to separate amino acids in protein hydrolysates and deproteinized biological samples on ion-exchange columns with postcolumn derivatization with ninhydrin or OPA or by precolumn derivatization followed by separation on reversed-phase octyl- or octadecylsilica.

(J) The simpler instrumentation for precolumn derivatization and the lower cost of such systems compared to postcolumn derivatization make the precolumn derivatization more desirable.

(K) Commercial systems based on precolumn derivatization with OPA and PITC (the two most commonly used reagents) have been developed.

(L) Despite the convenience of automated derivatization, high sensitivity and short analysis times (15 min for protein hyrolysates; 45 min for biological fluids), the precolumn derivatization with OPA has not achieved widespread acceptance in nutrition studies.

(M) The inability of OPA to directly react with proline and hydroxyproline and poor reproducibility for the determination of several nutritionally important amino acids such as lysine, cystine and glycine may be responsible for the lack of wide level of use initially predicted for the OPA-based amino acid analysis.

(N) The HPLC after precolumn derivatization with PITC has been used extensively for the analysis of all amino acids (including secondary amino acids) in hydrolysates of pure proteins and peptides, foods and feces and in deproteinized physiological samples due to its reproducibility, speed and sensitivity.

(O) The use of a PITC method enabled the accurate and rapid determination (12 min) of all amino acids except tryptophan. Tryptophan in alkaline hydrolysates was determined by a simple method requiring no derivatization in about 8 min.

(P) The PITC method for protein hydrolysates was modified for its application in rapid analysis (20 min) of 27 nutritionally important amino acids in serum and tissues such as liver, brain and heart.

(Q) The inter-laboratory variability of the PITC method has not been determined. The intra-laboratory variability of this HPLC method was, however, found to be similar to that of ion-exchange.

(R) When similar hydrolytic conditions were used in preparing protein hydrolysates, amino acid results obtained with the PITC method were generally in close agreement with those obtained by the ion-exchange method.

(S) The plasma amino acid profiles determined by reversed-phase HPLC of PTC amino acid derivatives and by IEC were also similar. There is, however, room for improvement in the analysis of amino acids in physiological samples, especially urine.

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