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Review

Advances in the high-performance liquid chromatographic determination of phenylthiocarbamyl amino acids

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ABSTRACT

Some of the general problems commonly encountered in the analysis of phenylthiocarbamyl amino acids are described. This review includes experiences associated with the preparation and storage of derivatives prior to analysis and those originating from the analytical procedure itself. The issue of the quantification of the phenylthiocarbamyl derivatives of tryptophan and cyst(e)ine, together with all other amino acids from the same hydrolysate, is also discussed. The possible reproducibility of the measurements, as a function of the conditions applied is considered.

CONTENTS

1. INTRODUCTION

Since the 1970s, the developments in high-performance liquid chromatography (HPLC) and the increased choice of reversed phases have extended its competitiveness with other chromatographic methods such as the classical ion-exchange chromatography (IEC) and gas chro-

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matography (GC) in the field of amino acid analysis. The main advantage of the IEC technique, which involves the direct elution of amino acid-containing samples, was offset in the amino acid analysis by greater time and cost requirements. HPLC also now seems to be superior to GC because in the latter instance the highly selective and necessarily quantitative derivatization procedures for all essential amino acids are time consuming methods consisting of two steps (acylation and esterification), the samples to be derivatized must be cleaned up to a much higher level than is needed in HPLC procedures and the derivatives, prior to and during their GC elution and quantification, must be present in almost water-free conditions.

1.1. Literature overview

In this paper, of the numerous possible precolumn derivatization procedures, the advances in the HPLC of amino acids as their phenylthiocarbamyl (PTC) derivatives [1–65] are followed in detail. PTC derivatives can be prepared from all essential amino acids, containing both primary and secondary amino groups, they can be obtained in quantitative yield over a wide concentration range, even at the low picomole level, they can be monitored by UV detection, before dissolution they can be stored in a refrigerator for an unlimited time, in dissolved form they are stable enough to make testing of the reproducibility of their derivatization and elution conditions possible and the automation of the procedure has been achieved, including the steps of hydrolysis, derivatization and the subsequent elution procedure.

After the first pioneering work [1], the required conditions for the precolumn derivatization method were published in 1984 [2–6]. A number of modifications and improvements have been suggested both at the basic research level [7–37], including comparative studies with other precolumn derivatization procedures for HPLC [32–37], and also in applications of the method [38–65] to the determination of free amino acids [38–47] present in various biological matrices, such as in pig plasma [38], human plasma [39,40–42,46], rat brain [41,45], urine [43] and microbially colonized sandstone [47]; the determination of the PTC derivatives of the important non-protein constituents of biological matrices, present in free forms [48,49], such as acidic opines [48], galactosamines and glucosamines [49], phosphorylated or sulphated amino acids [50], and the characteristic compounds in hydrolysates of special proteins [51–56], such as *trans*-hydroxyproline from collagen [51], hexitolamino acids from glycated proteins [52], methyl-substituted amino acids [53,54] (mono- and dimethylarginine, mono- and trimethyllsine and methylhistidine) from myelin basic protein [53], methylhistidine from actin [54], cross-linked glutamyllysine from complex biological systems, including tissue homogenates [55], and the losses of amino sugars in glycoproteins, monitoring the extent of Maillard reactions [56]; and the determination of PTC amino acids even in foods and feedstuffs [57–62] and in faeces [63]. Derivatization of the free acids in apple extract [57], in wine [58,59] and the components of hydrolysates obtained from molasses, infant formula, milk and isolated whey protein [60], peas and lentils [61], soya, sorghum, corn and peanut meals and their mixtures [62] has been reported.

2. SAMPLE PREPARATION AND STORAGE

In protein hydrolysates, amino acids to be derivatized are present in hydrochloric acid-containing solutions. Thus, after evaporating the excess of HCl acid, the samples are ready for derivatization.

Free amino acids present mostly in biological matrices are deprotonated by applying cation-exchange chromatographic clean-up [39], centrifugation [41], ultrafiltration [42] or treatment with sulphosalicylic acid [44,45], completed with centrifugation [45]. Electrochemical detection, with its higher selectivity in comparison with UV detection, makes easier and shortens the preparation of the sample; only mild and simple treatment of the sample with absolute ethanol is needed prior to derivatization [43].

2.1. Derivatization reaction

The derivatization reaction consists of two steps. The first, known as the redrying or coupling step, is performed under alkaline condi-

tions, in order to eliminate the last traces of water and to ensure that the amino groups of acids are in the "free" condition. The mainly used "coupling buffers" [1–4,6–10,12–25,27–30,32–37] contain ethanol–water–triethylamine (2:2:1, v/v/v) or (in other papers [5,11,26, 31]) acetonitrile–pyridine–triethylamine–water (10:5:2:3, v/v).

The second step, the real derivatization with phenyl isothiocyanate (PITC), is a very simple, fast and quantitative reaction requiring 5 min at ambient temperature; to be on the safe side, the suggested reaction time is 20 min. The composition of the mainly used reagent, following the coupling step with ethanol–water–triethylamine, is ethanol–triethylamine–water–PITC (7:1:1:1, v/v), and in other proposals [5,11,26,31] PITC is added to the above pyridine-containing coupling agent.

The most important and also the most time-consuming step of the derivatization procedure is the removal of the excess of PITC. For this purpose, according to the earlier work [1–6], a high vacuum is needed in supplying the end pressure of <60 mTorr (1 Torr = 133.322 Pa). This special requirement can be comfortably achieved by applying the Waters Pico Tag work station. Most workers use this apparatus as an evaporator in PTC derivatization; as an oven for protein hydrolysis it suffers from considerable limitations (see Section 2.2).

Later, in order to eliminate the excess of PITC [15,17,37] and those of its substitutes (1-naphthyl isothiocyanate [19] and 4-nitroisothiocyanate [25]), extraction procedures were recommended instead of the high-vacuum conditions, with diethyl ether [15], heptane [17,37], cyclohexane [19] or hexane [25] as extractant. The complete automation of the PTC derivatization procedure with the Varian Model 9095 autosampler also involves an extraction step instead of the high-vacuum removal of PITC.

2.1.1. Storage of derivatives

With regard to the stability of derivatized solid samples, in our experience they can be stored in vacuum-sealed flasks, or even in flasks closed with a ground-glass stopper, in a freezer for at least 6 months without any change; longer storage time are under study in our laboratory.

Regarding the stability of dissolved PTC derivatives, in general, they are considered to be stable compounds. Concerning the exact "lifetime" of the individual PTC amino acids there are contradictory experiences [2,10,14,16,26,27]: the statement that no loss could be detected after 3 days (in the cold) proved to be incorrect [10,16,26]. On keeping the dissolved PTC amino acids at 5–8°C their stability lasted 16 h [10], whereas others [16,26] reported a *ca.* 5% loss after storage for 48 h at 4°C. As a result of an exhaustive study in the author's laboratory [27], in which 21 PTC amino acids, dissolved in 0.05 *M* sodium acetate, were kept in a refrigerator at 4°C and tested immediately after preparation and after 0.5, 7, 18, 26, 44, 73, 102 and 190 h), revealed that the maximum time during which the dissolved derivatives retained their initial amounts was <7 h. After 18 h losses of serine (4%), histidine (7%), alanine (3%), arginine (9%), cyst(e)ine (5%) and ornithine (3%) could be determined. After 190 h the maximum loss for cyst(e)ine (30%), and the minimum loss for hydroxyproline was demonstrated.

2.2. Hydrolysis

It is very well known that hydrolysis is a crucial point in the amino acid analysis of protein hydrolysates: it is the limiting parameter for the reliable preparation of the sample for the chromatographic method. According to Pickering and Newton [65], in a paper entitled "Amino acid hydrolysis: old problems, new solutions", they stated that "With new instrumentation, amino acid hydrolysis is becoming a fast and automated process. But the problems of degradation, conversion, contamination and incomplete hydrolysis must be solved".

Prior to PTC derivatization, as the hydrolysis agent (with the only known exception of methanesulphonic acid (MSA) [13]) hydrochloric acid has been used, partly in the liquid phase and partly in the gas phase. In liquid-phase hydrolysis the acid is added directly to the sample tube, whereas in vapour-phase hydrolysis the dried sample-containing tubes are sealed in a larger vessel containing the acid. The gas-phase hydrolysis methods are regarded as preferable because their use excludes contamination of the

sample by the non-volatile impurities of the acid. When a limited amount of protein-containing sample is available, vapour-phase hydrolysis is the best choice.

2.3. The status of "difficult" amino acids

During hydrolysis, undesirable degradation, conversion and resistance of selected constituents must be taken into account [65,66]. Degradation of tryptophan, methionine, cystine, tyrosine, serine and threonine of >50%, >50%, >30%, >20%, >10% and >5% has been reported; the conversion of asparagine and glutamine to the corresponding acids proved to be quantitative. Because of the resistance of the peptide bonds between Ala–Ala, Val–Val, Ile–Ala and others, their incomplete hydrolysis necessitates a prolonged hydrolysis time. A number of remedies for the above phenomena have been proposed [67–79].

2.3.1. Tryptophan

For the determination of tryptophan [67–76], hydrolyses by alkali [67,68], by sulphur-containing organic acids [13,69–71] and by hydrochloric acid + additives [72–76] have been proposed. Alkaline hydrolyses (performed with NaOH [67], $Ba(OH)_2$ [67–68] or LiOH [68]), being extremely laborious and providing the quantification of tryptophan only, with an 80–90% recovery of the theoretical value, can be regarded as the worst solution to the problem. Sulphur-containing organic acids (non-volatile compounds such as *p*-toluenesulphonic acid [69], methanesulphonic acid [13,70] and mercaptoethanesulphonic acid [71]) lead to a number of shortcomings in the subsequent derivatization and chromatographic separation of the acids in hydrolysates. The most promising procedures are the classical hydrochloric acid hydrolyses in the presence of additives (thioglycolic acid [72], phenol [73], β-mercaptoethanol [74], ethanedithiol [75], thioglycolic acid + trifluoroacetic acid + indole [76] and tryptamine [28,29], providing the quantification of tryptophan simultaneously with all other amino acids; of the suggested additives, only the last two [28,29,76] proved to be satisfactory in vapour-phase hydrolyses.

Applying the combined additives [76], a >75% tryptophan yield from lysozyme, myoglobin and papain, monitored by conventional IEC, has been reported. Performing vapour-phase hydrolysis in the presence of tryptamine, with subsequent PITC derivatization, a >80% tryptophan recovery could be obtained, measured with different hydrolysis parameters, with a number of proteins and without any disturbing effect on the determination of the other amino acids [28,29]. The same results were achieved in vapour-phase hydrolysis in the author's laboratory using a CEM MDS-2000 microwave system with a protein hydrolysis accessory set (160°C, 110 psi, 65% power) (data will be presented at the 9th Danube Symposium on Chromatography, Budapest, Hungary, August 23rd–27th, 1993).

2.3.2. Cystine/cysteine

The determination of cystine/cysteine as their PTC derivatives proved to be an extremely complex problem, and in many papers this determination is not considered [30]. In those cases where it is also necessary to know the amount of cyst(e)ine, mainly the classical performic acid oxidation is applied, as detailed recently [61]. As a result of a 16-h reaction time, storing the tubes in an ice-bath at 5°C in a refrigerator, from cystine and cysteine the same product, *i.e.*, cysteic acid, and from methionine methionine sulphone are formed. The common, but very time-consuming and tedious (involving multiple reaction steps) procedure, suffers from a number of limitations [61,65]. With complex protein matrices, the performic acid oxidation results in substantial destruction of histidine (25%) and tyrosine (87%). Therefore, if cyst(e)ine, histidine and tyrosine determinations are required simultaneously, two separate hydrolyses need to be performed [61], with and without performic acid oxidation. Further possibilities for the determination total of cystine and cysteine, after reductive alkylation, in the form of the PTC derivatives of carboxymethylcysteine [16,77], pyridylethylcysteine [78] and sulphopropylcysteine [79] have been reported.

Our study (partly published [30] and discussed

[80], and partly to be presented very shortly) on the individual determination of all six possible racemates (L-, D- and DL-cystine and -cysteine) demonstrated that (i) the interaction of all six racemates and PITC results in two main and two trace PTC derivatives and the ratios of the derivatives are characteristic of the initial compound; and (ii) the total molar responses of the two main PTC derivatives are the same before and after hydrolysis and are independent of the original racemate form of cyst(e)ines. Thus, if the elution procedure permits the separation of the two main derivatives (their elution should be ensured by the gradient programme generally to be before isoleucine), the determination of the total cyst(e)ine does not need any previous pretreatment. The utility of this approach has been shown by the determination of the cyst(e)ine content in the hydrolysates of various proteins [30].

2.3.3. *Glutamine, asparagine*

Under hydrolysis conditions the glutamine and asparagine contents of proteins will be converted quantitatively into the corresponding acids. Thus, the PTC derivatives of aspartic and glutamic acid represent the total of the corresponding free acids and acid amides, respectively.

The treatment of several proteins with [bis-(trifluoroacetoxy)iodo]benzene (BTI) showed a possible way to determine separately their asparagine and glutamine contents through the determination of their corresponding 2,3-diaminopropionic and 2,4-diaminobutyric acids [32]. A rapid and quantitative conversion (30 min, 100%) has been reported for glutamine, but the reaction of asparagine could not be completed (2 h, 65%).

2.4. *Applicability to non-protein compounds*

Selected applications of the PTC derivatization of non-protein compounds [48–56] revealed without exception that these components did not need extra derivatization conditions. Only the elution parameters had to be adjusted to the separation of the particular derivatives present.

3. CHROMATOGRAPHIC CONDITIONS

This compilation contains various proposals given in selected papers cited above. The main differences regarding the size and filling of columns, the composition and pH values of eluents, the time of elution, and the number of components separated are presented (Table 1).

3.1. *Columns*

Table 1 indicates that the most advantageous conditions (short elution time, separation of the maximum number of amino acids) can be obtained by the use of the Waters Pico-Tag columns with any length (column filling of 3-μm particle size). A short column (15 cm $\times$ 3.9 mm I.D.) provides the resolution of 18 amino acids in 12 min [2,4,6,8,13–15,32], whereas on a longer column 29 amino acids have been determined in 20 min [16]. For laboratories with limited means even non-special columns can give results [27–30]. In our laboratory, two packings have been tested, and both proved to be satisfactory (for details, see Section 3.2).

3.2. *Eluents, gradient programmes*

The composition and pH of the eluents and the gradient programme have a considerable impact on the optimum resolution of the PTC amino acids in general and, in particular, on the "hardly" separable PTC derivatives such as those of histidine, threonine, alanine, proline and arginine, or phenylalanine and ammonia or ornithine and tryptophan. In order to overcome the poor resolution of these components, the use of a gradient programme with eluents [5], triethylamine (TEA) as an additive to eluent A, in different concentrations, and/or the optimization of the pH of the eluent for any given working conditions have been advised. In work at Waters, TEA has been applied in a small amount (0.05 ml/l [16] and 0.5 ml/l [2,4,6,8,13–15,32]), and its beneficial effect also up to 2.5 ml/l has been reported [11].

In our studies [27] (i) carried out with two different columns (Hypersil ODS bonded phase, Nucleosil 5 C_{18}, 5 μm), (ii) performed in parallel

TABLE 1

HPLC CONDITIONS SUGGESTED FOR THE ANALYSIS OF PTC AMINO ACIDS

Column (cm × mm I.D.)	Particle size (μm)	Product of	pH	Temperature (°C)	Eluents[a]		No. of amino acids measured	Analysis time (min)[b]	Ref.
					A	B			
15 × 3.9	3	Waters, Pico Tag	6.4	38	0.14 *M* NaOAc + 0.5 ml/l TEA	ACN–H_2O (6:4)	18	12	2, 4, 6, 8, 13–15, 32
25 × 4.6	5	IBM, DuPont, Altex	6.8	52	0.1 *M* NH_4OAc	ACN–MeOH–H_2O (44:10:46 and others)	16	35	5, 35
25 × 4.6	5	Waters, Pico Tag and others	6.4	46	0.7 *M* NaOAc + 2.5 ml/l TEA	H_2O; C = ACN–H_2O (80:20)	23	30	10
10 × 4.6	3	Thomson Instrument, Newark, DE	6.4 6.4	36	0.5 *M* NaOAc + 2.25 ml/l TEA	Solvent A: ACN–MeOH (5:4:1)	17	20	11
30 × 3.9	3	Pico Tag	6.4	40	0.14 *M* NaOAc + ACN (60 ml/l) + TEA (0.5 ml/l) + EDTA (1 ml of 10 m*M*)	ACN–H_2O (8:2)	16	25	17
15 × 3.9	3	Waters, Pico Tag	6.4	?	0.14 *M* NaOAc–ACN (9.4:0.6) + 0.05 ml/l TEA	ACN–H_2O (6:4)	18	14	39
15 × 3.9	3	Waters, Pico Tag	5.7	33	0.14 *M* NaOAc + 0.7 ml/l TEA	ACN–H_2O (6:4)	20	16	62
30 × 3.9	?	Waters, Pico Tag	6.4	46	0.14 *M* NaOAc–ACN (9.4:0.6) + 0.05 ml/l TEA	ACN–H_2O (6:4)	29	20	16, 17
25 × 4.6	5	Hypersil (?)	6.5	55	0.15 *M* NaOAc	ACN	16	20	31
25 × 4.6	5	Dynamex C_{18} (Rainin, Emeryville, CA, USA)	5.7	45	0.1 *M* NaOAc + 0.5 ml/l TEA	ACN–H_2O (6:4)	18	45	41
25 × 4.6	5	Hypersil (Jones, Hengoed, UK)	6.4	?	0.01 *M* NaOAc	0.01 *M* NaOAc–ACN (6:4)	17	60	43
25 × 4.6	5	Vydac C_{18} (?)	5.0–6.8	40–60	0.05 *M* NH_4OAc	0.023 *M* NH_4OAc–ACN–H_2O (44:46:10)	17	50	26
12.5 × 4.6 + 10 × 4.0 (guard column)	3	Spherisorb ODS	6.8	37	0.0125 *M* Na phosphate	ACN–0.0125 *M* Na phosphate (6:4)	21	25	36
15 × 4.0	5	Micro Pak SP-C_{18}	4.8	?	NaOAc (conc. ?)	ACN–NaOAc (conc. ?) (7:3)	18	20	37
15 × 4.6 2 × 2.6 (guard column)	5	Hypersil (Shandon) Nucleosil (Macherey–Nagel)	7.2	Ambient	0.05 M NaOAc or 0.05 *M* NH_4OAc	0.1 *M* NaOAc (NH_4OAc)–ACN–MeOH (46:44:10)	21	22	27, 30

[a] ACN = acetonitrile; MeOH = methanol; TEA = triethylamine; NaOAc = sodium acetate; NH_4OAc = ammonium acetate.

[b] Time from injection to elution of the last PTC derivative.

with sodium and ammonium salt-containing eluents of six pH values (5.6, 6.0, 6.4, 6.8, 7.2 and 7.6) and (iii) completed by TEA for eluent A of pH 7.2 in various concentrations (0.005, 0.05, 1, 2, 3 and 4 ml/l), the following results were obtained. (i) Concerning the two types of columns, under strictly identical elution conditions, no differences were found. (ii) The studies with eluents of lower pH, *i.e.* <7, showed disadvantageous changes; the retention times of the first nine components, eluting in the order aspartic acid, glutamic acid, hydroxyproline, serine, glycine, histidine, threonine, alanine and proline, increase in parallel with decreasing pH of the eluent, becoming increasingly closer to each other. At pH 7.2 and 7.6 optimum resolution could be obtained for the 21 PTC amino acids. Thus, in order to ensure a longer column lifetime, as the working pH a lower value of 7.2 was chosen. (iii) The effect of the presence of TEA proved to be of secondary importance. Although its presence increased the detector response of cyst(e)ine by 5–10%, at the same time the proline and arginine peaks became inseparable.

With regard to the column lifetime, in contrast to literature data [37], we have excellent experiences with both columns: on a 15 cm × 4.6 mm I.D. column filled with Hypersil, completed by a 2-cm long guard column, more than 1000 injections have been performed and it is still in satisfactory condition (the guard columns had to be changed after 50–150 injections, depending on the sample).

3.3. Quantification conditions

The reliability, repeatability and reproducibility of the determination of PTC amino acids are unambiguously accepted in the literature. Concerning the linearity of the detector responses and the statistical errors of measurements, different data can be found [13,14,16,22,27–30,34,36]. Linear detector responses have been reported with an error of <1.6% (R.S.D.) in wide concentration ranges: 20–500 pmol [14], 10–2000 pmol [13] and 10–5000 pmol [16]. These, excellent results were obtained with the use of Pico Tag columns [13,14,16]. Others [22,27–30,34,36], probably owing to the use of various, but not the special Pico Tag columns, reported higher error values, even in higher concentration ranges (R.S.D. <6.1% for 50–1000 pmol), while at the lower levels much higher error values were given [22] (R.S.D. 10% and 20% for 20 and 10 pmol, respectively).

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