



ELSEVIER

Journal of Chromatography A. 763 (1997) 1–10

JOURNAL OF  
CHROMATOGRAPHY A

## Review

# Tryptophan analysis in peptides and proteins, mainly by liquid chromatography

Ibolya Molnár-Perl

*Institute of Inorganic and Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112, Hungary*

### Abstract

Some of the general problems known in the analysis of tryptophan (both in its free form, alone or together with its metabolites, as well as in hydrolyzates of peptides or proteins, alone or together with all other amino acids) are described. This review includes an exhaustive literature overview using the author's experience with the preparation of derivatives and with various conditions arising from the analytical procedure itself. The special requirements of various tryptophan containing matrices (biological tissues or fluids, food and feed stuffs, etc.) are also taken into account. For the sake of completeness, in addition to the most common HPLC techniques, GC and spectrophotometry (in selected cases very important procedures), are also discussed.

**Keywords:** Reviews; Tryptophan; Peptides; Proteins; Amino acids

### Contents

1. Introduction .....	2
2. Free tryptophan and its metabolites .....	2
2.1. Gas chromatography .....	2
2.2. High-performance liquid chromatography .....	2
2.2.1. Tryptophan .....	2
2.2.2. Tryptophan together with its metabolites .....	3
3. Tryptophan in intact proteins: spectrophotometry .....	3
3.1. Derivative spectrophotometry without chemical derivatization .....	3
3.2. Spectrophotometric determinations subsequent to chemical derivatizations with aldehydes .....	3
4. Tryptophan in protein hydrolyzates .....	4
4.1. Chromatography of tryptophan .....	4
4.1.1. Ion-exchange chromatography .....	4
4.1.2. High-performance liquid chromatography .....	5
4.2. Chromatography of tryptophan together with all amino acids present in protein hydrolyzates obtained by different ways .....	6
4.2.1. By enzyme, such as pronase .....	6
4.2.2. By acids in the presence of additives/inhibitors .....	6
5. Conclusion .....	8
Acknowledgments .....	8
References .....	8

## 1. Introduction

The accurate and reliable analysis of tryptophan was, is and will be one of the most relevant and challenging task for analytical chemist, for two main reasons: (1) because of its importance in numerous aspects [1], (being nutritionally essential for many animals and for man and taking part in a number of biological functions), simultaneously and (2) because of methodological difficulties to maintain tryptophan in its intact form in protein hydrolyzates. (It is tremendously sensitive to redox interactions, particularly in strong acidic media needed for protein hydrolysis).

This last phenomenon proved to be the object of the overwhelming part of all those paramount efforts [2–84] which have been put into the field of tryptophan quantitation in this century; performed in order to find the optimum solution for its maximum recovery and selective determination. After an exhaustive general literature overview, based on the *Chemical Abstracts*, it became clear (i) that scientists are still looking for satisfactory alternatives and (ii) the increasing amount of newly developed analytical procedures are mainly chromatographic ones, representing—in the peak period of method development, in 1991—73% of the total: in accordance with the boom of high-performance liquid chromatography (HPLC). This distribution proved to be characteristic to methods published not only for quantitation of tryptophan in free form, including its metabolites, but also for those of proteins and peptides, together with the corresponding hydrolysis procedures.

Thus, based on the above detailed tendency, this paper aims to be a compilation with particular attention to the development of liquid chromatographic methods.

## 2. Free tryptophan and its metabolites

### 2.1. Gas chromatography

It is very well known that prior to gas chromatographic (GC) analyses amino acids should be present in impurity- and water-free condition in order of their acylation and esterification [3]. In addition, the quantitative acylation of amino- and indolyl groups

of tryptophan gave rise to special attention and proved to be critical—depending on the composition of acylation solution (solvent/acid anhydride ratio), temperature and time conditions. A single product could not be obtained. The ratio of the partly (monoacyl-) and fully (diacyl) acylated group containing derivatives, in favor of the fully acylated one, can be considerably (not quantitatively) influenced by the coelution of the acid anhydride with the derivatized sample. Due to the latter difficulties only few researchers have utilized the advantages of the very high sensitivity and selectivity of GC. The free tryptophan content of 20–100 mg crude extracts obtained from various plant samples, subsequently to solid-phase extraction and HPLC separation have been unambiguously identified and determined as their molecular ions, i.e., N-acetyl ( $m/z=M^+=260$ ) and N-diacetyl ( $m/z=M^+=302$ ) tryptophan methyl ester derivatives [4].

### 2.2. High-performance liquid chromatography

#### 2.2.1. Tryptophan

HPLC without derivatization [5–14] offers in quantitation of tryptophan [5–7] (i) particularly comfortable (previously only deproteinization had been carried out) and (ii) relatively selective conditions (both UV and fluorescence detection can be applied).

The tryptophan content of blood samples [5,6] was measured using isocratic elution, (column: 25 cm×4.6 mm), by UV detection, within 6 min, in the range of 0.1–100  $\mu\text{mol}$  [5], while miniaturized conditions (column: 8.3 cm×4.6 mm, 3  $\mu\text{m}$ ) provided considerably lower detection limit: 5 pmol using UV detection and 1 pmol applying fluorescence detection [6]. In order to determine the L-tryptophan content of tablets and capsules, (in comparison to the official AOAC microbiological method), its phenylisothiocyanate derivative was measured by HPLC [7]. Results revealed that out of the warm (solution autoclaved for 30 min, at 121–123 °C, 15 p.s.i.; 1 p.s.i.=6894.76 Pa) and cold (15 min sonication, at ambient temperature) microbiological procedures (if any), the cold is to be followed as the warm leads to 4–8% tryptophan losses; HPLC provides the advantages of using a smaller test portion and having a shorter analysis time.

### 2.2.2. Tryptophan together with its metabolites

Tryptophan being an essential amino acid whose metabolism involves several pathways, its quantitation by HPLC with its numerous metabolites [8–10], or with tyrosine [11] only and/or with 5-hydroxytryptamine [12] only, proved to be of primary importance [8–12].

Major tryptophan metabolites of kynurenine and of indole pathways have been determined by UV and fluorescence detections, simultaneously [8,9]: first [8] from 50–500  $\mu\text{l}$ , later [9] from 100  $\mu\text{l}$  direct plasma injections; Performing a special, automated precolumn deproteinization, within 40 min, 17 metabolites, present in human serum and/or plasma, were measured in pmol ranges: twelve of them by fluorescence and five by UV detection. Using a multiple ion detection system [10] (UV diode array detector connected on line with fluorescence one) which provided simultaneously UV spectra in the 190–450 nm range and fluorescence spectra setting two different excitation (280 and 340 nm) and emission (340 and 440 nm) wavelengths, 13 metabolites have been evaluated in 23 min. In spite of the fact that practical utilization was not given the procedure is worthy of mention being relatively fast and probably suitable for the analysis of real samples. Tryptophan and tyrosine content of 250  $\mu\text{l}$  blood [11], from perchloric acid deproteinized plasma, were quantitated by fluorescence detection.

Hydroxytryptamine and tryptophan [12] were determined in a 450  $\mu\text{l}$  whole blood sample within 10 min, (column: 15 cm $\times$ 4.6 mm) applying fluorescence detection. The method was utilized in the differentiation of healthy control subjects and depressed patients of major mental disorders.

## 3. Tryptophan in intact proteins: spectrophotometry

### 3.1. Derivative spectrophotometry without chemical derivatization

The characteristic absorbances of aromatic amino acids, in the low UV region, have been utilized in their quantitation applying second derivative spectrometry [13–16]. The ability to determine tryptophan by second derivative absorption became possible

from the relatively small overlap between the negative tryptophan bands at 290.5 nm and the positive tyrosyl peak at 289.5 nm. An early approach [13] is based on the mutual interference between the second derivative bands of the two major aromatic chromophores (tyrosine, tryptophan), at neutral pH. This interference has been evaluated in terms of the ratio between two peak-to-peak (tyrosine/tryptophan) distances observed in the second derivative spectrum. The ratio values proved to be well related for both model compound mixtures and proteins of known composition. According to an improved second derivative method [14] tryptophan, tyrosine and phenylalanine were measured by their second derivative absorbance values by means of the vertical distance from baseline to the derivative curve at a wavelength specifically assigned to each aromatic amino acid (tryptophan: 290 nm, tyrosine: 283 nm, phenylalanine: 258 and 264 nm). Recently [15,16] a substantially new principle has been reported for the quantitation of tryptophan and tyrosine in intact proteins based on their fourth derivative UV absorption spectra. Spectra evaluations obtained in 0.1 M sodium hydroxide solution or in supernatants of protein extracts provided excellent results. Tryptophan [15] and tyrosine [16] content both of standard proteins and those of protein containing matrices proved to be in accordance with literature data. Measured [16] and earlier published (in parentheses) tryptophan contents (tryptophan, g/100 g protein) for casein, wheat, barley and soy bean meals, in order of listing proved to be 1.38 (1.36, 1.31), 1.20 (1.14, 1.12, 1.20), 1.27 (1.12, 1.27, 1.49) and 1.66 (1.14–1.71) g tryptophan/100 g protein.

### 3.2. Spectrophotometric determinations subsequent to chemical derivatizations with aldehydes

Many spectrophotometric methods of tryptophan quantitation are based on the interaction of the indole ring with aldehydes [17–29]. The reaction of *p*-dimethylaminobenzaldehyde (DABA) in sulphuric acid media [17–20] has been exhaustively examined and widely utilized in its determination also in hydrolyzates [19,20]. A simplified version of the DABA method using 10 M hydrochloric acid instead of sulphuric acid was reported for free tryptophan [21]. Practical utilizations have been published [22–

24] based on the reaction between acetic acid/ferric chloride and tryptophan residues of proteins, in solutions of concentrated sulphuric acid, resulting in the formation of glyoxylic acid. The tryptophan content of  $\gamma$ -globulins and casein [22], as well as those of numerous cereal grains [23] and potatoes [24] have been quantitated by measuring the absorbances of the resulting violet solution at 545 nm in all cases [22–24].

The most promising tryptophan/aldehyde reaction performed with the intact protein matrix proved to be the acid ninhydrin method, because of its selectivity, general applicability and outstanding reproducibility. Earlier works [25–27] proposed long lasting (16–20 h) incubation of intact proteins and insufficient reaction time for the quantitative interaction of tryptophan (residue) with the ninhydrin reagent. Recently [28,29], an exhaustive kinetic study was performed (with tryptophan, tyrosine and lysozyme) in order to obtain optimum analytical conditions providing: (i) stoichiometric reaction both with free tryptophan and tryptophan residues of proteins (including also protein containing matrices, such as feed- and food stuffs), (ii) quantitative elimination of the disturbing effect of tyrosine. Applying optimum analytical conditions [35 °C, 90 min, 1% ninhydrin prepared with a solution containing HCl (37%) and formic acid (82–92%) in a volume ratio of 2:3]. Optimum conditions allowed the direct interaction of samples, using the same molar absorptivity values for free and peptide linked tryptophan. The practical utilization of the improved procedure was shown by the tryptophan quantitation of numerous standard proteins (lysozyme, human and bovine serum albumin, casein,  $\alpha$ -chymotrypsin, trypsin) and protein matrices (soy bean, wheat, maize, barley, rye, sun flower, fish and meat meals).

#### 4. Tryptophan in protein hydrolyzates

Those efforts which aim to determine tryptophan in hydrolyzates should be divided into two basically different groups. Aiming at the quantitation of tryptophan only, the use of various alkalis [NaOH, KOH, LiOH, Ba(OH)<sub>2</sub>] have been investigated and proposed. In order to determine tryptophan together with all other amino acids, hydrolyses by enzymes,

by organic acids and also by HCl with different, tryptophan protecting additives/inhibitors have been suggested. In general, the cumbersome, time consuming and tedious hydrolyses procedures proved worthwhile only in those cases where hydrolyses were followed by one of the selective chromatographic techniques. Tryptophan monitoring in hydrolyzates by any of the colour reactions, followed by spectrophotometry can be regarded as an unnecessary luxury. Consequently, special problems in chromatography of tryptophan alone and together with all other amino acids —subsequent to their various hydrolysis techniques— being the most relevant topic, will be given in detail, applying ion-exchange chromatography (IEC), (carried out with amino acid analyzers), some GC and mainly HPLC.

##### 4.1. Chromatography of tryptophan

This field of research, including inter-laboratory comparative studies, covers optimization tests concerning simultaneously the quality and quantity of alkalis, the temperature and time of hydrolyses and the conditions of chromatographic elution and quantitation procedures.

###### 4.1.1. Ion-exchange chromatography

The selective identification and quantitation of tryptophan in alkaline hydrolyzates started in the early seventies by means of IEC [30–33]. At that time, taking into account the relatively large amount of sample required for IEC analyses, (elutions have been performed with 10–1000  $\mu$ g amino acids), sodium hydroxide providing 85–95% tryptophan recovery [30–32] (4.2 M, at 110 °C, 22 h [30] and 20 h [31,32]), was preferred to barium hydroxide [33] (4.2 M, 110 °C, 16 h). The reason was that elimination of excess barium hydroxide needed an extra precipitation step, in form of BaSO<sub>4</sub>, or BaCO<sub>3</sub>, resulting in additional 4–10% loss of tryptophan, prior to its elution by IEC. The elution procedure only, by IEC, carried out at 52 °C, in all four cited cases, needed on short columns (7×0.9 cm), as an optimum achievement, 30 min [33], while others, remaining on the safe side, for the sake of better resolution, used 90 min [31,32] and 120 min [30] elution times (regeneration not included).

#### 4.1.2. High-performance liquid chromatography

In the light of the above (Section 4.1.1), a considerably faster chromatographic technique of higher sensitivity, (elutions have been carried out in the ng, pg ranges), opened a new chapter for tryptophan analysis alone, in alkaline protein hydrolyzates, for both practical and scientific reasons. The increased availability of chromatographic information also gave rise to review of optimization [34–41] and of comparison [42–49] of hydrolysis techniques in connection with chromatography.

Lithium hydroxide (4 M, 110–120 °C, 22–26 h) [34] has been recommended for the quantitation of tryptophan in numerous foods and feed-stuff hydrolyzates, obtained by direct injections, with linear responses, by fluorescence detection, in the 0.2–100 ng range. Tryptophan was eluted with 5 min retention time, with water–methanol (2:1, v/v), acidified with phosphoric acid to pH 3 (recovery of the method was not reported).

Sodium hydroxide was proposed (4.2 M, 110 °C, 16 h, in autoclave) [35], for hydrolysis of feed stuffs in the presence of lactose (applied as protecting agent against tryptophan losses). In calculation of tryptophan values, in order to correct losses,  $\alpha$ -methyltryptophan was applied as internal standard. Recovery data were not given.

The reevaluation of barium hydroxide digestions [36–41] was associated with the development of HPLC, in particular by fluorescence detection. In the possession of this very sensitive and selective technique—due to the possibility of direct injections of diluted hydrolyzates—barium ion elimination became unnecessary. Consequently, the main disadvantage, the additional tryptophan loss due to its adsorption on barium precipitates no longer counted. The poor solubility of barium hydroxide still remained a problem. Exhaustive basic studies [36,37] performed by barium hydroxide hydrolyses, without any previous pretreatment, clarified important characteristics. Investigating the peak purity parameters by diode array detection [36] of tryptophan, (obtained from feedstuffs, rich in UV absorbing constituents), it could be stated, that no UV interfering substance was coeluted with tryptophan [ $\sim$ 1.66 M Ba(OH)<sub>2</sub>, 125 °C, 16 h, injected after neutralization directly]. The advantages of HPLC with fluorescence detection has been repeatedly shown, low

detection levels (20–2000 ng) and short analysis time (5 sample/h). In the hydrolysates of 14 different feed stuffs  $99.7 \pm 1\%$ , i.e., quantitative tryptophan recoveries were reported, indicating that neither losses nor incomplete liberation occurred at the stated conditions (based on additive hydrolysis of lysozyme) [37]. The statement of quantitative tryptophan recovery has been later on modified by others [42,43]. Several simplifications [38–40], such as, decreasing the injection volume [38], the hydrolysis time [39,40] (16, 8, 4 h) increasing the hydrolysis temperature [39,40] (110, 125, 140 °C) at various barium hydroxide concentrations (2.7 M [39,40] and 3.3 M [41]), simultaneously were added. The use of internal standards (5-methyltryptophan [38], or both,  $\alpha$ -methyl- and 5-methyltryptophan [40]) was ultimately introduced. Comparisons, based on intra- [42,43,47,49] and inter- [44–46,48] laboratory studies revealed that: (i) 100% tryptophan recovery cannot be obtained by any of the alkaline hydrolysis procedures [42–49]; (ii) while performing IEC [42], subsequent to the hydrolysis (4–8 h), best results (5% losses) have been obtained by 4 M LiOH, in the presence of 5% SnCl<sub>2</sub>. In separation and quantitation by HPLC, subsequent to sodium hydroxide hydrolyses, although the same internal standard (5-methyltryptophan) was used, contradictory experiences have been reported: according to earlier studies [43] NaOH and LiOH provided similar tryptophan recoveries, (between 88.1% and 98.5%), while recently published data reported also by the same authors [46,49] revealed the outstanding necessity of oxygen elimination. Without applying autoclave purging, comparison of 1.3 M Ba(OH)<sub>2</sub> (125 °C, 16 h) and 4 M LiOH (110 °C, 24 h) proved they were as alkalies of identical value [46]: ensuring 82.7–85.2% recovery, without correction. Later on, using Ba(OH)<sub>2</sub>, allowing the quantitative oxygen elimination before hydrolysis, by autoclave purging at 100 °C, peak tryptophan recoveries were reported (96.1–102.4%) [47].

Inter laboratory studies [44,45,48] presented less promising results. In a comprehensive work [44] applying partly IEC, partly HPLC, using hydrolyses uniformly by 4.2 M NaOH, within and between laboratories analyzing the same samples, quite the same variabilities have been reported. Reproducibilities, within and between laboratories, proved to be

3.87–16.1% and 3.87–16.5%, relative standard deviation (RSD) percentages). These low and identical reproducibilities could be explained by the many steps containing procedures covering subjective errors. Regarding the data of 14 laboratories [45], as a function of the matrix analyzed, they varied between 3.87 and 26.4% (RSD). The overall reproducibility, which means the average of averages obtained in 23 laboratories [48], proved to be  $86.5 \pm 5.6\%$ .

#### 4.2. Chromatography of tryptophan together with all amino acids present in protein hydrolyzates obtained by different ways

##### 4.2.1. By enzyme, such as pronase

Neglecting the earlier efforts [1,2], relying on a recently presented work only [50], pronase digestion provided excellent characteristics. Enzymatic digestions have been accomplished in less than 6 h under mild conditions (pH 8.5, 50 °C). Spectacular HPLC conditions were shown: injection-to-injection times of 8 min, including elution of tryptophan and 5-methyltryptophan (internal standard). Quantitation based both on UV (diode array proven) and fluorescence spectra could be carried out without disturbing interferences.

##### 4.2.2. By acids in the presence of additives/inhibitors

(i) The additives or inhibitors are the collective designation of all those compounds which decrease or inhibit tryptophan decomposition under peptide/protein hydrolyses in acidic media. It is known for a long time [51] that when oxygen is removed by evacuation, the rate of tryptophan decomposition decreases dramatically. These results show that oxygen concentration, during acidic hydrolyses, is the main contributor to the destruction. Performing hydrolysis in sealed tubes under vacuum or nitrogen, traces of oxygen in the medium can contribute to the destruction. Under these presumably “oxygen free” conditions, addition of thioglycolic acid [51] or phenylmethanethiol [52] or any others detailed in Sections 4.2.2.1 and 4.2.2.2 decrease the occurrence of free radical chain reactions responsible for tryptophan decomposition. All additives/inhibitors, including also tryptamine, probably act as free

radical scavengers during hydrolysis procedures, preventing tryptophan degradation by any residual oxygen in the hydrolysis tube. (ii) As to the mechanism of tryptophan's destruction it was shown [51,53–56] that most of the essential amino acids do not affect the stability of tryptophan during hydrochloric acid hydrolysis with the exception of cystine. In the presence of traces of oxygen tryptophan reacts with cystine resulting in the 2,3-dihydro-2-oxo-tryptophan, cysteine and cystine [51,52]. In the course of this transformation, arising from cystine oxidation, bis(2-amino-2-carboxy-ethyl) trisulfide, sulfenic/sulfinic acids have been identified, which proved to be responsible for tryptophan losses [54]. It has been also shown that oxidized chlorine derivatives [55] and carbohydrates [56] may also contribute to the degradation of tryptophan.

*By sulphur containing organic acids+additives (methanesulfonic acid+tryptamine, p-toluenesulfonic acid+tryptamine/thioglycolic acid, mercaptoethanesulfonic acid without additive)*

Methanesulfonic acid: Since its first proposal [57] 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (henceforth: tryptamine) has been used also in subsequent papers [59–61]. Since a competition has been observed between the molecular breakdown of serine, threonine, tryptophan and the release of valine, as optimum conditions 115 °C and 22–72 h were suggested. The quantitation of 18 amino acids, including cysteine and tryptophan, has been reported by IEC, from lysozyme hydrolyzate: theoretical values were indicated without reproducibility data [57]. Performing microwave irradiation [58,59] followed by IEC, the time of hydrolyses could be dramatically shortened: at 160 °C, 45 min [58], while at 178 °C and at 186 °C, 4 min [59] proved to be satisfactory. ~98% tryptophan recoveries have been reported in both cases [58,59]. Methanesulfonic acid hydrolysis of cytochrome *c* [60] was carried out at 115 °C for 22 and 70 h and at 150 °C for 22 h. Results proved that 115 °C for 22 h should be chosen as the optimum conditions. 16 amino acids, including cysteine and tryptophan have been quantitated by HPLC, subsequent to their direct derivatization in the hydrolyzate by 9-fluorenylmethylchloroformate.

*p*-Toluenesulfonic acid: The principle of the use of 3 *M* *p*-toluenesulfonic- [61] and 4 *M* methanesulfonic acids [57], both in the presence of 0.2% tryptamine, have been introduced by the same researchers: they stated that *p*-toluenesulfonic acid is inferior to methanesulfonic acid providing lower tryptophan recovery which proved to be by IEC between 91 and 98%. This early experience [61] has been repeatedly tested by microwave irradiation followed by HPLC after precolumn derivatization with *o*-phthalaldehyde [62]. Tryptophan recovery of model solution, irradiated by 2450 MHz frequency for 15 min (corresponding temperature was not given), proved to be 98.6%.

Mercaptoethanesulfonic acid: The advantages of the use of 3 *M* mercaptoethanesulfonic acid without additives [63] has been presented in comparison with *p*-toluenesulfonic acid +0.2% tryptamine and with 6 *M* HCl+4% thioglycolic acid: all three types of hydrolyses have been followed for 22 and 72 h, at  $110 \pm 2$  °C with lysozyme, chymotrypsin and cytochrome *c*. Data obtained proved that in all cases tested, maximum tryptophan recovery was obtained at 110 °C after 22 h by mercaptoethanesulfonic acid: in order of listing above, tryptophan contents proved to be 94.5%, 96% and 93%, while hydrochloric acid with thioglycolic acid provided the lowest recovery, at 86.5%, 81.5% and 75%, respectively. Later on difficulties have been reported [64,65] in quantitation by IEC. Due (i) to the formation of a mixed disulfide from mercaptoethanesulfonic acid and cysteine [65], as well as (ii) to the coelution of cysteine with proline [66]. These difficulties could be solved by modifying the elution program of IEC [65,66].

*By hydrochloric acid+additives (pyridine borane, thioglycolic acid,  $\beta$ -mercaptoethanol, phenol, tryptamine)*

In the classical 6 *M* HCl hydrolyses the overwhelming part of tryptophan (70–100% of the total present in protein) gets destroyed. The reason for that needs still further explanation. Since HCl is the best hydrolysing agent from numerous points of view, researchers tried to prevent tryptophan destruction by applying HCl+different inhibitors.

1,2-Ethanedithiol [67], in absence of oxygen, was proposed for preventing destruction of cyst(e)in,

tryptophan and histidine during HCl acid hydrolyses of lysozyme as model compound. Resulting amino acids have been measured by GC as their *N*-acetylated methyl esters. Correction for losses during hydrolysis have been done on the base of isotope dilution assay. The recovery of tryptophan was 75.4%.

Pyridine borane [68] reduces tryptophan in the presence of trifluoroacetic acid to dihydrotryptophan which appeared as a doublet between lysine and histidine measured by IEC. As to their HPLC quantitation difficulties have been reported: its phenylthiocarbonyl derivatives could not be detected, (likely covered by the reagent peaks), while as dabsyl derivatives the first eluting member of the doublet coeluted with tyrosine. Thus, this type of hydrolysis study remained proven by IEC only, confirmed quite quantitative recoveries of tryptophan as dihydrotryptophan compared to theoretical values in the case of twelve standard proteins [68].

Thioglycolic acid [52,54,69–71] was tested at first [52] in 0.5–6% (v/v) concentration added to 6 *M* HCl. Maximum tryptophan yield from lysozyme (84%) and cytochrome *c* (86%) were obtained with 2% thioglycolic acid containing HCl, performing hydrolyses in evacuated tubes for 24 h at 108–110 °C, measured by IEC. With shortened hydrolysis time [69] (4 h, 145 °C), applying the first introduced procedure [52], the method has been extended for the analysis of various meat proteins by IEC and by HPLC, simultaneously [69]. Hydrolyses in vapour phase [70] with 7 *M* HCl containing 10% trifluoroacetic and 20% thioglycolic acids were carried out at 166 °C, for 15 min and 30 min. After 15 min hydrolysis time tryptophan recovery from model solutions proved to be 86%, while in the case of myoglobin, papain and lysozyme, 95, 80 and 80% respectively were obtained. From reducing hydrolyzates [71], with 6 *M* HCl, containing 5% thioglycolic acid +0.1% phenol, at 118 °C, after 18 h 77, 86 and 82% tryptophan recoveries have been reported from chymotrypsin, trypsin and  $\alpha_2$ -macroglobulin subunit.

$\beta$ -Mercaptoethanol (0.2%) in 6 *M* HCl [72], was used for 24 h, at 110 °C in order to determine all amino acids, including tryptophan in numerous protein hydrolyzates. Amino acid constituents of several proteins were quantitated as their phenylthiocar-

bamyl derivatives by HPLC: tryptophan recoveries varied between 93 and 102%.

Phenol [73] added in 3% (v/v) to 6 M HCl largely prevented the destruction of tryptophan during rapid hydrolyses at 166 °C, for 25 min, or at 145 °C, for 4 h. The largely prevented status indicates at least 80% recovery.

Hydrochloric acid+tryptamine [74–84]: Exhaustive studies have been performed in order to optimize the preferred (i) chromatographic procedure, (GC [74–77] or HPLC [78,79]), (ii) the ratios of tryptamine to proteins [76,81], [1:1 (w/w) ratios proved to be the optimum], (iii) the shortest hydrolysis time coupled with the lowest necessary temperature in liquid phase [76,77] and in vapour phase [81,82,84] equally, (iv) both by simple heat and by microwave irradiation [83] as well. Based on hundreds and hundreds of hydrolyses, followed by GC of the N,O,(S)trifluoroacetyl *n*- and isobutyl amino acid esters [74–77] and, by HPLC of the phenylthiocarbamyl amino acid derivatives [78–84], (carried out with standard proteins and protein matrices, such as feed and food stuffs), it has been proved that: (i) Tryptamine protection can be achieved between 80 and 100%. (ii) In liquid phase hydrolyses, followed by GC, the tryptophan contents of standard proteins and protein matrices have been recovered equally: because of the coelution of the lysine and tryptamine derivatives by GC, prior to derivatization, the cation-exchange clean up of the hydrolyzates, to eliminate tryptamine, could not be avoided. (iii) Applying vapour phase hydrolyses and HPLC, continued by the quantitation of phenylthiocarbamyl derivatives, no interferences of tryptamine could be detected [81,82]: tryptophan recovery of pure proteins and peptides proved to be  $\geq 80\%$ . (iv) Concerning microwave digestion [83] its advantages, reported by others [58,59], were not confirmed because uniformly optimum parameters for all components present in hydrolyzates were not found and the reproducibility in the quantitation of amino acids, characterized by the RSD proved to be higher ( $\leq 8.0\%$ ) than in previous experiments [78,79] ( $\leq 5.0\%$ ,  $\leq 5.0\%$ ) and the CEM microwave hydrolysis system works reproducibly up to 0.689 MPa (100 p.s.i.g.) only, which means that the hydrolysis time could not be shortened as reported

## 5. Conclusion

In summary it can be stated that

(1) Selective chromatographic techniques, preferably HPLC should be followed for pure proteins and/or peptides, to measure the amount of free tryptophan and its metabolites, or to find the quality and quantity of all amino acids, including tryptophan, present in hydrolyzates.

(2) The tryptophan quantitation of feed and food-stuffs, in particular those of high saccharide content, can be performed most advantageously with intact proteins: applying the acid-ninhydrin method [4].

## Acknowledgments

This work was supported by the Hungarian Academy of Sciences (Project No.: OTKA T 5073, T 01639, T 016006).

## References

- [1] G. Curzon, in I. Ishiguro, R. Kido, T. Nagatsu, Y. Nagamura and Y. Ohta (Editors), *Advances in Tryptophan Research*, Fujita Health University Press, 1992, pp. 297–302.
- [2] M. Friedman and J.-L. Cuq, *J. Agric. Food Chem.*, 36 (1988) 1079–1093.
- [3] I. Molnár-Perl, M. Pintér-Szakács, M. Morvai and V. Fábián, in I. Ishiguro, R. Kido, T. Nagatsu, Y. Nagamura and Y. Ohta (Editors), *Advances in Tryptophan Research*, Fujita Health University Press, 1992, pp. 195–198.
- [4] L. Michalczuk, K. Bialek and J.D. Cohen, *J. Chromatogr.*, 596 (1992) 294–298.
- [5] H. Gjerde, P.T. Normann and J. Morland, *Biomed. Biochim. Acta*, 46 (1987) 53–57.
- [6] L.C. Peterson, M.E. Dwyer and P.R. Brown, *Chromatographia*, 24 (1987) 309–312.
- [7] H.S. Kim and G. Angyal, *J. AOAC Int.*, 76 (1993) 414–417.
- [8] I. Morita, T. Masujima, H. Yoshida and H. Imai, *Anal. Biochem.*, 151 (1985) 358–364.
- [9] I. Morita, M. Kawamoto, M. Hattori, K. Eguchi, K. Sekiba and H. Yoshida, *J. Chromatogr.*, 526 (1990) 367–374.
- [10] U. Caruso, B. Fowler, G. Minniti and C. Romano, *J. Chromatogr. A*, 661 (1994) 101–104.
- [11] M.B. Berardino, F.C. Roingard and N.K. Fukagawa, *J. Nutr. Biochem.*, 1 (1990) 220–222.
- [12] N. Aymard, P. Honore and I. Carbuccia, *Prog. Neuro-Psychopharmacol. Biol. Psychiat.*, 18 (1994) 77–86.



- [13] L. Servillo, G. Colonna, C. Balestrieri, R. Ragone and G. Irace, *Anal. Biochem.*, 126 (1982) 251–257.
- [14] Y. Nozaki, *Arch Biochem. Biophys.*, 277 (1990) 324–333.
- [15] D.J. Fletouris, N.A. Botsoglou, G.E. Papageorgiou and A.J. Mantis, *J. AOAC Int.*, 76 (1993) 1168–1173.
- [16] N.A. Botsoglou, D.J. Fletouris, G.E. Papageorgiou and A.J. Mantis, *J. Agric. Food Chem.*, 41 (1993) 1635–1639.
- [17] J.R. Spies and D.C. Chambers, *Anal. Chem.*, 21 (1949) 1249–1266.
- [18] J.R. Spies and D.C. Chambers, *Anal. Chem.*, 22 (1950) 1209–1210.
- [19] J.R. Spies, *Anal. Chem.*, 22 (1950) 1447–1449.
- [20] J.R. Spies, *Anal. Chem.*, 39 (1967) 1412–1416.
- [21] S.A. Al-Tamrah, *Anal. Lett.*, 22 (1989) 387–401.
- [22] J. Opienska-Blauth, M. Charezinski and H. Berbec, *Anal. Biochem.*, 6 (1963) 69–76.
- [23] J.M. Concon, *Anal. Biochem.*, 67 (1975) 206–219.
- [24] S. Gorinstein, Y. Shin, D. Hadziyev, *Nutr. Rep. Int.*, 37 (1988) 397–408.
- [25] M.K. Gaitonde and T. Dovey, *Biochem. J.*, 117 (1970) 907–911.
- [26] J.C. Zahnley and J.G. Davis, *Biochem. J.*, 135 (1973) 59–61.
- [27] L. Sodek, P.T.D. Vecchia and M.L.G.P. Lima, *Food Chem.*, 23 (1975) 1147–1150.
- [28] I. Molnár-Perl and M. Pintér-Szakács, *Anal. Biochem.*, 177 (1989) 16–19.
- [29] M. Pintér-Szakács and I. Molnár-Perl, *J. Agric. Food Chem.*, 38 (1990) 720–726.
- [30] T.E. Hugly and S. Moore, *J. Biol. Chem.*, 247 (1972) 2828–2834.
- [31] H. Sato, T. Seino, T. Kobayashi, A. Murai and Y. Yugari, *Agric. Biol. Chem.*, 48 (1984) 2961–2969.
- [32] H. Sato, T. Kobayashi, R.W. Jones and R.A. Easter, *J. Anim. Sci.*, 64 (1987) 191–200.
- [33] R. Knox, G.O. Kohler, R. Palter and H.G. Walker, *Anal. Biochem.*, 36 (1970) 136–143.
- [34] G. Werner, *Landwirtsch. Forsch.*, 39 (1986) 109–117.
- [35] S. Bech-Andersen, *Acta Agric. Scand.*, 41 (1991) 305–309.
- [36] S. Delhaye and J. Landry, *Anal. Biochem.*, 159 (1986) 175–178.
- [37] J. Landry, S. Delhaye and G. Viroben, *J. Agric. Food Chem.*, 36 (1988) 51–52.
- [38] J. Landry and S. Delhaye, *J. Agric. Food Chem.*, 40 (1992) 776–779.
- [39] S. Delhaye and J. Landry, *J. Agric. Food Chem.*, 41 (1993) 1633–1634.
- [40] J. Landry and S. Delhaye, *Amino Acids*, 5 (1993) 317–321.
- [41] K. Aiple und K.H. Menke, *Agribiol. Res.*, 43 (1990) 200–208.
- [42] B. Lucas and A. Sotelo, *Anal. Biochem.*, 109 (1980) 192–197.
- [43] H.K. Nielsen and R.F. Hurrell, *J. Sci. Food Agric.*, 36 (1985) 893–907.
- [44] M.C. Allred and J.L. MacDonald, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 603–606.
- [45] K. Ranfft and U. Faure, *Agribiol. Res.*, 46 (1993) 65–72.
- [46] J. Landry, S. Delhaye and D.G. Jones, *J. Sci. Food Agric.*, 58 (1992) 439–441.
- [47] S. Delhaye and J. Landry, *Analyst*, 117 (1992) 1875–1877.
- [48] J. Landry and S. Delhaye, *J. Agric. Food Chem.*, 42 (1994) 1717–1721.
- [49] J. Landry and S. Delhaye, *Food Chem.*, 49 (1994) 95–97.
- [50] S.E. Garcia and J.H. Baxter, *J. AOAC Int.*, 75 (1992) 1112–1119.
- [51] M. Stewart and C.H. Nicholls, *Aust. J. Chem.*, 25 (1972) 1595–1599; 25 (1972) 2139–2144.
- [52] H. Matsubara and R.M. Sasaki, *Biochem. Biophys. Res. Commun.*, 35 (1969) 175–181.
- [53] L.C. Gruen, *Aust. J. Biol. Sci.*, 26 (1973) 287–290.
- [54] T. Ohta and T. Nakai, *Agric. Biol. Chem.*, 43 (1979) 2419–2423.
- [55] T. Ohta, S. Susuki, M. Todo and T. Kurechi, *Chem. Pharm. Bull.*, 29 (1981) 1767–1771.
- [56] H. Olcott and H. Fraenkel-Conrat, *J. Biol. Chem.*, 171 (1947) 583–594.
- [57] R.J. Simpson, M.R. Neuberger and T.-Y. Liu, *J. Biol. Chem.*, 251 (1976) 1936–1940.
- [58] S.H. Chiou and K.T. Wang, *J. Chromatogr.*, 448 (1988) 404–410.
- [59] S.H. Chiou and K.T. Wang, *J. Chromatogr.*, 491 (1989) 424–431.
- [60] M.F. Malmer and L.A. Schroeder, *J. Chromatogr.*, 514 (1990) 227–239.
- [61] T.-Y. Liu and Y.H. Chang, *J. Biol. Chem.*, 246 (1971) 2842–2848.
- [62] A. Pecavar, M. Prosek, D.F. Temeljotov and J. Marsel, *Chromatographia*, 30 (1990) 159–162.
- [63] B. Penke, R. Ferenczi and K. Kovács, *Anal. Biochem.*, 60 (1974) 45–50.
- [64] M.L.G. Gardner, *Anal. Biochem.*, 141 (1984) 429–431.
- [65] J. Csapó, B. Penke, I. Tóth-Pósfai and Zs. Csapó-Kiss, *Acta Alimentaria*, 15 (1986) 227–235.
- [66] J. Csapó, Zs. Csapó-Kiss, S. Folestad and A. Tivesten, *Anal. Chim. Acta*, 289 (1994) 105–111.
- [67] P. Felker, *Anal. Biochem.*, 76 (1976) 192–213.
- [68] W.S.D. Wong, D.T. Osuga, T.S. Burcham and R.E. Feeney, *Anal. Biochem.*, 143 (1984) 62–70.
- [69] R.B. Ashworth, *J. Assoc. Off. Anal. Chem.*, 70 (1987) 80–85.
- [70] H. Yano, K. Aso and A. Tsugita, *J. Biochem.*, 108 (1990) 579–582.
- [71] L. Sottrup-Jensen, *Biochem. Molecular Biol. Int.*, 30 (1993) 789–794.
- [72] L.T. Ng, A. Pascaud and M. Pascaud, *Anal. Biochem.*, 167 (1987) 47–52.
- [73] K. Muramoto and H. Kamiya, *Anal. Biochem.*, 189 (1990) 223–230.
- [74] I. Molnár-Perl and V. Fábíán-Vonsik, *J. Chromatogr.*, 446 (1988) 231–236.
- [75] I. Molnár-Perl, M. Pintér-Szakács and V. Fábíán-Vonsik, *J. Chromatogr.*, 93 (1987) 434–438.
- [76] V. Fábíán, M. Pintér-Szakács and I. Molnár-Perl, *J. Chromatogr.*, 520 (1990) 193–199.

- [77] V. Fábán, M. Morvai, M. Pintér-Szakács and I. Molnár-Perl, *J. Chromatogr.*, 553 (1991) 87–92.
- [78] M. Morvai, V. Fábán and I. Molnár-Perl, *J. Chromatogr.*, 600 (1992) 87–92.
- [79] I. Molnár-Perl and M. Morvai: *Chromatographia*, 34 (1992) 132–136.
- [80] I. Molnár-Perl, in G. Allegri Filippini and C. Costa (Editors), *Recent Advances in Tryptophan Research*, Plenum Press, 1996, pp. 635–648.
- [81] I. Molnár-Perl, M. Pintér-Szakács and M. Khalifa, *J. Chromatogr.*, 632 (1993) 57–61.
- [82] I. Molnár-Perl and M. Khalifa, *Chromatographia*, 36 (1993) 43–46.
- [83] E. Tatár, M. Khalifa, Gy. Záray and I. Molnár-Perl, *J. Chromatogr. A*, 672 (1994) 109–115.
- [84] I. Molnár-Perl, *J. Chromatogr. A*, 661 (1994) 43–50.