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Review

Hydrolysis and amino acid composition analysis of proteins

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Abstract

Amino acid composition analysis is a classical protein analysis method, which finds a wide application in medical and food science research and is indispensable for protein quantification. It is a complex technique, comprising two steps, hydrolysis of the substrate and chromatographic separation and detection of the residues. A properly performed hydrolysis is a prerequisite of a successful analysis. The most significant developments of the technology in the last decade consist in the (i) reduction of the hydrolysis time by the use of microwave radiation energy; (ii) improvement in the sensitivity of the residue detection, the quantification of the sensitive residues and separation of the enantiomeric forms of the amino acids; (iii) application of amino acid analysis in the large-scale protein identification by database search; and (iv) gradual replacement of the original ion exchange residue separation by reversed-phase high-performance liquid chromatography. Amino acid analysis is currently facing an enormous competition in the determination of the identity of proteins and amino acid homologs by the essentially faster mass spectrometry techniques. The amino acid analysis technology needs further simplification and automation of the hydrolysis, chromatography and detection steps to withstand the pressure exerted by the other technologies.

Keywords: Reviews; Hydrolysis; Amino acids; Proteins

Contents

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1. Introduction

Amino acid compositional analysis is a classical protein analysis technique. It is based on the principle that the composition of the amino acids, i.e., the number of the individual residues present in a polypeptide, is characteristic for each peptide and protein. Since the appearance of the first automated analyzer of Spackman et al. 40 years ago [1], the method has been an important part of protein science. Amino acid composition analysis finds wide applications in biochemistry, food science, microbiology, clinical and diagnostic studies. It is indispensable in the quantification of peptides and proteins, as it is the only method to determine absolute protein quantities in solution [2]. Compositional analysis is usually included in the quality control studies of pharmaceutical proteins intended for human use. Its contribution consists in the determination of the protein concentration, and subsequently of the administered dose, and the detection of amino acid homologs.

In its early years, amino acid composition analysis was essential in the determination of the protein identity. Nowadays, it has been substantially displaced in this application by more modern and faster techniques such as mass spectrometry. In biochemistry, amino acid analysis is in addition applied to the determination of the protein–protein interaction ratio in various complexes and to the identification by database search of proteins separated by one- or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Except for their basic function as peptide building units, amino acids and their analogs exert important functions as neurotransmitters, signal transducers and free radical scavengers. Taking into consideration the limitations of the fast mass spectrometry techniques in identifying and quantifying free low-molecular-mass compounds, amino acid analysis remains one of the principal detection and quantification methods for this class of molecules.

The determination of the amino acid composition of a polypeptide is a relatively complex analytical process, consisting of two steps, complete hydrolysis of the substrate to liberate the residues, followed by chromatographic analysis and quantification of the liberated amino acids. Hydrolysis is an important step and its proper performance is a prerequisite of a successful analysis. Comparative studies have shown that errors in the performance of the hydrolysis are primarily responsible for variations in the determined compositions [3,4]. Whereas improvements leading to higher sensitivity, precision in residue quantification, speed and automation of analysis have been reported, no single hydrolysis method for all residues still exists, so that hydrolysis represents the least controllable part of the whole analysis.

The scientific contributions to amino acid com-

position analysis in the last years are relatively fewer in comparison with other methodologies. The most important developments of the technology are the use of microwave radiation for sample hydrolysis, the ongoing replacement of the ''classical'' ion exchange by reversed-phase chromatographic analysis and the application of the amino acid composition analysis for large-scale protein identification. Here we provide a review of the articles that appeared mainly in the last ten years, on protein hydrolysis techniques and their effects on the determination of the composition of peptides and proteins.

2. Hydrolysis

2.1. Hydrolysis methods

The aim of every hydrolysis method is the quantitative liberation of all amino acids of the substrate and the quantitative recovery of them in the hydrolyzate. No hydrolysis method can accomplish such a task, as hydrolysis and destruction of the liberated residues proceed simultaneously. Several factors affect the completeness of the hydrolysis process, such as temperature, time, hydrolysis agent and additives. The combination of these factors is critical for the determination of the sensitive residues and their influence is largely investigated by ''trial and error'' approaches. It is relatively easy to obtain an accurate analysis of a mixture of amino acid standards or of a short synthetic peptide after hydrolysis, but it is often difficult to obtain an accurate analysis of a ''real'' protein, having been subjected to several purification steps and carrying post-translational modifications.

Because a large number of diverse samples are usually analyzed in a laboratory, the choice of the right hydrolysis method largely depends on the aim of the particular analysis. In most cases, a compromising approach is chosen. Polypeptide hydrolysis can be accomplished by either chemical or enzymatic means. Chemical hydrolysis can be performed under either acidic or basic conditions. The acidic hydrolysis itself can be performed in a liquid- or a gas-phase mode. In their majority, the recent articles deal with hydrolysis under acidic conditions, whereas only a small number of modifications on the basic or the enzymatic digestion methods have been reported.

2.2. Acidic hydrolysis

2.2.1. Hydrolysis with hydrochloric acid

Detailed descriptions of the conditions of acid hydrolysis can be found in numerous classical articles and reviews, of which a partial list only is presented here [5–24]. Treatment with hydrochloric acid (HCl) is the most common hydrolysis method. This is due to the convenience of application of this reagent, as it can be used in both the liquid- and gas-phase modes and can be evaporated afterwards, so that the hydrolyzate is recovered in a small volume of the reconstitution buffer. The analysis can be performed with small amounts of substrate, a factor which is often critical if only limited quantities of the protein sample are available.

The conventional acidic hydrolysis uses 6 *M* HCl for 20 or 24 h at 110°C in vacuo [5,14]. Under the conditions of the conventional acidic hydrolysis, asparagine and glutamine are completely hydrolyzed to aspartic acid and glutamic acid, respectively. Tryptophan is completely destroyed and cysteine can not be directly determined from the acid-hydrolyzed samples. Tyrosine is partially destroyed by traces of impurities present in the hydrolysis agent. Serine and threonine are partially hydrolyzed as well and usually losses of about 10 and 5%, respectively, occur [20]. For the last amino acids, correction factors can be employed if a precise quantification is desired [18,25–27]. The simplest correction employs extrapolation to zero time [14]. Darragh et al. applied a series of hydrolysis times to study the amino acid losses during acid hydrolysis. Most of the residues underwent some degree of loss during hydrolysis. The greatest losses were observed with cysteic acid and serine [27]. Protective agents, such as phenol, thioglycolic acid, mercaptoethanol, indole or tryptamine, are added to the sample solution to reduce losses of the various residues during hydrolysis [14] (see also Section 2.9).

The amino acid sequence of the substrate is critical for the outcome of the hydrolysis. The side chains of the aliphatic residues cause a steric hindrance and the tertiary structure of the protein affects the degree of unfolding as well. The most difficult to

cleave bonds are those between aliphatic residue-containing amino acids, such as in the sequences Ile–Ile, Val–Val, Ile–Val, which are particularly resistant to hydrolysis and are cleaved to about 50–70% at 110°C in 24 h. Hydrolysis for 92 h or even 120 h may be necessary to achieve a quantitative cleavage of these bonds [20]. Complete digestion of such bonds can be achieved in a shorter time (48 or 72 h, [28]). During such a prolonged hydrolysis, the more sensitive residues undergo even larger losses than mentioned above.

Gas-phase hydrolysis delivers results comparable to those of the liquid-phase hydrolysis. In addition, it reduces hydrolyzing agent-dependent contamination and with the use of specially designed hydrolysis vessels, accommodating a large number of vials, it allows the simultaneous hydrolysis of a great number of samples [16,19,24]. A brief comparison of the benefits of the various hydrolysis approaches is given by Davidson [22].

2.2.2. Hydrolysis with sulfonic acids and other agents

Sulfonic acids are strong, non-oxidizing reagents and find wide applications as hydrolyzing agents [14,20,29–32]. The newer articles on hydrolysis deal to a large extent with the use of these reagents. Table 1 provides a list of the most common acid hydrolysis agents.

Simpson et al. used methanesulfonic acid (MSA) and performed hydrolysis in the presence of low concentrations of 3-(2-aminoethyl)indole [32]. The

Table 1
Agents used in acidic hydrolysis of proteins

	Hydrolysis agent	Hydrolysis conditions	Additives	Method specific for determination of	Ref.
1	6 *M* HCl	110°C, 24 h	0.02% Phenol	All residues except of Cys, Trp	[5,14,20,24]
2	6 *M* HCl or 4 *M* MSA	110°C, 24 h	0.2% Sodium azide	Cys	[102]
3	6 *M* HCl	110°C, 18 h	5% Thioglycolic acid, 0.1% phenol, 3,3′-dithiodipropionic acid	Cys	[105,106]
4	6 *M* HCl		3-Bromopropylamine	Cys	[103]
5	6 *M* HCl	145°C, 4 h	Samples previously oxidized with performic acid	Cys, Met, Lys	[107,108]
6	4 *M* MSA		3-(2-Aminoethyl)indole	Trp, methionine sulfoxide	[32]
7	4 *M* MSA	115°C, 22 h	Samples previously alkylated, tryptamine	All residues	[15]
8	4 *M* MSA	160°C, 45 min		All residues	[33,34]
9	4 *M* MSA or 5.7 *M* HCl	150°C, 90 min	Oxidation with performic acid 50°C, 10 min	All residues	[36]
10	3 *M* *p*-Toluenesulfonic acid			Methionine sulfoxide	[35]
11	12 *M* HCl–propionic acid (1:1)	150°C, 90 min		Resin-bound peptides	[23]
12	12 *M* HCl–propionic acid (1:1)	840 W, 1–7 min, microwave		Resin-bound peptides	[38,40]
13	*p*-Toluenesulfonic acid	15 min, microwave			[44]
14	DCl	medium power, 30 min, microwave		Sensitive residues	[46]
15	2.5 *M* Mercaptoethanesulfonic acid	176°C, 12.5 min	*S*-Pyridylethylated samples	Cys, Trp	[109]
16	6 *M* HCl–TFA (6:3)	120°C, 16 h	Dithiodiglycolic acid, 1% phenol	Cys	[110]
17	HCl		Thioglycolic acid	Trp	[113–115]
18	HCl	110°C, 24 h	0.4% β-Mercaptoethanol	Trp	[116]
19	HCl	166°C, 25 min or 145°C, 4 h	3% Phenol	Trp	[94]
20	HCl	145°C, 4 h	Tryptamine	Trp	[117]
21	6 *M* HCl	145°C, 4 h gas-phase	Tryptamine[3-(2-aminoethyl)]indole	Trp	[118]
22	TFA–HCl (1:2)	166°C, 25–50 min	5% Thioglycolic acid	Trp, Met	[115]
23	7 *M* HCl, 10% TFA		10% Thioglycolic acid, indole	Trp	[119]
24	3 *M* Mercaptoethanesulfonic acid	176°C, 25 min, gas-phase		Trp	[95]

major advantage of methanesulfonic acid, in comparison to HCl, is that, in addition to the other residues, it allows the determination of tryptophan and methionine sulfoxide. Tryptophan is destroyed during conventional hydrolysis with HCl. Methionine sulfoxide, the most common and easily to generate oxidation product of methionine, cannot be determined following hydrolysis with HCl, as it is partially reduced to methionine during hydrolysis. Inglis reported a single hydrolysis method for all amino acids including tryptophan and cysteine. The method involves alkylation with iodoacetic acid or 4-vinylpyridine and hydrolysis of the derivatives with 4 *M* methanesulfonic acid at 115°C for 22 h in the presence of 0.02% tryptamine [15]. The approach, however, includes many steps and is impractical for serial analyses. It is usually applied if a particular reason exists, for example introduction of radioactive labels into proteins.

Chiou and Wang applied hydrolysis with 4 *M* methanesulfonic acid at 160°C for 45 min. Oxygen was removed from the hydrolysis mixture by flushing with nitrogen instead of removal by evaporation. They obtained amino acid recoveries similar to those achieved following hydrolysis at 110°C for 24 h [33,34]. In a recent study, Weiss et al. used model proteins and compared the effect of the hydrolyzing agents, HCl and methanesulfonic acid, on the determination of their composition. They used as criterium the success of identification of the substrate protein by search in databases introducing the data derived from the particular analysis. Amino acid compositions obtained following hydrolysis with 4 *M* methanesulfonic acid were closer to the theoretical ones compared to those obtained after hydrolysis with 6 *M* HCl [24].

Methanesulfonic acid is not volatile, so it cannot be evaporated after hydrolysis. For chromatographic analysis of the digestion products, the hydrolyzate needs to be diluted (usually four-fold) and the pH to be adjusted to a value near 2.3, with the consequence that larger sample amounts have to be used for hydrolysis. Proteins blotted onto membranes are not usually hydrolyzed with methanesulfonic acid because the reagent as non-volatile cannot be used in the gas-phase hydrolysis mode. Use of the reagent in the liquid phase could result in contamination of the small amounts of the substrate. Thus, in spite of its obvious advantages, routine use of methanesulfonic acid in protein hydrolysis is less popular than that of HCl.

Hydrolysis with 3 *M* *p*-toluenesulfonic acid at 110°C for 22 h was applied for the determination of methionine sulfoxide in protein and food, in addition to the other residues [35]. Using this hydrolysis agent, methionine and methionine sulfone were quantitatively recovered, whereas methionine sulfoxide was partially converted to methionine during hydrolysis (about 94% recovery). The presence of carbohydrates did not affect the yield [35]. Methionine sulfone is stable and can be determined following hydrolysis with HCl as well.

Liu and Boykins reported the use of 4 *M* methanesulfonic acid or 5.7 *M* HCl as hydrolysis agents and the performance of the digestion in hermetically sealed microcapillary tubes, thus, reducing the air to liquid ratio. The method makes unnecessary the time-consuming tube sealing under vacuum or the flushing with nitrogen. Oxidation of the samples with performic acid at 50°C for 10 min instead of at 0°C for 4 h, before hydrolysis, was 30-fold faster and yielded good recoveries of cysteic acid and methionine sulfone [36]. Hydrolysis of resin- bound peptides, for monitoring peptide synthesis, was performed with a mixture of 12 *M* HCl–propionic acid (1:1, v/v) at 150°C for 90 min [23].

2.3. Microwave radiation-induced hydrolysis

One of the most significant, recent developments in the performance of compositional analysis is the use of microwave radiation energy for hydrolysis of protein samples. This usually takes place in specially designed pressurized apparatuses. The mechanism by which microwave heating occurs offers certain advantages to conventional decomposition. Energy transfer takes place by polarization instead of collision of the molecules. The magnitude of the polarization effect depends on the dipole character of the molecules involved and is very high for water molecules [37].

The substrates can be hydrolyzed in either the liquid- or the gas-phase mode with HCl, methanesulfonic acid (in the liquid-phase only) or other reagents. The instant uptake of the radiation energy results in reduction of the overall hydrolysis time

from many hours, required for conventional hydrolysis, to a few minutes, usually 1–30 min for liquid-phase and 20–45 min for gas-phase hydrolysis, so that the protein samples can be hydrolyzed and analyzed within a few hours and this represents the major advantage of the method [24,38–49]. Simultaneously, certain residues may be recovered in higher yields in the hydrolyzate, in particular when protective substances, such as phenol and thioglycolic acid, are added during hydrolysis [49].

Various modifications of the conventional hydrolysis have been introduced which can be more advantageous for the particular application. Joergensen and Thestrup hydrolyzed purified proteins and ''real'' proteins, containing carbohydrates, lipids, nucleic acids and minerals, with HCl in a microwave oven at 150°C for 10–30 min. The results were similar to those of conventional hydrolysis. Addition of the scavenger phenol stabilized tyrosine, phenylalanine and histidine in performic acid-oxidized samples. Addition of thioglycolic acid stabilized methionine and partially tryptophan [49]. Yu et al. used 6-mm thick-wall hydrolysis vials of PTFE and placed them in containers of the same PTFE material of 75 mm internal diameter. Hydrolysis was performed with propionic acid–12 *M* HCl (1:1, v/v) at 840 W for 1–7 min. They achieved a fast cleavage and an accurate analysis of the first residue released from Merrifield solid-phase resins [40].

Pecavar et al. used liquid-phase hydrolysis with *p*-toluenesulfonic acid in a microwave oven for 15 min to completely hydrolyze peptide bonds. The results were comparable to the results of conventional hydrolysis for 48 h [44]. Microwave hydrolysis was also applied to specifically cleave peptide bonds at the carboxyl- and amino-terminal ends of aspartyl residues [45].

Peter at al. used deuterium chloride as hydrolysis agent and found that at an intermediate radiation power for 30 min, the recoveries of the sensitive residues were optimal and the degree of racemization minimal [46]. Tatar et al. studied the effects of pressure and time of hydrolysis on the recovery of the hydrolyzate components. Depending on the pressure and time applied, the recoveries varied from 63 to 111% [47].

In a comparative study, Weiss et al. found that the results derived from the HCl and methanesulfonic acid hydrolysis using microwave radiation are equally reliable with those of the conventional hydrolysis except for slightly higher losses for threonine and serine [24]. It is possible that with the use of a mixture of hydrolysis agents, such as HCl–propionic acid, the recoveries of threonine and serine will improve. Because one single autosampler vial can be used for sample hydrolysis and residue analysis, a significant reduction of sample contamination, reagent consumption and handling time can be achieved by the microwave hydrolysis approach, all of which represent important steps towards process automation [42].

2.4. Alkaline hydrolysis

Alkaline hydrolysis is almost exclusively used for determination of tryptophan which is stable under basic conditions. Alkaline hydrolysis is also applied if the protein sample contains a large percentage of carbohydrates, as it is the case with foods [14] and in the formulation solutions of pharmaceutical proteins, which usually include high percentages of monosaccharides [50]. The major drawback of the method is that serine, threonine, arginine and cysteine are destroyed and the other amino acids are racemized. Alkaline hydrolysis is usually performed with NaOH, KOH or more seldom with barium hydroxide [51–53]. In the last years, only few articles on alkaline hydrolysis of proteins for amino acid analysis were published, whereas several contributions appeared dealing with tryptophan determination, following acid hydrolysis in the presence of various scavengers (see Section 2.10.2). Acidic hydrolysis is applied when no high accuracy is aimed in the tryptophan determination. Delhaye and Landry reported, however, that 5–10% of the tryptophan present in the various foods are lost during alkaline hydrolysis prior to analysis [54].

Base hydrolysis is often applied in the determination of phospho-amino acids. Hydrolysis with KOH was applied for the quantification of phosphorylated and sulfated tyrosine [55–57]. Alkaline hydrolysis was also used for analysis of phosphohistidine [58], for the release of phosphate from phospho-serinyl and threonyl residues [59] and the analysis of phospho-amino acids of blotted proteins [60].

By varying the conditions of the alkaline hy-

drolysis, certain substances, like vitamins, amphetamines, thiophenol, *N*-acetylcysteine, present in physiological samples, were detected [61–67]. Alkaline hydrolysis is applied in nucleic acid research [68–70] and in general, for the digestion of a wide variety of substrates in order to release products of interest, such as carbohydrate moieties, lipids etc., [71–73]. Following alkaline hydrolysis of proteins, aminomalonic acid was detected, which had been probably destroyed during acidic hydrolysis [73,74].

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of proteins for amino acid analysis is seldom applied, when the investigation of the degree of racemization in a polypeptide is the aim of the analysis. Enzymatic hydrolysis has the advantage that it allows the quantification of asparagine and glutamine and of other sensitive residues, which are otherwise destroyed during chemical digestion, of residues carrying side-chain modifications and that it does not induce racemization during digestion. Its widespread application has been hampered by the relative specificity of the proteases for certain residues, thus, skewing quantitative results, in particular, if only small amounts of sample are available. For a complete digestion, incubation with several enzymes is required and the reactions usually last long, therefore, the method is not applied for serial analyses.

Church et al. used a system of immobilized proteases and obtained a complete hydrolysis within 18–24 h and recoveries 92–103% in comparison with standard acid hydrolyzates [75]. Treatment of the substrate with a side-chain non-specific L-amino acid oxidase was employed to specifically degrade L-amino acids. The remaining residues were in the D-form and could be quantified with one of the standard analytical techniques (see Section 2.11) [76]. D'Aniello et al. developed a hydrolysis method comprising a partial chemical digestion with 6 *M* HCl at 80–90°C for 15 min and a sequential enzymatic digestion with pronase at 50°C for 12–16 h, followed by digestion with leucine aminopeptidase and peptidyl-D-amino acid hydrolase for 24 h. The total hydrolysis yielded average recoveries of 97–100% and the racemization was less than 0.002% [77]. However, the method is complex and lengthy and represents the method of choice when no introduction of racemization is desired.

Thoma and Crimmins used a system of proteases in a fully automated exopeptidase digestion procedure for the determination of partial sequences from the N- and C-terminal ends of proteins [78]. With the exception of articles dealing with the generation of partial N- or C-terminal sequences (sequence ladders) and specific cleavages along a peptidic chain [79,80], otherwise few contributions to complete enzymatic hydrolysis leading to amino acid analysis appeared in the last years. Smith and Wheeler provide a summary of the common enzymatic digestions employed for analytical purposes [81].

2.6. Specific cleavages

In addition to the acidic, alkaline and enzymatic hydrolysis, specific cleavages between certain peptide residues have been reported. Allen and Campbell reported a specific cleavage with copper(II) between the sequences Ser–His or Thr–His, at the N-terminal side of the hydroxyaminoacyl residue. Cleavage of other histidine-containing peptides also occurs at a 10- to 110-fold lower rate. The reaction is inhibited by amine-containing buffer components like Tris [82]. Smith summarizes certain types of chemical cleavage of peptide bonds, mainly involving tryptophan and methionine residues, of proteins in solution and blotted onto poly(vinylidene difluoride) (PVDF) membranes [83]. However, these types of cleavage do not lead to a complete digestion, so they are of limited interest for compositional analysis.

2.7. Hydrolysis of proteins blotted onto membranes

A significant development in protein analysis during the last decades is the electrotransfer of proteins separated by one-dimensional (1D) or 2D-PAGE to membranes and the analysis of the blotted proteins by Edmann degradation and compositional analysis. This is particularly important since it is the most efficient approach to identify by physicochemical means proteins in complex mixtures which are only available in small quantities. Ozols reported that if the protein band is going to be used for amino acid

analysis only, the protein transfer to nitrocellulose membrane or nylon-based support is more advantageous for the protein recovery, compared to the sequencing-resistant PVDF membranes [20]. However, the recent articles almost exclusively report the use of PVDF membranes for both N-terminal sequencing and compositional analysis.

N-Terminal sequence analysis and composition analysis represent today's routine techniques of any protein analytical laboratory. Serial analysis of blotted proteins, previously separated by 2D-PAGE, made possible the large-scale identification by amino acid analysis (see Section 4). There are numerous articles on the use of protein blotting as part of a protein characterization process, as well as for determination of cysteine, tryptophan, and detection of post-translational modifications [60,84–92].

Blotted proteins are usually hydrolyzed with HCl in the gas-phase mode to avoid contamination from the reagent. Lottspeich et al. [90] and Yan et al. [91] studied several sources of contamination during hydrolysis of blotted proteins. The most frequent amino acid contaminants present in PVDF membrane extracts are glycine and glutamic acid [20,93]. Quantification of the less frequent residues histidine and methionine is often imprecise. Determination of the very low-abundance residue tryptophan from PVDF membranes has been reported [94], however, on account of possible interference of background peaks with the peak corresponding to this low-frequency amino acid, the accuracy of the determination can be ambiguous. We occasionally met difficulties with the quantification of phenylalanine from blotted proteins, following post-column derivatization with *o*-phthalaldehyde (OPA) [93].

A critical step in the analysis is the equal extraction of the liberated residues so that the composition is not altered. Hydrolyzate extraction has been reported with various solvents, among others with 0.1 *M* HCl–30% methanol [88,95], 60% acetonitrile, containing 0.01% trifluroacetic acid (TFA) [91], 0.1 *M* HCl, containing 60% acetonitrile [93] and 88% formic acid [20]. Weiss et al. used 0.1 *M* HCl or 0.1 *M* HCl, containing 60% acetonitrile and compared the hydrolyzate extraction employing the two solvents. They used the compositions determined to identify the substrates by search in databases. Following HCl extraction, no protein identification could be assigned, whereas after inclusion of the organic solvent, the proteins were successfully identified [24]. In general, more accurate compositions were obtained from proteins in solution in comparison with blotted proteins. For blotted proteins, conventional hydrolysis, in the liquid- or gas-phase mode, delivered compositions closer to the theoretical ones, in comparison with microwave radiation-induced hydrolysis [24]. Protein quantification resulting from the amino acid analysis of blotted proteins is not very reliable since the hydrolyzate recovery is not quantitative (Section 5.1).

2.8. Hydrolysis of proteins in polyacrylamide gels

Amino acid analysis of proteins hydrolyzed in-gels has been reported a long time ago [96]. The method has not found a wide application, thus, it is of minor importance for amino acid analysis today. Hydrolysis of proteins in gels, following separation by one- or two-dimensional electrophoresis could result in a simplification of the analysis, as the protein electrotransfer to membranes would be avoided. This could be of great advantage for large-scale protein identification. Transfer of proteins from gels to membranes represents one additional, laborious step, the transfer is seldom a quantitative process, especially for complex protein mixtures, spots on membranes are often more diffuse in comparison with spots in gels and a precise correlation between these two types of spots is sometimes difficult.

There probably exist technical difficulties that did not yet allow the method to be applied in serial, large-scale protein analysis. The major drawbacks of the method are the large amounts of ammonia produced during hydrolysis, affecting the detection of several residues, and the large quantities of acrylamide-derived material entering the analytical columns. To partially overcome the problem of the ammonia production, fluorescamine detection of the amino acids has been employed during the analysis [96,97]. Interestingly, Hashimoto et al. reported the amino acid analysis of protein bands excised from gels, following post-column derivatization with OPA. They hydrolyzed the proteins together with the gel, analyzed the hydrolyzate by high-performance liquid chromatography (HPLC), and could identify 16 residues, including cysteine. The methionine re-

covery was inconsistent. Thioglycolic acid protected the amino acids tryptophan, cysteine and methionine [98].

An alternative approach to the direct in-gel hydrolysis of proteins is the electroelution of the protein from the gel slice and hydrolysis of the eluate after lyophilization and reconstitution [99]. Brown and Howard investigated the background contaminations, the amounts of which were analogous to the gel volume. Typically, serine, aspartic acid, alanine and threonine were determined as background residues of gels [99]. The method is laborious and the eluate recovery is often poor.

2.9. Effect of time and temperature on the hydrolysis performance

Whatever the hydrolysis conditions are, they represent a compromise between performance of reaction and conservation of the reaction products, aiming to deliver the best results, depending on the goal of the analysis. Several intra- and interlaboratory studies deal with the effects of time and temperature on the completeness of hydrolysis. Collaborative research studies showed that hydrolysis is the major source of inaccuracy in an amino acid analysis process [4,17,18]. Automated hydrolysis instrumentation may help to control the conditions more precisely.

Gehrke and Zumwalt found that hydrolysis with 6 *M* HCl at 145°C for 4 h gives results comparable to those of the conventional hydrolysis at 110°C for 24 h [100]. The precision of analysis increases with the amino acid concentration in the sample solution. The values for isoleucine, valine and serine should be increased by 6% to correct for incomplete recovery of these residues. They consider that two hydrolyzates with different times are sufficient for a precise quantification of valine and isoleucine, with extrapolation to infinite, and to zero time for serine and threonine [17,18]. They also found that PTFE-lined screw-cap tubes give comparable results to those obtained with sealed ampoules [100].

Darragh et al. used up to 18 hydrolysis intervals ranging from 2 to 141 h for accurate determination of the residues. They considered 10 hydrolysis intervals with one interval greater than 100 h as a good compromise between accuracy and cost of analysis. Such an approach is expected to yield a mean recovery of 100% [27]. The best mean yields were observed for methionine sulfone, followed by valine and histidine with almost quantitative recoveries after 141 h. The greatest loss values were found for cysteic acid, serine, threonine, arginine and aspartic acid [27].

Malner and Schroeder found that hydrolysis with methanesulfonic acid at 115°C for 22 h yielded recoveries equal or higher than those obtained at 115°C for 70 h or at 150°C for 22 h. Triple evacuation of the hydrolysis tube, alternated with nitrogen flush, gave recovery improvements over single evacuation [101].

As the reaction rate doubles for every 10°C increase, heating at 160°C for 45 min corresponds to heating at 110°C for 24 h. Chiou studied the effect of time on the hydrolysis performance at 160°C in nitrogen-flushed screw-cap tubes. The recoveries of threonine and serine decreased to about 50% in 4 h, whereas the recoveries of valine and isoleucine increased to about 100% in the same time [34]. In general, elevated temperatures and shorter hydrolysis times gave results similar or superior to those of conventional hydrolysis at 110°C for 24 h [34]. In addition to residue recovery, the duration of hydrolysis affects the degree of racemization of the hydrolyzate (see Section 2.11).

2.10. Quantification of sensitive residues

A great percentage of the recent contributions to compositional analysis concerns the hydrolysis conditions for the determination of the sensitive residues cysteine and tryptophan. The reported methods represent alternatives to established determination methods for these residues (Table 1). There is not yet a single and simple liberation method for all residues simultaneously.

2.10.1. Cysteine determination

Cysteine cannot be accurately determined as a free amino acid on account of side reactions during hydrolysis and of insufficient determination on the amino acid analyzer. Cysteine is usually determined as cysteic acid, following oxidation with performic acid [9] or as alkylation product with iodoacetate or 4-vinylpyridine [15]. The oxidation, carboxymethyl-

ation or *S*-pyridylethylation are labor- and time-demanding approaches. An efficient and simple quantification method for cysteine, consisting in the addition of 0.2% sodium azide in the hydrolysis solution, was reported by Manneberg et al. [102]. Hydrolysis with 6 *M* HCl or 4 *M* methanesulfonic acid resulted in practically quantitative oxidation of cysteine to cysteic acid, that was efficiently separated from the other residues and quantified [methanesulfonic acid should be used in the absence of the scavenger 3-(2-aminoethyl)indole]. The addition of azide resulted in partial oxidation of methionine to methionine sulfone, so that the analysis needs to be performed with and without azide for the determination of methionine. The recoveries of the other residues were not affected. The cysteine quantification in the presence of sodium azide is superior to the performic acid method in that it is faster, more convenient to perform and allows a more precise determination of the cysteine, tyrosine and histidine residues [102] (warning: concentrated solutions of azides, upon contact with strong acids, can lead to explosions).

Hale et al. reported the use of 3-bromopropylamine to derivatize cysteines [103]. Cysteine was determined as *S*-3-aminopropylcysteine which elutes in a unique position on four different amino acid analysis systems. The recovery was about 90% [103]. Alkylation with 3-bromopropylamine can be applied for cysteine detection during N-terminal sequence analysis as well [104]. Cysteine was also determined as a 3,3′-dithiodipropionic acid derivative [105] or after hydrolysis with 6 *M* HCl at 110°C for 18 h in the presence of 5% thioglycolic acid and 0.1% phenol in one step together with the other residues [106]. Ploug and co-workers applied in situ cysteine alkylation of proteins blotted onto PVDF membranes with a recovery of 90%. The modified residues were analyzed by both N-terminal sequence and amino acid composition analysis. The protein transfer to the membrane was necessary to avoid a desalting step before derivatization [84,85].

Cysteine and methionine, after oxidation with performic acid, were determined as cysteic acid and methionine sulfone, with recoveries of 95 and 101%, respectively [107]. Gehrke et al. applied oxidation with performic acid and hydrolysis with 6 *M* HCl at 145°C for 4 h and could determine cysteine, methionine and lysine in a single preoxidized hydrolysate [108]. Yamada et al. reported a 90% or higher recovery of both tryptophan and cysteine, in addition to the other amino acids, following hydrolysis of *S*-pyridylethylated samples with 2.5 *M* mercaptoethanesulfonic acid at 176°C for 12.5 min [109]. Hoogerheide and Campbell applied hydrolysis in the presence of dithiodiglycolic acid which allowed the determination of cysteine with a recovery of over 90% in addition to the other amino acids [110]. Hydrolysis of proteins with mercaptoethanesulfonic acid or hydrolysis with HCl in the presence of mercaptoethanol or thioglycolic acid, if care is not taken to ensure cysteine oxidation before amino acid analysis, can lead to overestimation of proline, as one hydrolysis product comigrates with proline in many ion exchange analytical systems [111].

2.10.2. Tryptophan determination

Tryptophan is a low-abundance amino acid and its determination can strongly facilitate protein identification by compositional analysis. This amino acid is destroyed during HCl digestion, in particular in the presence of carbohydrates [14,15,20]. It is usually determined following hydrolysis with methanesulfonic acid [32] or mercaptoethanesulfonic acid [30]. If the major goal of the analysis is the determination of tryptophan, alkaline hydrolysis is the most proper digestion method [51,52]. In a collaborative study, tryptophan was determined after hydrolysis of the substrates with 4.2 *M* NaOH with a mean recovery of 85% (59–102%) [112]. Tryptophan is well separated from the other residues by ion-exchange chromatography (Fig. 1B). Following base hydrolysis with barium hydroxide, Huet and Pernollet used Fractogel TSK for tryptophan separation [53].

Hydrolysis with methanesulfonic acid is usually performed at 110°C for 24 h in the presence of 3-(2-aminoethyl)indole [14,24,32]. Chiou and Wang applied hydrolysis with 4 *M* methanesulfonic acid at 160°C for 45 min to determine all residues including tryptophan [33]. Yamada et al. determined tryptophan by hydrolysing *S*-pyridylethylated samples with mercaptoethanesulfonic acid [109].

In addition to the sulfonic acids, hydrolysis has also been performed with HCl in the presence of various scavengers added to protect tryptophan. As scavengers have been used thioglycolic acid

[49,113–115], mercaptoethanol [116], phenol [94], tryptamine [117] and tryptamine[3-(2-aminoethyl)indole] [118]. The last additive allowed an 80–98% recovery of tryptophan, following gas-phase hydrolysis with 6 *M* HCl at 145°C for 4 h.

Muramoto and Kamiya added 3% (w/v) phenol to the hydrolysis mixture to protect tryptophan which was recovered to 80%, following digestion with 6 *M* HCl at 166°C for 25 min or at 145°C for 4 h. The method allowed the determination of tryptophan from proteins blotted onto PVDF membranes. However, background peaks can disturb the quantification of this low-abundance residue [94]. Yamada et al. tried the method of Muramoto and Kamiya for tryptophan determination without being able to reproduce their high yields. Yamada et al. achieved recoveries of 42–58% for tryptophan released from proteins and of 77% for free tryptophan [109]. Ng et al. added 0.4% β-mercaptoethanol and reported an 100% tryptophan recovery after hydrolysis of non-glycosylated proteins at 110°C for 24 h [116]. The method is not applicable to carbohydrate-rich samples. Fábián et al. found that addition of tryptamine at 0.08–0.33 mg per 1 mg of amino acids protected tryptophan during digestion with HCl at 145°C for 4 h [117].

Yokote et al. hydrolyzed carboxymethylated protein samples with a TFA–HCl mixture (1:2, v/v) at 166°C for 25–50 min in the presence of 5% thioglycolic acid and obtained tryptophan recoveries of 73–88%. The thioglycolic acid addition improved the methionine recovery as well [115]. Yano et al. applied gas-phase hydrolysis with a mixture of 7 *M* HCl, 10% TFA and 20% thioglycolic acid in the presence of indole and obtained a tryptophan recovery of 75% [119]. Tryptophan determination from proteins blotted onto PVDF membranes was also achieved by gas-phase hydrolysis with 3 *M* mercaptoethanesulfonic acid at 176°C for 25 min. The hydrolyzate was extracted with 0.1 *M* HCl, containing 30% methanol [95].

2.11. Racemization

Amino acid analysis is indispensable for the determination of amino acid enantiomers. Racemization of amino acids takes place during chemical hydrolysis of proteins. Determination of the real value of the D-amino acids in proteins (before hydrolysis) is of importance for pharmaceutical and food science. Many microorganisms, in particular marine species, contain large percentages of D-forms of amino acids and food treatment at elevated temperatures may occasionally induce a racemization. Imai et al. reviewed the distribution of D-amino acids in the human body, their roles in various diseases and the methods of their analysis [120]. The hydrolysis-induced racemization of free amino acids is lower (20–80%) than that of the residues in a peptide. The quantification of the D-amino acid forms in a polypeptide requires the use of specific hydrolysis methods, such as that reported by D'Aniello et al. [77]. This and similar methods usually involve multiple enzymatic digestions, are lengthy and consequently impractical for routine analysis.

Csapó et al. studied the effect of temperature on the racemization of amino acids during acidic hydrolysis. They found that treatment with 6 *M* HCl at 160°C for 1 h or at 170°C for 45 min caused a lower racemization (about 50%) compared to that produced by conventional hydrolysis (110°C, 24 h). Alkaline hydrolysis with 4 *M* barium hydroxide at 110°C for 48 h resulted in a complete racemization of all residues, including tryptophan [121]. Peter et al. applied microwave hydrolysis and obtained a minimal racemization at an intermediate irradiation power and a hydrolysis time of 30 min [46]. Microwave-induced hydrolysis was found to be responsible for a higher degree of racemization in comparison with conventional hydrolysis [122]. Weiss et al. found that liquid-phase hydrolysis generated a higher degree of racemization when microwave radiation energy was used. The D-forms represented about 7.5% of total residues after liquid-phase hydrolysis, compared to 4.5% of D-forms generated in the gas-phase hydrolysis mode. In comparison, conventional thermal hydrolysis resulted in racemization of 4.1% of total amino acids in the liquid- and 4.5% in the gas-phase mode [24]. The percentages of the D-forms of the various residues, with few exceptions, ranged between 0 and 10%. Hydrolysis with methanesulfonic acid (liquid-phase) produced less racemates (total D-amino acids 2.6%) than hydrolysis with HCl. Addition of phenol to the protein solution did not exert any effect on the degree of racemization during conventional hydrolysis, whereas it significantly

reduced the generation of racemates during microwave hydrolysis. In the absence of phenol, the total D-forms counted about 30 and 9%, following liquid- and gas-phase microwave hydrolysis, respectively. When phenol was included, these values decreased to about 7.5 and 3.7%, respectively [24]. Larger amounts of D-forms were generated during the liquid-phase hydrolysis using microwave radiation, in comparison to the gas-phase, because in the former case, the samples came in direct contact with the liquid reagent.

An efficient method for determination of D- and L-forms of amino acids involves the derivatization of the residues with OPA and a chiral N-acylated cysteine, like *N*-isobutyryl-L-cysteine. The diastereomeric derivatives are separated by reversed-phase HPLC, abolishing the necessity to utilize an expensive chiral stationary phase [24,123–125]. The method allows the detection of all D- and L-amino acids, including the achiral glycine. Cysteine enantiomers can be determined after previous oxidation to cysteic acid. The quantification of the tryptophan enantiomers is performed after hydrolysis with methanesulfonic acid. As the reagent OPA does not react with secondary amines, 9-fluorenylmethylchloroformate (Fmoc-Cl) is used in a second step to derivatize proline. This demanding pre-column procedure is no longer a problem with modern autosamplers. Synthetic peptide antibiotics and pharmaceutical formulations of L-amino acids for parenteral nutrition were investigated using this technique [125]. Low amounts of D-forms (0.1–0.9%) were determined in aqueous formulations of L-amino acids.

For the separation of the cysteine and methionine enantiomers, Griffith and Campbell used C_{18} reversed-phase silica columns, developed with a Cu^{2+}–L-proline chiral mobile phase, followed by derivatization with OPA [126]. An alternative method for the pre-column derivatization employs reaction with 1-fluoro-2,4-dinitrophenyl-5-alanine amide, separation on microbore columns and UV detection with a sensitivity at the picomole level [127].

The enantiomeric separation of phenylthiocarbamoyl amino acids (PTC-amino acids) separated by Edman sequencing was achieved on a tandem column of octyl silica and the chiral phase 3.3 phenylcarbamoylated β-cyclodextrins. Under optimal conditions, all individual PTC-amino acids were separated in 150 min, confining the method to very specific applications [128]. Liu et al. used metalchelate chiral capillary electrophoresis (CE) with laser-induced detection of D- and L-amino acid derivatives of fluorescein isothiocyanate. The method allows the determination of racemization in pharmaceutical products [129].

2.12. Effect of the components present in the hydrolysis solution on the amino acid quantification

Proteins to be subjected to amino acid composition analysis should possibly be free of any buffer components, that can interfere with the amino acid quantification, such as salts, metal ions, antibacterials and stabilizers. Otherwise hydrolysis can give variable yields for several residues. Under the drastic conditions of hydrolysis, agents present in the protein solution or in impure hydrolyzing reagents may react or catalyze reactions and thus affect the quantification of certain residues, in particular tyrosine, methionine and histidine. Several common contamination sources and their effects on residue recovery are described in various articles and notebooks [14,15,20], others are less known. Except of the agents present in the hydrolysis solution, many other precautions to avoid external contamination should be taken, such as the use of clean and pyrolized glassware, the wearing of gloves throughout the operation etc. The amino acid analysis operation procedures can be found in the various manuals and the references mentioned here.

Presence of NaCl, an ingredient of many buffers, in the hydrolysis solution affects the quantification of tyrosine [14,130]. Sodium azide, a widely used bacteriostatic agent, affects the quantification of methionine which is partially oxidized to methionine sulfone [131]. Simultaneously, cysteine is quantitatively oxidized to cysteic acid. Oxidation in the presence of 0.2% azide was used for cysteine determination [102]. Presence of glycerol, a stabilizing and protein folding promoting agent, in the hydrolysis solution affects the quantification of aspartate and glutamate. These residues were quantified to only 6–60% in the presence of glycerol [132]. A wide spectrum of esterification products between primary and secondary hydroxyl groups of

glycerol and both carboxyl groups of aspartate and glutamate were identified [132].

Moreover, protein samples should be free of ammonia salts or urea as these substances generate large amounts of ammonia during hydrolysis which can affect the chromatographic analysis. Carbohydrates leaching from the column material into the protein solution during chromatography, may affect the quantification of the protein by amino acid analysis [133]. In order to reduce variations in the amino acid determination, caused by the presence of salt, Inglis et al. added a standard amount of salt to the hydrolyzate before drying it [134].

The proper preparation of the samples is of decisive importance for a successful analysis. Whereas analysis of a standard mixture of amino acids can be accomplished reproducibly, to obtain accurate results from a hydrolyzed protein sample is much more difficult. Ideally, the protein solution should be dialyzed against water before hydrolysis. Dialysis of large protein quantities may not essentially affect the protein concentration in the dialyzate in comparison with that before dialysis, however, dialysis of small and dilute protein samples will certainly affect the protein concentration of the sample, so that the analysis cannot deliver reliable information about the protein concentration in the original solution.

Except for dialysis, the disturbing factors can be removed from the protein solution by employing other techniques, such as ultrafiltration or precipitation with organic solvents, like acetone, containing 0.2% HCl or with trifluoroacetic or trichloroacetic acid. The acid–acetone precipitation removes salts, glycerol and detergents. The TFA precipitation can be improved by the addition of deoxycholate [20]. Ultrafiltration with specific membranes, such as Centricon, can result in concentration of the sample and removal of most of the interfering substances by employment of repeated dilution and centrifugation steps. Similarly to dialysis, the precipitation or ultrafiltration approaches can alter the protein concentration in the reconstituted solution, so that these methods should be used with caution where protein quantification is the major aim of the analysis.

3. Amino acid separation techniques

Many chromatographic and electrophoretic techniques have been developed to resolve amino acids. The amino acids of the hydrolyzate need to be derivatized to be detected. The most popular methods are categorized into two groups, in relation to the chromatographic separation, the post-column and the pre-column derivatization methods of the free amino acids. Historically, the post-column technique has been developed first. It involves the separation of the residues on a cation-exchange column, followed by reaction of the effluent with a chromophore or fluorophore [1]. In a pre-column system, the amino acids are derivatized first in the hydrolyzate and then the derivatives are separated on a reversed-phase HPLC column.

The post-column technique has the advantage of chromatographically separating interfering analytes prior to reaction. This constitutes an advantage when dealing with unknown, complex mixtures. In general, the ion-exchange columns last longer and allow the separation of a larger number of amino acid homologs. On the other hand, post-column derivatization requires an on-line reactor, which adds complexity to the system, causes peak-broadening and additional baseline noise. Ion-exchange separation is the method of choice if a large number of amino acids has to be separated in a single run (about 40 amino acids and homologs). In particular, the ion-exchange method is applied to the separation of amino acids and derivatives in physiological fluids.

Resolution on reversed-phase columns is superior and their run times are substantially shorter in comparison with the ion-exchange chromatography. However, the performance of reversed-phase columns varies, the columns lose resolution and develop backpressure faster than ion-exchange columns [20]. Reversed-phase HPLC is the method of choice if a lower number of residues (about 20) has to be separated in one run [135].

Since the introduction of the HPLC pre-column derivatization methods, most of the amino acid analyzer suppliers have left the field of the ion-exchangers. To a great extent, HPLC instruments have replaced the ion-exchangers [136]. The ion exchangers in use are mostly older instruments. According to a survey by the Association of Biomolecular Resource Facilities, the post-column derivatization methods are used now about equally often, with a tendency of increase in the pre-column techniques over the past few years. The most popular

post-column method appears to be the ninhydrin method (39%) and of the pre-column techniques, the phenylisothiocyanate (PTC) method (40%) [137]. Here a review of reported developments in the separation techniques is given. More information regarding type and dimension of columns, run times, buffer and detection systems and instrument suppliers can be found in the references mentioned and in handbooks.

3.1. Post-column residue derivatization

Of the reagents used for residue derivatization in the post-column approach, ninhydrin is the one most frequently applied. It is still considered the standard method of the technology. The advantages of the method are that it is robust, reliable, reproducible and can detect a large number of amino acids and homologs. The major disadvantage is that the method is not sensitive. A minor disadvantage is that it requires two wavelengths for detection, 440 nm for proline and 570 nm for the other residues [138]. Analyses above a level of 100 pmol are very reliable.

In search for higher sensitivity, ninhydrin was replaced first by flurescamine [139]. Fluorescamine-derivatization lowers the detection limit to about 20 pmol. The high specificity of flurescamine for amino acids makes it a very versatile reagent [97]. Its reactivity with ammonia is about three orders of magnitude lower compared to that of ninhydrin, opening up the possibility to analyze proteins directly from polyacrylamide gels (Section 2.8).

Use of OPA as a post-column derivatizing agent [140] has the benefit of substantial savings in the instrument design and cost and the retainment of high sensitivity (1 pmol of an amino acid), however, it produces an absorbing baseline, which needs to be subtracted from the sample spectra (Fig. 1A). Fig. 1B shows an example of a chromatographic separation of amino acid standards on a cation-exchange column, followed by post-column derivatization with OPA. Both fluorescamine and OPA have the disadvantage over ninhydrin that they do not form a fluorophor with proline unless the imidazole ring is first opened by oxidation [141].

3.2. Pre-column residue derivatization

Various reagents are used to derivatize the hydrolyzate prior to separation. Derivatization with OPA, in the presence of a reducing agent, for primary amino acids, and with 9-fluorenylmethyl chloroformate (Fmoc-Cl), for secondary amino acids, are the most widespread pre-column methods and are available in automated instruments. The derivatives are separated on reversed-phase HPLC columns and the quantification is based on the fluorescent properties of the derivatives [101,142]. The total analysis time, including pre- column derivatization, separation, column washing and reequilibration cycles, is approximately 20 min.

The reagent naphthalene-2,3-dicarboxaldehyde (NDA) is a modification product of OPA and yields slightly more stable derivatives [143]. The effect of matrices in the sample, such as salts, buffers and surfactants, are similar to those using Fmoc derivatization.

A widespread application finds the derivatization with PTC. The derivatives are detected at 254 nm and the detection limit is about 1 pmol [144–147]. The PTC amino acid analysis is also available on automated equipment. Cohen and Strydom compared this method with the others in use [145]. Another non-fluorescent derivatization reagent is diethyl(ethoxymethylene)malonate. As derivatives of this reagent were determined proline and cystine, on a standard HPLC system, in addition to the other residues, at a level of sensitivity of 3 pmol, with spectrophotometric detection at 280 nm and within 35 min. The reaction time was 50 min and the derivatives were stable at room temperature [148]. Pre-column residue derivatization with dansyl chloride and chromatography on a C_{18} reversed-phase column has also been used for analysis of hydrolyzates of collagen and elastin [149].

An improved method for derivatization with Fmoc has been recently described [150]. The amino acid mixture was derivatized in borate buffer, pH 11.4 at room temperature for 40 min. This yielded exclusively monosubstituted histidine and disubstituted tyrosine and considerably less of the hydrolysis products of Fmoc. The detection limits were in the femtomole range and the derivatives were stable for 48 h.

Stocchi et al. used pre-column derivatization with 4-*N*,*N*-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) and separated the amino acid derivatives on a reversed-phase HPLC system in 25 min.

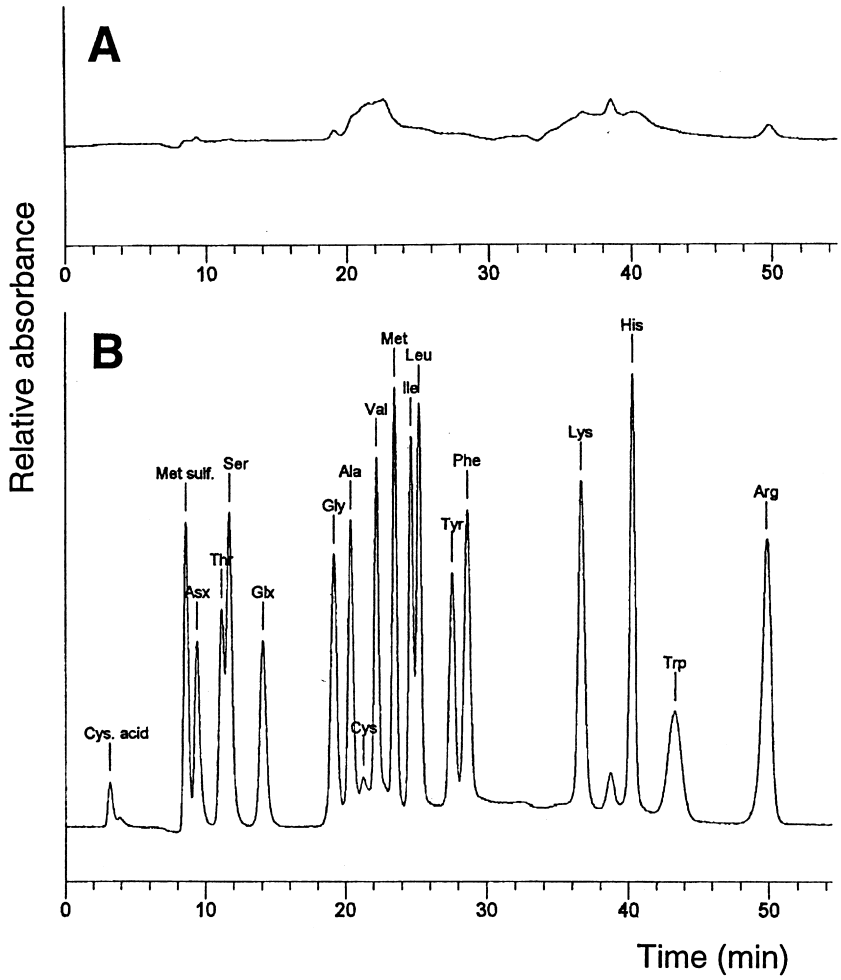


Fig. 1. Baseline (A) and amino acid separation (B) on a sodium cation-exchange column, following injection of 50 μl of loading buffer (A) or of 100 pmol amino acid standards, including tryptophan, cysteic acid and methionine sulfoxide. The separation was performed in a Dionex BioLC amino acid analyzer equipped with post-column derivatization with OPA. (B) The chromatogram was not corrected for baseline absorption.

The reaction was performed at 70°C for 10 min. The method allowed the determination of primary and secondary amino acids [151]. For the reaction of DABS-Cl with phosphoamino acid- and aminosugar-containing (*N*-acetylglucosamine and *N*- acetylgalactosamine) peptides, the hydrolysis time is critical. A 1-h gas-phase hydrolysis time was found to be suitable for the majority of glycopeptides and 1.5 h for the majority of phosphopeptides [152].

Analysis of unusual and complex amino acid mixtures can be performed following derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and separation of the derivatives by reversed-phase HPLC [153]. Strydom and Cohen compared the performance of chromatography during separation of PTC and AQC derivatives using amino acid standards. Similar coefficients of variation were observed only when freshly prepared derivatives were analyzed. With prolonged time, the AQC derivatives were more stable and the AQC methodology was found to be superior to the PTC approach [153]. However, for the analysis of complex samples originating for example from cell culture fluids, a complicated quaternary gradient is needed to precisely control eluent pH and organic solvent gradient [154].

Oates and Jorgenson derivatized the hydrolyzate derived from the gas-phase mode with naphthalene-2,3-dicarboxaldehyde and analyzed the derivatives by open tubular liquid chromatography with amperometric detection. Fourteen amino acids present in the hydrolyzate of 0.1 ng (4 fmol) of protein were quantified within an error range of 5–8.5% [155]. The separation of amino acids as their 1-methoxycarbonylindolizine-3,5-dicarbaldehyde (LDA) derivatives requires a sophisticated binary gradient to achieve detection limits in the range from 0.2 fmol for leucine to 200 fmol for methionine. Proline, tyrosine and tryptophan could not be determined with this method [156].

Of the many proposed techniques only a few are being utilized in a day-to-day operation in a laboratory. In a collaborative study, aiming to discriminate between hydrolysis and the chromatographic analysis as a possible major source of error, it was found (i) that hydrolysis is to a higher extent responsible for errors in the amino acid analysis and (ii) the post-column methods yielded more accurate results than the pre-column ones [4]. Post-column derivatization with OPA gave the lowest correlation error. Simultaneously, this method offers the advantage of efficient separation of the residues by ion-exchange chromatography and sensitive detection. We applied both pre- and post-column derivatization with OPA for amino acid analysis of blotted proteins. Both approaches resulted in the efficient identification of more than 110 proteins of *Haemophilus influenzae* by database search (Section 4). The post-column approach led in most cases to a more confident protein identification [93].

3.3. Other analysis techniques

Schmeeer et al. used liquid chromatography atmosphere pressure ionization mass spectrometry (LC–API-MS) to determine D/L-cystines and cysteines as their phenylthiocarbamyl derivatives. The fragmentation patterns of seventeen PTC-amino acids and of six PTC-cysteines were obtained [157]. Phenylthiocarnalyl derivatives of amino acids were analyzed by thermospray LC–MS [158].

There are many reports dealing with separation of amino acid derivatives by CE techniques [158,159]. The advantages of the method are improved resolution and sensitivity, however, the potentials have not yet been fully realized. A Fmoc-derivatized amino acid standard mixture was separated by CE and detected by laser-induced fluorescence at a detection limit of 0.5 n*M* for Fmoc-alanine with a signal-to-noise ratio of 2 [160]. The recent developments of the method are summarized by Smith [161]. Pre-, post- and on-column derivatization techniques have been employed for separation of mixtures and enantiomeric forms of amino acids [161]. CE is still in a growth stage in biochemical analysis.

Using cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) of amino acids derivatized with NDA, a detection limit of 0.8 amol was reported for leucine at a signal-to-noise ratio of 2 [162]. Aromatic and sulfur-containing amino acids, as well as peptides and proteins, were determined by HPLC, using on-line, post-column, continuous photolytic derivatization with electrochemical detection [163,164].

Gas chromatography (GC) for determination of derivatized amino acids has been used for a long time [165]. Gehrke et al. compared gas chromatography with the ion-exchange chromatographic analysis and the results found were similar in both cases [3]. Husek reported the simultaneous gas chromatographic analysis of 20 amino acids and 30 non-amino organic acids abundant in plasma. Less than 30 min were required for the sample preparation prior to analysis [166]. GC–MS is a powerful alternative analytical method, which allows the simultaneous determination of post-translational modifications [132,167].

4. Identification of proteins by amino acid analysis

Amino acid analysis enjoys a renaissance nowadays which is mainly due to its application in large-scale protein identification by search in databases. Relatedness between proteins on the basis of amino acid composition was first studied by Cornish-Bowden [168,169]. Composition cannot lead to precise conclusions like sequence, still it can provide a reliable indication of relatedness. The factors which were decisive for the application of the method in protein identification were the reproducible quantifi-

cation of the residues and the development of sensitive detection methods (OPA, Fmoc), the reproducible separation of large protein mixtures by 2D-PAGE, the development of efficient and user-friendly software for protein search in databases and in particular, the immense information derived from the complete sequencing of the genomes of various organisms. Simultaneously, other more empirical techniques were further improved, like the efficient electrotransfer of proteins from gels to membranes, the large-scale hydrolysis of blotted proteins, the equal extraction of the hydrolyzate residues, and the identification of the sources of external contamination. Up to now, the genomes of 12 microorganisms have been completely sequenced and published, including *Haemophilus influenzae*, *Mycoplasma genitalum*, *Bacillus subtilis*, *Archaeoglobus fulgidus*, *Borrelia burgdorferi*, *Escherichia coli, Helicobacter pylori*, *Saccharomyces cerevisiae* and others [170–178]. The sequencing of the genomes of many other microorganisms is expected to be completed in the near future.

The two-dimensional gel technology is capable of separating large numbers of proteins in a fairly reproducible manner [93,179–192]. It finds wide applications in biochemistry and in medical diagnosis [193], in particular, since the introduction of immobilized pH gradient strips increased the convenience of performance [194,195]. The new term Proteomics mainly denotes the application of the 2D-PAGE technology in the investigation of the proteins expressed by the various organisms and of their modifications, providing us with data enabling the proper target selection for drug discovery. A much larger contribution of Proteomics to proteome analysis and therapeutics discovery is anticipated for the next years, which is assisted by the ongoing genome sequencing and the further development of efficient software and analytical instrumentation.

For amino acid analysis, the proteins, following separation by 2D-PAGE, are electrotransferred to membranes. The membranes are usually stained with amidoblack or Coomassie blue and the protein spots are excised and subjected to gas-phase hydrolysis with HCl according to the conventional method [24] or at elevated temperatures for a shorter time (155°C for 1 h) [90,91,196]. Lottspeich et al. [90], Golaz et al. [197] and Yan et al. [91] investigated the conditions of hydrolysis of blotted proteins and identified various sources of experimental errors. Weiss et al. found that microwave hydrolysis of proteins blotted onto PVDF membranes can affect the equal extraction of the hydrolyzate residues [24]. For large-scale hydrolysis, we constructed specific hydrolysis vessels, each accommodating 56 vials. We used several vessels at the time so that prolonged hydrolysis did not affect the overall performance [24].

Golaz et al. classified the amino acids into ''reliable'' (Asp, Glu, Ser, Thr, Ala, Pro, Arg, Val, Ile, Leu) and ''non-reliable'' (His, Gly, Tyr, Met, Lys, Phe) [197]. The non-reliable residues could have been oxidized during hydrolysis (His, Met) [Met most likely during the sodium dodecyl sulfate (SDS)-PAGE] or contaminated from the gels (Gly) or not efficiently extracted from the PVDF membrane (Lys). In our hands, HPLC separation of the pre-column derivatized residues occasionally resulted in irreproducible quantification of Asp, Glu and sometimes His. Gly and Glu were in most cases overestimated, as well as the phenylalanine residues in proteins represented by weak spots, following post-column derivatization with OPA. In general, the quantification of the low-abundance residues His, Met and occasionally Phe, from weak spots, was imprecise [93].

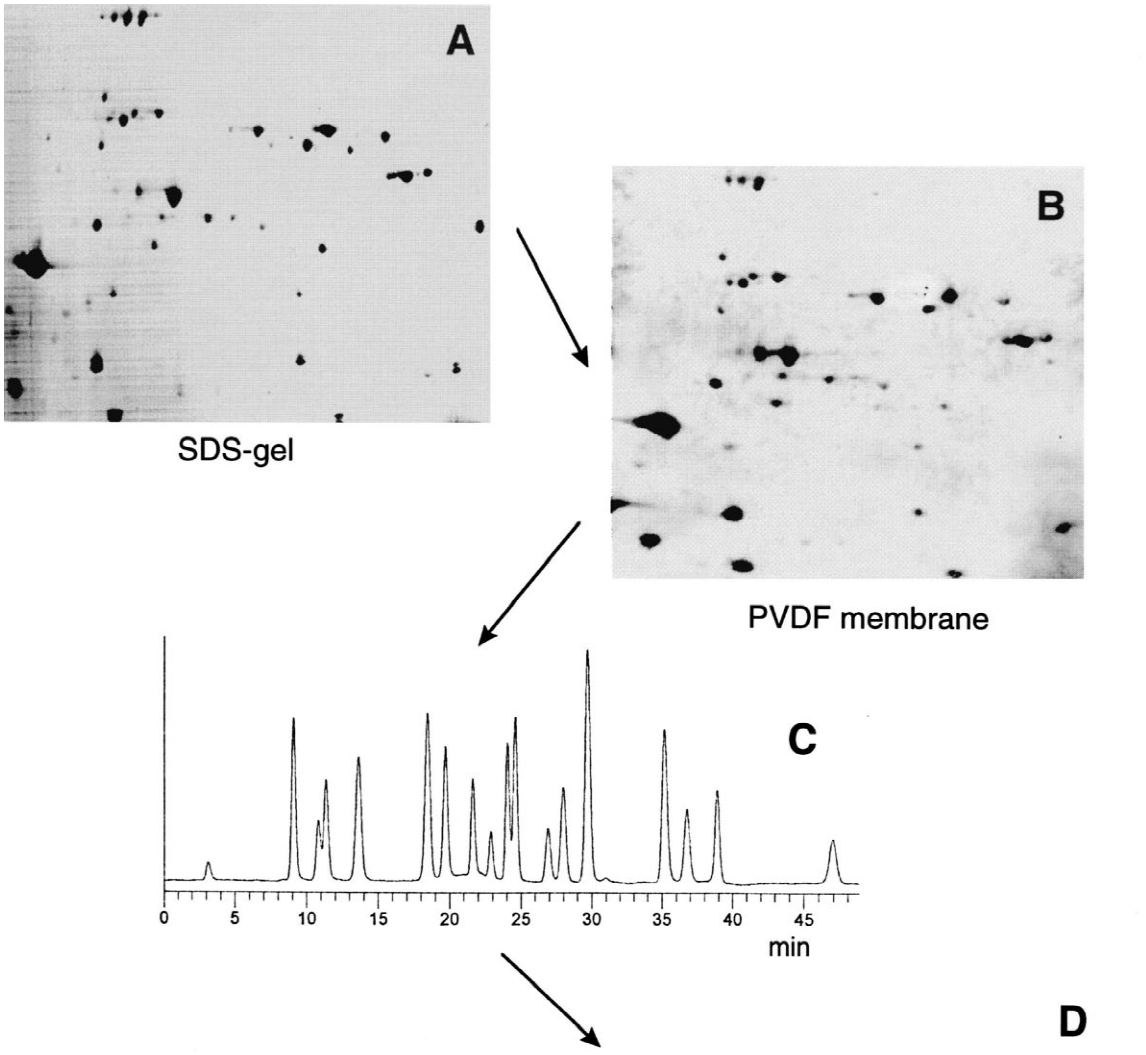
Specific software have been developed to assist the protein identification process by search in databases [88,198–205]. Eckerskorn et al. identified mouse brain proteins by amino acid analysis and confirmed the identifications by N-terminal sequence determination [199]. Using amino acid compositional analysis and the AACompIdent software, Wilkins et al. identified 20 *E. coli* proteins [196]. Protein search considering the observed molecular mass (M_r) and isoelectric point (pI) values resulted in correct identification of 75% of the proteins, whereas without considering them, only 40% of the proteins were correctly identified. Similarly, Wheeler et al. identified confidently 8 out of 12 cardial proteins. They consider the identifications resulting from the amino acid analysis data as more confident compared to the peptide mass fingerprinting data [206]. This may be due to insufficient digestion or inefficient peptide recovery or disturbing post-translation protein modifications.

Garrels et al. identified *S. cerevisiae* proteins, single- or double-labeled with ^{14}C and ^{35}S. For 70 out of 86 spots, with single and double labeling, the identifications assigned by amino acid analysis were also confirmed by other approaches. The other cases were either ambiguous or no prediction could be made [207]. Applying amino acid analysis, we identified 110 proteins of *H. influenzae* with a success rate of approximately 52% [93]. Ninety five identifications were confirmed by MS. For the remaining proteins, no clear correlation could be made between spots seen in blots (used for amino acid compositional analysis) and spots seen in gels (used for mass spectrometry analysis). Fig. 2 gives a schematic representation of the steps followed for identification of proteins separated on 2D-gels by amino acid analysis and search in data bases.

The precise quantification of the less frequently abundant residues, such as Trp, His, Met, Cys, is more critical (and simultaneously more difficult) for the correct identification of a protein by composition database search than the quantification of the more abundant residues, such as Gly, Ala, Ser, Val. On the other hand, the distribution of the more abundant is relatively more narrow than that of the less frequently abundant residues. This means that fewer proteins exist in a certain range of the more abundant residues [93]. Table 2 shows the theoretical abundance frequency of the various amino acids in all proteins of *H. influenzae* (1742 predicted coding regions). The most abundant amino acids in the microorganism are Leu, Ala, Val and Ile. Okayasu et al. determined the amino acid compositions in various mammalian cells and found that Gly, Ala and Leu are the most frequent residues [208]. They observed significant differences between compositions of bacterial and mammalian cells.

The most significant advantages of the compositional analysis in protein identification are: (i) it is an error robust method, tolerating some imprecision in the residue quantification. If the residues are determined with an error of 2%, the likelihood of an average combination of 14 amino acids is less than 1:1000. The quantification of 12 residues is usually sufficient for an unambiguous protein identification [93]. (ii) It is fast, allowing the analysis of up to 60 samples per day. The method can be significantly further improved with the availability of software for the automatic quantification of the residues, the automatic data introduction and search in the databases. (iii) It does not require very expensive instrumentation and can be practically established in any biochemical laboratory, however, it requires skilled personnel. (iv) Homologous proteins from other species can be easily identified [24,209–211]. (v) It is independent of post-translational modifications.

In comparison with MS, the composition analysis shows certain weaknesses: (i) it is much slower than matric-assisted laser desorption ionization (MALDI) MS by which today up to 500 samples can be easily analyzed by one person daily, following in-gel protein digestion in low-salt buffer, simplified peptide recovery in a small volume and direct measurement without previous desalting [212]. (ii) It is less sensitive than MS, taking into consideration the extraction yield (for example, MALDI-MS requires approximately 100 ng of a starting protein quantity for a successful identification, amino acid analysis requires approximately 1 μg). (iii) One additional step is included in the amino acid analysis, the electrotransfer of the proteins from the gel to a membrane; the spots on the membrane are often diffuse and a correlation between spots seen in membranes and in gels is sometimes difficult. (iv) The performance of hydrolysis, the extraction of the hydrolyzate and the avoidance of external contamination are critical and difficult to control parameters. (v) In general, identifications on the basis of compositional analysis include a higher ambiguity and the identification has to be supported by additional data, such as the M_r and pI values of the particular protein [93,196]. In MS, internal standards can be used during the analysis, increasing, thus, the precision of the mass determination and consequently the confidence of identification. Moreover, in cases of doubt, MS can produce a partial sequence of major matching peptides [213]. Compositional analysis has been combined with a partial N-terminal end sequencing of 3-4 residues, which increases the confidence of identification, and after which the protein is subjected to amino acid analysis [214,215]. In spite of the introduced automation, Edman degradation is relatively slow. However, it is useful in proteome analysis as it delivers information about the N-terminal residues of processed proteins which is not



The SWISS-PROT entries having pI and Mw values in the specified range:

Rank	Score	Protein	(pI	Mw)	Description
✓ 1	5	DEOD_HAEIN	5.29	25885	PURINE NUCLEOSIDE PHOSPHORYLASE (EC 2.4
2	45	UREG_HAEIN	5.12	24688	UREASE ACCESSORY PROTEIN UREG.
3	50	DAPD_HAEIN	5.46	29723	2,3,4,5-TETRAHYDROPYRIDINE-2
4	52	DAPB_HAEIN	5.94	28869	DIHYDRODIPICOLINATE REDUCTASE (EC 1.3.1
5	52	PYRH_HAEIN	5.81	25726	URIDYLATE KINASE (EC 2.7.4.-) (UK)
6	56	RPIA_HAEIN	5.08	23093	RIBOSE 5-PHOSPHATE ISOMERASE A (EC 5.3
7	56	PFS_HAEIN	5.04	23964	PFS PROTEIN HOMOLOG.
8	56	KAD_HAEIN	5.18	23508	ADENYLATE KINASE (EC 2.7.4.3) (ATP-AMP
9	58	HIS6_HAEIN	5.24	28626	HISF PROTEIN (CYCLASE).
10	65	YEBC_HAEIN	4.57	26383	HYPOTHETICAL PROTEIN HI0315.

Fig. 2. Schematic representation of the major steps leading to protein identification by amino acid analysis. (A) Protein separation by 2D-PAGE. In this example, a partially purified soluble protein fraction of *H. influenzae* was used. The gel was stained with Coomassie blue. (B) Electrotransfer of proteins onto PVDF membrane. The membrane was stained with amidoblack. (C) Amino acid chromatogram of a protein spot excised from the membrane and hydrolyzed with HCl in the vapor phase. (D) List of proteins with compositions resembling the one of the analyzed spot, supplied by the AACompIdent software. The first ranking protein with a (score of rank No. 2 protein):(score of rank No. 1 protein) ratio of 9 was successfully identified.

Table 2
Theoretical amino acid composition of total proteins of *H. influenzae*

Residue	(%)
Glycine	6.50
Alanine	9.50
Asparagine	5.45
Aspartic acid	5.30
Arginine	3.00
Isoleucine	7.00
Glutamine	4.80
Glutamic acid	6.80
Cystine	0.75
Serine	6.30
Tyrosine	3.00
Tryptophan	1.00
Threonine	5.00
Valine	7.10
Histidine	2.00
Phenylalanine	4.00
Proline	3.50
Methionine	2.50
Lysine	5.50
Leucine	11.00

The approximate amino acid composition was calculated for all predicted coding regions of *H. influenzae* [171] and is expressed as the percent contribution of each residue to the composition of the microorganism. The predicted composition is based on the assumption that all proteins are expressed at a certain time point.

obtained by the other techniques. (vi) The chromatography columns are expensive and since relatively new columns deliver efficient residue separation, they need to be replaced regularly. The working life of certain reversed-phase columns recommended by the manufacturer is about 50 cycles, so that for serial analyses, new columns should be installed daily.

5. Other applications of the amino acid analysis

5.1. Protein quantification

The major contribution of amino acid analysis to protein science today is the determination of the protein quantity in solution. The compositional analysis is the most reliable protein quantification method, significantly superior to the colorimetric methods which usually determine the amount of a protein in comparison with a third, unrelated standard protein [2]. Amino acid analysis is primarily used to calibrate the other protein quantification assays, like optical density, fluorescence and colorimetric methods. Use of microwave hydrolysis has reduced the total analysis time [37].

Amino acid derivatization with OPA or Fmoc allows a confident determination of the protein concentration in the range of 10 ng/ml or lower, whereas the colorimetric methods determine concentrations in the range of about 1 μg/ml or higher. Use of internal standards, such as norleucin, during hydrolysis or consideration of the recoveries of only the stable amino acids increases the confidence of protein quantification by amino acid analysis. It is most reliable for small-to-medium size proteins (M_r 2000–22 000), whereas it can underestimate the high-molecular-mass proteins, such as immunoglobulins (M_r 150 000) [216].

Factors affecting the quantification of the various residues, as described in the corresponding section (Section 2.12), should be considered. Their removal by any method may affect the protein concentration in the original solution, especially in dilute solutions. Amino acids, in particular tryptophan, are destroyed during hydrolysis in the presence of large amounts of carbohydrates [15]. Compositional analysis is of limited value in the quantification of glycoproteins, carrying large carbohydrate moieties (30% or more). Chung described a quantification method for membrane proteins, following derivatization with fluorescamine and spectroscopic detection in a mixed solvent system [217]. Proteins blotted onto membranes can be quantified as well. However, here the hydrolyzate recovery has to be taken into consideration. In our hands, approximately 30% of the hydrolyzate were recovered from PVDF membranes, following gas-phase hydrolysis with 6 *M* HCl at 110°C for 24 h and extraction by sonication in 60% acetonitrile, containing 0.1% TFA, for 5 min [24].

5.2. Determination of the protein–protein interaction ratio

Amino acid composition analysis has been used for the determination of the stoichiometry of protein–protein interaction in protein complexes, like those between ligand–receptor or antigen–antibody. Antoni and Presentini developed a software for the calculation of the ratio of the components in a

complex [218]. The protein complexes are usually purified over a sizing column or even more efficiently on non-denaturing polyacrylamide gels [219,220], prior to analysis. The method was applied for the determination of the stoichiometry of interaction between various cytokines, such as interferon γ of human and mouse species [220,221], interleucin-5 [222], tumor necrosis factor α [223] and their corresponding receptors.

A prerequisite of a successful protein ratio determination is that the amino acid composition of the complex is substantially different from the compositions of the components. If one of the components is much shorter in comparison with the other binding protein, as for example is leptin compared to its receptor [224], the contribution of its amino acids to the composition of the complex is insignificant and can fall within experimental error. Amino acid analysis is a reliable approach for the determination of the protein–protein interaction ratio, however, it has not found a wide application, most likely on account of the complexity of the analysis.

5.3. Analysis of amino sugars

Hydrolysis under mild conditions is widely used in the carbohydrate analysis of glycoproteins and other oligosaccharide-carrying compounds. Carbohydrate analysis is largely assisted by the more selective enzymatic digestion, so that the role of the non-selective chemical hydrolysis is mainly limited to the sugar release from glycoproteins and glycoconjugates. Palladino et al. investigated the conditions of hydrolysis and chromatography leading to a reduced interference of carbohydrates in the accuracy of the amino acid composition analysis of glycoproteins [225]. Here selected applications of hydrolysis in the sugar analysis are discussed.

Manneberg et al. applied hydrolysis with 2 *M* TFA at 100°C for 4 h to produce monosaccharides from the oligosaccharide moieties released from a recombinant interferon γ receptor, following treatment with glycosidase F [226]. Lagana et al. used microwave hydrolysis in 2 *M* acetic acid to release neuraminic acids from glycoconjugates within 10 min. Prolonged digestion beyond this time resulted in a partial destruction of the sialic acids, depending on the final concentration of the hydrolyzing agent [227]. For monosaccharide and oligosaccharide analysis of blotted proteins, Weitzhandler et al. [228] and Packer et al. [92] used a two-step hydrolysis procedure: (i) treatment with 0.1 *M* TFA at 80°C for 40 min to remove sialic acid residues, and (ii) treatment with 2 *M* TFA at 100°C for 4 h to release neutral and amino sugars. The deglycosylated proteins were further hydrolyzed with 5.7 *M* HCl at 115°C for 1 h for identification by database search [92].

Tams and Welinder treated horseradish peroxidase isoenzyme C with anhydrous trifluromethanesulfonic acid in the presence of methanol and removed most of the carbohydrates of the protein which had not been removed by a previous enzymatic digestion [229]. Gorbics et al. investigated the conditions of hydrolysis and analysis of phosphopeptides and *N*-acetylglucosamine- and *N*-acetylgalactosamine-containing glycopeptides. The phospho- and glycopeptides were subjected to gas-phase hydrolysis for 1 and 1.5 h, respectively [152,230].

5.4. Specific applications

As already mentioned, amino acid analysis finds wide applications in the diagnostic medicine and food science for the detection of amino acids and their homologs. GC–MS and CE are alternative methods for the analysis of this class of molecules. Amino acid analysis has been used in numerous specific applications, such as determination of post-translational modifications of amino acids, determination of sulfur-containing amino acids in hair, determination of total body protein, quantification of total free and bound D- and L-amino acids in food, like cheese, milk, meat, determination of free amino acids in hemofiltrate, plasma and cerebrospinal fluid, evaluation of the polymer content in poly(ethylene glycol)-modified proteins, quantification of total protein deposits on contact lenses, quantification of cyclosporine and its metabolites, determination of amino acids in critically ill patients, just to mention a few [231–243]. Essentially, the mentioned or slightly modified hydrolysis, chromatography and detection techniques have been employed for the determination of large numbers of amino acid modifications [244–246]. The description of these approaches is beyond the scope of this study.

6. Conclusions

The present article provides a summary of the most significant developments in the amino acid composition analysis technology in the last 10 years. The number of publications in this field is significant, but certainly smaller than those on other technologies, like mass spectrometry. Many of them concerned microwave hydrolysis and the application of amino acid analysis for protein identification. The rest dealt with the quantification of the sensitive residues, modifications and minor improvements of the chromatographic techniques. Hydrolysis is the most critical part of the analysis and mainly responsible for analysis errors. In the spirit of performance improvement, the slower, classical ion exchangers are gradually replaced by the faster reversed-phase HPLC instruments. The method requires further automation in hydrolysis and residue quantification, improvements in the detection approach and possibly combination with another, efficient analytical technique, to be able to compete with the fast developing MS technologies.

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